Published in final edited form as:

Neurochem Int. 2020 October ; 139: 104787. doi:10.1016/j.neuint.2020.104787.

Reciprocal communication between astrocytes and endothelial cells is required for astrocytic glutamate transporter 1 (GLT-1) expression

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Abstract

Astrocytes have diverse functions that are supported by their anatomic localization between neurons and blood vessels. One of these functions is the clearance of extracellular glutamate. Astrocytes clear glutamate using two Na⁺-dependent glutamate transporters, GLT-1 (also called EAAT2) and GLAST (also called EAAT1). GLT-1 expression increases during synaptogenesis and is a marker of astrocyte maturation. Over 20 years ago, several groups demonstrated that astrocytes in culture express little or no GLT-1 and that neurons induce expression. We recently demonstrated that co-culturing endothelia with mouse astrocytes also induced expression of GLT-1 and GLAST. These increases were blocked by an inhibitor of γ -secretase. This and other observations are consistent with the hypothesis that Notch signaling is required, but the ligands involved were not identified. In the present study, we used rat astrocyte cultures to further define the mechanisms by which endothelia induce expression of GLT-1 and GLAST. We found that co-cultures of astrocytes and endothelia express higher levels of GLT-1 and GLAST protein and mRNA. That endothelia activate Hes5, a transcription factor target of Notch, in astrocytes. Using recombinant Notch ligands, anti-Notch ligand neutralizing antibodies, and shRNAs, we provide evidence that both Dll1 and Dll4 contribute to endothelia-dependent regulation of GLT-1. We also provide evidence that astrocytes secrete a factor(s) that induces expression of Dll4 in endothelia and that this effect is required for Notch-dependent induction of GLT-1. Together these studies indicate that reciprocal communication between astrocytes and endothelia is required for appropriate astrocyte maturation and that endothelia likely deploy additional non-Notch signals to induce GLT-1.

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7. Competing Interest

The authors declare no competing interests.

Keywords

Astrocytes; GLT-1; GLAST; blood-brain barrier; endothelia; Notch

1. Introduction

Astrocytes, the most abundant glial cell type in the mammalian nervous system, have a privileged location that allows them to contact synapses and blood vessels. This anatomic localization facilitates astrocyte functions. For example, astrocytes clear and release neurotransmitters, regulate the distribution of water, participate in ion buffering, produce glutathione and remove reactive oxygen species, synthesize and release trophic factors, and contribute to neurovascular coupling (Abbott, 2002; Dringen et al., 2015; Nuriya and Hirase, 2016; Potokar et al., 2016; Sahlender et al., 2014; Santello and Volterra, 2009; Wang et al., 2020). In addition to shaping their environment through these functions, they are also regulated by their environment. In fact, there is ample evidence for bi-directional communication between neurons and astrocytes that regulates diverse functions including synapse formation, astrocyte morphology, and glutamate transporter expression (Gegelashvili et al., 1997; Ghosh et al., 2011; Hasel et al., 2017; Levy et al., 1995; Schlag et al., 1998; Stogsdill et al., 2017; Swanson et al., 1997); for reviews, see (Allen and Eroglu, 2017; Allen and Lyons, 2018; Chung et al., 2015; Clarke and Barres, 2013; Durkee and Araque, 2019; Lee and Chung, 2019; Mederos et al., 2018). In addition to contacting synapses, astrocytes extend endfeet that contact the vasculature (Kubotera et al., 2019; Mathiisen et al., 2010). This anatomic interaction allows astrocytes to directly control the supply of glucose and oxygen to the brain (Angelova et al., 2015; Hertz et al., 2007; Kacem et al., 1998; Lee et al., 2016; Weber and Barros, 2015). In addition, endothelia have diverse effects on astrocytes, including: increasing their proliferation through the effects of a secreted molecule (Estrada et al., 1990), increasing glutamine synthetase activity (Spoerri et al., 1997), regulating calcium signaling (Yoder, 2002), inducing astrocyte differentiation and maturation through LIF and BMP/Smad signaling (Imura et al., 2008; Mi et al., 2001; Sakimoto et al., 2012), increasing aquaporin 4 (AQP4) expression in endfeet (Camassa et al., 2015) and increasing expression of the astrocytic glutamate transporters (Lee et al., 2017).

Glutamate, the main excitatory neurotransmitter in the central nervous system, is also a neurotoxin and therefore needs to be rapidly cleared (Frandsen et al., 1989; Roberts and Davies, 1987). Clearance is mediated by a family of five Na⁺-dependent excitatory amino acid transporters; only two of these transporters are found in astrocytes, glutamate transporter 1 (GLT-1) and glutamate/aspartate transporter (GLAST), also called EAAT2 and EAAT1 respectively (Danbolt, 2001; Divito and Underhill, 2014; Grewer et al., 2014; Kandasamy et al., 2018; Magi et al., 2019; Malik and Willnow, 2019; Martinez-Lozada et al., 2016; Murphy-Royal et al., 2017; Vandenberg and Ryan, 2013). In contrast to the effects of deletion of the other forebrain transporters, deletion of GLT-1 causes seizures and premature death (Aoyama et al., 2006; Karlsson et al., 2008; Peghini et al., 1997; Robinson, 1998; Tanaka et al., 1997 for review, see Danbolt, 2001). GLT-1 expression increases during synaptogenesis and correlates with astrocyte maturation (Chaudhry et al., 1995; Danbolt, 1994; Furuta et al., 1997; Rothstein et al., 1994; Shibata et al., 1996; Sims and Robinson,

1999; Ullensvang et al., 1997). GLT-1 is highly enriched in astrocytes *in vivo* (Danbolt et al., 1992; Rothstein et al., 1994), but cultured astrocytes express almost no GLT-1 unless they are co-cultured with neurons (Gegelashvili et al., 1997; Hasel et al., 2017; Schlag et al., 1998; Swanson et al., 1997; Yang et al., 2009).

We recently reported that co-culturing with brain endothelial cells also increases GLT-1 and GLAST expression in astrocytes (Lee et al., 2017). Results from transwell experiments in which astrocytes and endothelia were separated by a semi-permeable membrane demonstrate that contact is required for this effect. We found that inhibition of the Notch pathway using a γ -secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) or by knocking-down RBPJ, a transcriptional regulator of Notch target genes, blocks the effect of endothelia. The Notch pathway has been implicated in the regulation of cell proliferation, cell fate and differentiation in different organs. In the brain, Notch is a major regulator of neural stem cell maintenance and glia specification (Louvi and Artavanis-Tsakonas, 2006; Lundkvist and Lendahl, 2001; Miller and Gauthier, 2007; Wang and Barres, 2000; Zhou et al., 2010).

Canonical Notch signaling starts when the Notch ligand, expressed on the plasma membrane of the sending cell, interacts with the Notch receptor on the membrane of the receiving cell; signaling can then be activated in both cells. In fact, the ligand and receptor can be expressed in a single cell to have cell autonomous effects (Nandagopal et al., 2019; Sainson et al., 2005; Sakamoto et al., 2002). Notch ligands contain a Delta, Serrate and Lag2 (DSL) domain. In mammals, there are four Notch ligands that activate Notch receptors that are classified in one of two groups: Delta-like (Dll1 and Dll4) and Serrate (Jagged)-like (Jag1 and Jag2) (Kopan and Ilagan, 2009). The interaction between Notch receptor with its ligand allows sequential cleavages of the receptor. First, ADAM10 cleaves the extracellular domain; then γ -secretase releases the Notch Intracellular Domain (NICD). NICD translocates to the nucleus, interacts with RBPJ (also known as CBF1), and recruits transcriptional activators (Kopan, 2012; van Tetering and Vooijs, 2011). The NICD/RBPJ complex with other coactivators increases transcription of target genes which contain a basic helix-loop-helix (bHLH) motif, for example, Hes and Hey gene family (Ables et al., 2011; Lai, 2004; Mumm and Kopan, 2000; Sjöqvist and Andersson, 2019). This is a non-amplifying stoichiometric process, in which one receptor/ligand interaction results in one transcription activator complex (Andersson et al., 2011).

In the present study, we investigated the components of the Notch pathway that are responsible for the endothelia-dependent effects on astrocytic glutamate transporters. We found that both delta-like Notch ligands Dll1 and Dll4 contribute to regulation of GLT-1. In addition, we show that astrocytes secrete a soluble signal that increases expression of Dll4 in endothelia. These results show that endothelia-dependent induction of GLT-1 and GLAST requires bidirectional communication between astrocytes and endothelial cells.

2. Materials and Methods

2.1 Animals.

A colony of Sprague-Dawley rats was maintained at the animal facility of the Children's Hospital of Philadelphia. A new male breeder was introduced every year (Charles River Laboratories, MA, USA, RRID:RGD_734476) and the females were bred four times only to limit inbreeding. All studies were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were housed in standard controlled temperature, humidity, and light, and had *ad libitum* access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Primary enriched astrocyte cultures.

Both sexes of neonatal rats 1-3 days of age were used to prepare enriched primary astrocyte cultures as previously described (Zelenaia et al., 2000). Briefly, after dissection and removal of meninges, rat brain cortices were dissociated by trypsin, triturated into a single-cell suspension and plated at a density of 2.5×10^5 cells/mL in 75 cm² flasks. Astrocytes were maintained in Dulbecco's modified eagle's media (DMEM) (Gibco 11960-014), supplemented with 10% Ham's F12 (Gibco 11765-047), 10% defined heat-inactivated fetal bovine serum (FBS) (HyClone SH30070.03) and 0.24% penicillin/Streptomycin (Gibco 15140-122). The media was exchanged every 3-4 days. After 7-10 days (when cells were ~90% confluent) A2B5 positive cells were eliminated using A2B5 hybridoma supernatant (1:50; from the laboratory of Dr. Judy Grinspan, Children's Hospital of Philadelphia) and Low Tox-M rabbit complement (Cederlane CL3005). Two days later, the astrocytes were split (1 to 1.5 surface area to surface area) into 6-, 12- or 24-well culture plates for experiments as indicated in each figure. Under these conditions, >95% of these cells are GFAP positive, indicating that they are astrocyte cultures.

2.3 Primary cultures of rat brain endothelial cells (RBEC).

Primary cultures of endothelial cells were prepared as described previously (Lee et al., 2017; Takata et al., 2013; Welser-Alves et al., 2014). Briefly, two adolescent rats (3 weeks old, both sexes) were anesthetized with isoflurane and euthanized by decapitation. The brains were harvested into ice-cold HBSS, the brainstem, cerebellum, white matter and meninges were removed. The remaining tissue was cross-chopped into small pieces using a razor blade and incubated in 10 mL 1X PBS containing 1mg/mL collagenase/dispase (Roche 10269638001) and 100µg/mL DNase I (Roche 04716728001) for 1.5 h at 37°C; this suspension was gently inverted every 30 min. The tissue was triturated 10 times with each of the following: a glass Pasteur pipette, a 1mL plastic pipette tip on the end of a 10mL pipette, followed by a 200µL plastic pipette tip. This homogeneous suspension was centrifugated at 600 x g for 10 min. The pellet was resuspended in 15 mL DMEM (Gibco 11960-014) containing 25% bovine serum albumin (Sigma 05470) and centrifugated at 600 x g for 10 min. The pellet containing the endothelial cells were washed in RBEC media consisting of: DMEM/F12 (Gibco 11320-033) supplemented with 10% heat inactivated FBS (HyClone, SH30070.3), 1% penicillin/streptomycin (Gibco 15140-122), and 20 mg of endothelial cell

growth supplement from bovine neural tissue (Sigma E2759). They were then plated in the same media supplemented with 4µg/mL puromycin (Gibco A1113803) on culture dishes coated with 75 µg/mL collagen I (Corning 354236) and 15µg/mL fibronectin (Corning 356008) (cells obtained from two rat brains into a 10cm dish). Three days later media was replaced with fresh RBEC media without puromycin followed by complete media exchanges every 3-4 days until the cells were confluent (~10-14 days). Cells were then split (1:5 surface area to surface area) onto 6-well coated plates for further studies.

2.4 Culture of the bEND.3 endothelioma cell line.

The mouse brain endothelioma cell line bEND.3 was purchased from American Type Culture Collection (cat. # CRL-2299, RRID:CVCL_0170). The advantages of this cell line are that it is a homogeneous population of cells, and it allowed us to use fewer animals. These cells were maintained in DMEM media containing 4.5 g/L D-glucose (Gibco 11960-014) and supplemented with 10% defined heat-inactivated FBS (HyClone, SH30070.3), 4 mM L-glutamine (Gibco 25030-081), and 1 mM sterile filtered sodium pyruvate at 37°C and 5% CO₂. This “endothelial” media was exchanged every 3-4 days. Cells were never used past passage 30 as per the vendor’s recommendation to limit genetic drift.

2.5 Co-culture of astrocytes and endothelial cells.

In experiments in which astrocytes were cultured on top of endothelial cells, bEND.3 cells (ATCC, cat. # CRL-2299; RRID:CVCL_0170) from a confluent 10 cm dish were suspended in 30 ml of “endothelial” media and plated onto 6-, 12- or 24-well plates (2, 1 or 0.5 mL per well respectively). Two days later bEND.3 were ~80% confluent and astrocytes were re-plated (1:1.5 surface area to surface area, from a fully-confluent flask) directly into empty wells (control) or on top of endothelia. These cultures were maintained in astrocyte media that was changed every 3-4 days for 7 to 10 days. In a subset of experiments/wells, 10 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Santa Cruz SC201315) was included in the media and with every media exchange. In a subset of experiments, bEND.3 were allowed to become confluent and fixed as previously described (Stogsdill et al., 2017) with ice-cold methanol for 5 or 15 min, followed by air-drying for 10 to 15 min before astrocytes were layered on top of these cells as indicated above.

2.6 Conditioned media treatments.

Media from astrocytes or endothelia was taken during normal media exchanges (having been on cells for 3 to 4 days), cleared by centrifugation at 600 x g for 10 minutes, and then supplemented with 10% FBS prior to addition to cells. Uncubated media was used as a control.

2.7. Western blot analysis.

Cells were lysed and proteins were collected as described previously (Ghosh et al., 2016; Ghosh et al., 2011; Li et al., 2006). In brief, cells were rapidly rinsed in 1X PBS with Ca²⁺/Mg²⁺ and then incubated in ice cold RIPA on an orbital shaker platform at 4° C for 45 min. Protein was measured using bicinchoninic acid (Pierce BCA protein assay kit, Thermo

Fischer Scientific). For Western blot analysis, total proteins (15µg for GLT-1 and GLAST, 25µg for Dll1 and Dll4) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene fluoride membranes (transblot apparatus, Bio-Rad) and blocked for 30 minutes in blocking buffer (5% non-fat dry milk in 1X TBS 0.1% Tween 20). Membranes were then probed with rabbit anti-GLT-1 (C-terminal-directed, 1:5000, final concentration of 0.25µg/mL, Cat#GLT-1a RRID:AB_2314565) (Rothstein et al., 1994), mouse anti-GLAST (1:500, final concentration of 200ng/mL, anti-ACSA-1 pure Miltenyi Biotec 130-095-822 RRID:AB_10829302), rabbit anti-Dll1 (1:500, final concentration of 1µg/mL, abcam ab84620 RRID:AB_1860333), rabbit anti-Dll4 (1:500, final concentration of 2µg/mL, abcam ab7280 RRID:AB_449562), mouse anti-actin (1:10,000, Cell Signaling 3700S RRID:AB_2242334), or a combination of these antibodies in 1% blocking buffer. In all cases, GLT-1 and GLAST antibodies were combined and developed in separate channels. These proteins migrate to different molecular weights, and we confirmed that the two signals do not overlap. After washing three times in 1% blocking buffer, the membranes were probed with secondary antibodies (either anti-mouse or anti-rabbit fluorescently conjugated antibodies, 1:10,000; LI-COR Biosystems RRID:AB_10953628 and RRID:AB_621848 respectively) for 45 minutes in 1% blocking buffer. The membranes were washed three more times and visualized using a LI-COR Odyssey Infrared Imaging System. Images were quantified and analyzed using Image Studio Lite (LI-COR Biosystems software RRID:SCR_013715). Linearity of signal was assayed examining optical density as a function of protein loaded curves for antibodies directed against GLT-1, GLAST, Dll1 and actin; these were found to be linear under the conditions used in these experiments. Glutamate transporters variably form homomultimers that are not disassociated in a laemmli buffer (Haugeto et al., 1996) (see Fig. 1A for an example). Therefore, we quantify monomers (lower molecular weight) and multimer (higher molecular weight band), and report the sum of the immunoreactivity observed in the two bands. We compared the levels of actin observed in every experiment; there were no differences between groups except in figures 1 and 10 where endothelial monocultures consistently have lower levels of actin. Therefore, although equal amounts of total protein were loaded in each lane, we also normalized the optical densities for each protein of interest to the optical density for actin measured on the same immunoblot.

2.8 Immunocytochemistry.

Primary astrocytes, bEND.3 cells, or co-cultures were grown as indicated above on sterile coverslips coated with poly-D-lysine (50µg/mL, Sigma-Aldrich P0899). Cultures were washed once in 1X PBS and then fixed with 4% paraformaldehyde in 1X PBS (10 min). After three 10 min rinses in 1X PBS, cells were treated with ICC-blocking solution (5% goat serum, 0.4% Triton X-100, in 1X PBS) for 1 h at room temperature. Cells were incubated overnight at 4°C with primary antibodies diluted in ICC-blocking solution, a mixture of the following: rabbit anti-GLT-1 (C-terminal-directed; 1:250, final concentration of 5µg/mL, Cat#GLT-1a RRID:AB_2314565)(Rothstein et al., 1994), mouse anti-GLAST (1:250, final concentration of 100ng/mL, anti-ACSA-1 pure Miltenyi Biotec 130-095-822 RRID:AB_10829302), rabbit anti-Dll1 (1:200, final concentration of 2.5µg/mL, abcam ab84620 RRID:AB_1860333), or rabbit anti-Dll4 (1:200, final concentration of 5µg/mL, abcam ab7280 RRID:AB_449562) plus rat anti-CD31 (1:200, final concentration of 78

ng/mL, BD Pharmingen Clone MEC 13.3 550274 RRID:AB_393571), and chicken anti-GFAP (1:500, Millipore AB5541 RRID:AB_177521). After three 10 min rinses in 1X PBS, coverslips were incubated in ICC-blocking solution containing goat anti-rabbit Alexa Fluor 488 (A11034, RRID:AB_2576217) or goat anti-mouse Alexa Fluor 488 (A11029, RRID:AB_138404), with goat anti-rat Alexa Fluor 594 (A11007, RRID:AB_10561522) and goat anti-chicken Alexa 633 conjugates (A21103, RRID:AB_2535756) (all at 1:500, final concentration of 4µg/mL, Invitrogen Thermo Fisher Scientific) for 1 h at room temperature. Nuclei were counterstained using a mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc). Pictures were taken with a DMi8 Leica confocal microscope equipped with a 40X objective (Leica Microsystems) using the 405, 488, 594 and 633 nm laser lines. For some images an additional 2X optical zoom was used. To blind the analysis, fields were selected using the DAPI, CD31, or GFAP signals (see individual figure legends). The images were taken using sequential mode to avoid contamination of the signals from others fluorophores. For each experiment, at least two coverslips and three fields per coverslip were imaged. At least three independent experiments were conducted.

2.9 Isolation of mRNA and Quantitative PCR.

Astrocytes and bEND.3 cells were cultured as described above, and mRNA was harvested after 7-10 days using the RNeasy Mini Kit (Qiagen 74104) according to the manufacturer's instructions. Equal amounts of isolated mRNA were reverse transcribed into cDNA using QuantiTect Reverse Transcription kit (Qiagen 205311), and one tenth of this reaction was used per quantitative polymerase chain reaction (qPCR). Quantitative PCR was performed using TaqMan primer-probe mixes (Applied Biosystems, TaqMan gene expression assay numbers: Mm01275814_m1 for Slc1a2, Mm00600697_m1 for Slc1a3, and Mm99999915_g1 for Gapdh), and an Applied biosystem quantflex 7 was used for detection. Gapdh mRNA was measured in multiplex assays. There were no differences between samples in gapdh levels; therefore, data generated from qPCR are presented as those normalized to Gapdh. TaqMan gene expression assays have a PCR efficiency of 100% ($\pm 10\%$) as validated by Applied Biosystems. To determine the relative expression levels, the comparative C_T ($-C_T$) method was used (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

2.10 Transient transfection and Luciferase assay.

Two-three days after splitting, astrocytes in 12 well plates (70-80% confluent) were transfected with a mixture of Hes5 promoter firefly luciferase reporter construct (1µg, RRID:Addgene_26869) and HSV-thymidine kinase promoter renilla luciferase reporter construct (pRL-TK 500ng, Promega E2241) using lipofectamine 2000 according to manufacturer's instructions. In parallel, the efficiency of transfection was evaluated using an eGFP construct (Ghosh et al., 2011). Data from experiments in which fewer than 15 to 20% of cells expressed detectable eGFP were excluded (1 of 6 experiments in Fig. 3, and 2 of 7 experiments in Fig. 6B); in these cases, the levels of luciferase activity were indistinguishable from the non-transfected astrocytes. Two days post transfection, bEND.3 cells were split 1:3 on top of astrocytes. Seven days later, cells were harvested and firefly and renilla luciferase activities were analyzed using dual-luciferase reporter assay (Promega E1910). There were no differences in thymidine kinase renilla luciferase activities between

samples. Therefore, data are presented as firefly luciferase activity normalized to renilla luciferase activity.

2.11 Recombinant-Notch Ligand treatment.

Treatments with Notch ligands were performed as described previously (Ilagan and Kopan, 2014). Briefly, culture plates were coated with 5µg/mL solution of the extracellular domain of Notch ligand fused at the C-terminal to the Fc portion of human IgG1 (Dll1 (mouse):Fc (human) recombinant protein AdipoGen AG-40A-0148Y, Dll4 (mouse):Fc (human) recombinant protein AdipoGen AG-40A-0145, Jag1 (mouse):Fc (human) recombinant protein AdipoGen AG-40A-0157T, or Jag2 (mouse):Fc (human) recombinant protein R&D Systems 4748-JG). The recombinant Fc portion of human IgG1 was used as control (recombinant AdipoGen CHI-HF-210IG1) in sterile 1X PBS overnight at 4°C. To remove unbound ligand, that is known to inhibit Notch signaling (Small et al., 2001), plates were washed once with sterile 1X PBS prior to astrocyte seeding. Astrocytes were maintained for 7-10 days, with complete media changes every 3-4 days.

2.12 Inhibition of Notch signaling with neutralizing antibodies.

Confluent bEND.3 cells were incubated with medium containing 5µg/mL of anti-Dll1 (BioXCell Clone HMD1-5 BE0155, RRID:AB_10950546), anti-Dll4 (BioXCell Clone HMD4-2 BE0127, RRID:AB_10950366), anti-Jag1 (BioLegend Clone HMJ1-29 130902, RRID:AB_2561301), anti-Jag2 (BioXCell Clone HMJ2-1 BE0125, RRID:AB_10949305), or a non-immune IgG of the same isotype (Armenian Hamster IgG BioXCell BE0091, RRID:AB_1107773) for 1 hour. Media was aspirated and astrocytes were layered over these cells (Koga and Aikawa, 2014). The co-cultures were maintained in astrocyte media for 7-10 days with complete media changes every 3-4 days that included the antibodies at the same concentration.

2.13 Lentiviral vector production and bEND.3 infection.

Lentivirus were prepared as previously described (Ghosh et al., 2011). Briefly, HEK-293T cells were grown on poly-D-lysine (500µg/mL, MP Biomedicals 27964-99-4) coated plates and transfected using Ca²⁺ phosphate transfection kit (Takara Bio, USA formerly known as Clontech Laboratories 631312) with a mixture of 15µg of packing plasmid pCMV r8.2, 5µg of the envelope plasmid JS-86, and 20µg of the transfer plasmid pLKO.1 (Sigma-Aldrich) containing either shRNA directed against Dll1 (CCGGCTCGGGCTGTTCAACTTCAAACCTCGAGTTTGAAGTTGAACAGCCCGAGTT TTTG), shRNA directed against Dll4 (CCGGGATGACCACTTCGGACATTATCTCGAGATAATGTCCGAAG-TGGTCATCTTTTT), or a scrambled shRNA control (CCGGCAACAAGATGAAGAGCACCAA-CTCGAGTTGGTGCTCTTCATCTTGTGTTTTT) that has no known mammalian target genes. Sixteen hours post-transfection, cells were washed with fresh media. Media containing the lentiviral particles were collected 24 and 48 hours later and filtered through 0.45µm filters followed by centrifugation at 50,000 x g for 2 hours in a SW28 rotor (Beckman Coulter, Brea, CA, USA) at 4°C. The pellet was resuspended in 500µL of ice-cold endothelial media and aliquots were maintained at -80°C, and used within two months. The

concentration of the lentiviral particles was determined using the HIV-p24 Elisa assay (Perkin Elmer, Waltham, MA, USA). For transduction of endothelia, lentiviruses were thawed on ice, mixed with media to a final concentration of 400 ng p24/mL, and added to bEND.3 cultures 2-3 days after plating (~confluency of 70-80%). The media was replaced with fresh endothelia media 24 hours later, and the next day astrocytes were split on top of transduced bEND.3 cells as described above. Cells were maintained in astrocyte media, with complete media exchanges every 3-4 days, and lysed for western blot analysis 7-10 days after astrocytes were plated. A lentivirus that expresses eGFP was used in parallel to assay transduction efficiency; it was consistently between 80 and 90% (data not shown).

2.15 Statistical analyses.

All independent experiments were conducted with different primary cultures and performed on different days. All statistics were performed using Graphpad Prism software (RRID:SCR_002798). We performed a non-parametric test Kruskal-Wallis one-way analysis of variance (ANOVA) followed by a Mann-Whitney-Wilcoxon test with a Bonferroni correction for multiple comparisons or one sample t-test and Wilcoxon test for comparisons of two groups.

3. Results

3.1 Endothelial cells induce expression of GLT-1 and GLAST by two distinct mechanisms

Previously, we showed that co-culturing primary endothelia or an endothelial cell line with mouse cortical astrocytes increases astrocytic expression of the glutamate transporters GLT-1 and GLAST, increases Notch intracellular domain (NICD) in nuclei of astrocytes, and that DAPT or siRNA-mediated inhibition of RBPJ block the effect of endothelia (Lee et al., 2017). In this study, the goal was to investigate the components of the Notch signaling pathway involved. In earlier studies, we found essentially no detectable GLT-1 protein in rat astrocytes (Schlag et al., 1998) and low levels in mouse astrocytes (Ghosh et al., 2011). We reproduced this result (data not shown, n = 10) and decided to use rat astrocytes for the current study. As previously observed in mouse astrocytes (Lee et al., 2017), we found that co-culturing rat astrocytes on top of the brain endothelial cell line, bEND.3, for 7 to 10 days results in higher levels of GLT-1 and GLAST protein (Fig. 1A). In our earlier study, we also demonstrated that essentially all GLT-1 can be modified with a membrane impermeant biotinylation reagent, indicating that it is on the plasma membrane (Lee et al., 2017). In the present study, we only measured total transporter. In the co-cultures, total protein is near twice the observed in the monocultures. Therefore, the reported changes in GLT-1 or GLAST are an under-estimation of the increased levels of these transporters because the same amounts of total protein were loaded from each set of cultures. Continuous incubation with the γ -secretase inhibitor DAPT significantly attenuated the increase in GLT-1, but the levels of GLT-1 are still higher than in astrocyte monocultures (Fig. 1A). In these same samples, DAPT completely blocked endothelia-dependent induction of GLAST. We consistently found a shift in the migration of GLAST in co-cultures of astrocytes with endothelia. This suggests that in addition to regulating GLAST expression, endothelia also regulate post-translational modifications of GLAST.

We measured the levels of *Slc1a2* (GLT-1) and *Slc1a3* (GLAST) mRNAs in astrocytes, endothelia, astrocyte-endothelia co-cultures, and co-cultures treated with DAPT. We found that astrocyte-endothelia co-cultures have higher *Slc1a2* and *Slc1a3* levels than pure astrocyte cultures, and that treatment with DAPT completely blocks the effect of endothelia on both mRNAs (Fig. 1B).

There is some evidence that porcine brain endothelial cells express glutamate transporters (Cohen-Kashi-Malina et al., 2012), and it is possible that the observed increases were due to endothelial expression of these transporters. Therefore, we used specific antibodies to determine which cells express the transporters in these co-cultures. We used antibodies directed against CD31 (also known as platelet-endothelial cell adhesion molecule-1 or PECAM-1) or glial fibrillary acidic protein (GFAP) to label endothelia and astrocytes, respectively (Fig. 1 C&D). The expression of both GLT-1 and GLAST was restricted to GFAP⁺ cells. Furthermore, we observed higher levels of GLT-1 and GLAST immunoreactivity in astrocytes that were in close proximity to CD31⁺ cells (endothelia) (Fig. 1 C&D).

Using transwells and mouse astrocytes, we found that endothelia only induce GLT-1 expression when they are in contact (Lee et al., 2017). We also found that endothelia-conditioned media did not increase GLT-1 or GLAST protein when using mouse astrocytes (Lee/Robinson unpublished observation, n=3). Here, we found that bEND.3 conditioned media increases GLT-1 protein in rat astrocytes (Fig. 2A) but had no effect on GLAST protein (Fig. 2B). Together, these studies demonstrate that, as was observed in mouse astrocytes, endothelia cause an increase in GLT-1 and GLAST proteins in rat astrocytes. Unlike mouse astrocytes, DAPT only partially blocks the increase in GLT-1 protein. These results are also consistent with the hypothesis that endothelia regulate GLT-1 expression in rat astrocytes by two mechanisms. First, they increase GLT-1 mRNA and protein by a DAPT-sensitive, presumably Notch-dependent mechanism. Second, they also increase GLT-1 protein by a mechanism that is apparently independent of an increase in mRNA, consistent with a post-transcriptional mechanism. These results also show that endothelia increase GLAST mRNA and protein; these effects are both DAPT-sensitive.

Notch signaling is associated with activation of transcription factors that contain the basic helix-loop-helix (bHLH) domain, like Hes and Hey genes (Kamakura et al., 2004). To determine if endothelia activate Hes5 in astrocytes, we transduced astrocytes with Hes5 firefly and thymidine kinase renilla luciferase reporter constructs with lipofectamine. Two days later, endothelia were plated on top of these astrocytes. Transfection of the Notch Intracellular Domain (NICD) was used as a positive control of Hes5 activation. Under these conditions, endothelia cause a DAPT-sensitive increase in Hes5 activity (Fig. 3). It should be noted that although this experiment demonstrates that endothelia activates Notch and Hes5 in astrocytes, that this activation is completely block by DAPT, it does not prove that endothelia-dependent GLT-1 expression is due to direct interaction of Hes5 with the GLT-1 promoter.

3.2 Delta-like Notch ligands contribute to endothelia-dependent induction of GLT-1

In mammals, there are four different Notch ligands capable of Notch activation: delta-like 1 (Dll1), delta-like 4 (Dll4), jagged 1 (Jag1), and jagged 2 (Jag2). Transcriptomic analyses of freshly isolated mouse brain cells (Zhang et al., 2014) show that the mRNA of all four Notch ligands are found in endothelial cells (Fig 4). To determine which of these Notch ligands are required for endothelia-dependent induction of GLT-1 and GLAST, we used antibodies directed against Dll1, Dll4, Jag1, or Jag2; an IgG isotype was used as a negative control. Endothelia were pre-incubated with these antibodies for 1 h, prior to introduction of the astrocytes. In these experiments, the IgG negative control significantly decreased the endothelia-dependent increase in GLT-1; we hypothesize that this effect could be due to the sodium azide used as a preservative. In future studies, this can be tested by dialyzing the antibodies prior to their use. Anti-Dll1 was the only antibody that significantly decreased the endothelia-dependent induction of GLT-1 beyond the effect observed with IgG control (Fig. 5A). As observed with GLT-1, IgG control decreased the endothelia-dependent increase in GLAST, and none of the anti-Notch ligand antibodies further decreased the endothelia-dependent increase in GLAST (data not shown, n=7). Dll1 and Dll4 are frequently redundant, causing the same effect (Tveriaikhina et al., 2018), and in the brain, Dll4 is almost exclusively expressed by endothelial cells (Fig. 4). Therefore, we used lentiviral vectors that were designed to express shRNAs against Dll1 or Dll4. Two to three days after transduction of endothelia, astrocytes were introduced and these co-cultures were maintained for 7 to 10 days. With this approach we found that shRNAs directed against either Dll1 or Dll4 block the endothelia-dependent increase in GLT-1 protein (Fig 5B).

To determine if Notch ligands are sufficient to induce expression of GLT-1 or GLAST, astrocytes were seeded onto culture plates that were pre-coated with Fc-fusion recombinant Notch ligands Dll1, Dll4, Jag1, Jag2 or a Fc-control. We found that only Dll4 was sufficient to induce GLT-1 expression (Fig. 6A), while none of the Notch ligands increased GLAST levels in these same cultures (data not shown, n=5). Given that anti-Dll1 antibody or shRNA directed against Dll1 blocked the endothelia-dependent increase in GLT-1 (Fig. 5), we were concerned that recombinant Dll1 might not be active. To address this possibility, we tested these same batches of recombinant ligands Dll1 and Dll4 for Hes5 reporter activation; the effects of Dll1 and Dll4 were comparable to that observed for endothelia demonstrating that both were active (Fig. 6B). Together, these studies suggest that both Dll1 and Dll4 contribute to the endothelia-dependent increase in GLT-1. We did not evaluate effectiveness of recombinant Jag1 or Jag2. Therefore, we cannot rule out the possibility that these Notch might also cause an increase in GLT-1 expression.

3.3 Delta-like Notch ligands are expressed in endothelia and their expression is regulated by astrocytes

Dll1 expression has been reported in endothelial cells from spleen and bone marrow using genetic Dll1 reporter mice and immunostaining (Gamrekashvili et al., 2016); however, brain endothelia are transcriptionally distinct from endothelia from other tissues (Daneman et al., 2010). Transcriptomic data shown that *Dll1* mRNA levels in brain endothelia are low (Fig. 4) (Zhang et al., 2014). Therefore, we evaluated if bEND.3 cells express Dll1 and Dll4. Using polyclonal antibodies, we observed bands of the appropriate molecular weight for

both Dll1 and Dll4 in the endothelioma cell line bEND.3 (Fig. 7A). To confirm that their expression is not unique to this cell line, we evaluated the expression of Dll1 and Dll4 in lysates from primary cultures of rat brain endothelial cells (RBEC). As observed with bEND.3, we observed immunoreactive bands for Dll1 and Dll4 of the appropriate molecular weight (Fig. 7B). Using fluorescence immunocytochemistry, we detected immunoreactivity for Dll1 but not Dll4 in astrocytes (top rows of Fig. 8). Both ligands were also observed in bEND.3 endothelioma cells (middle rows of Fig. 8). As indicated in the methods, these images were analyzed using CD31 immunoreactivity to randomly choose fields for comparison of bEND.3 alone versus co-cultures of astrocytes with bEND.3. We found that the bEND.3 cells consistently express higher levels of Dll1 and Dll4 when they are co-cultured with astrocytes (bottom rows Fig 8).

When brain endothelia or peripheral endothelial cells are co-cultured with astrocytes or treated with astrocyte conditioned media, they undergo morphological changes, form tight junctions reminiscent of those observed *in vivo*, and increase expression of proteins associated with mature brain endothelia (Abbott, 2002; Alvarez et al., 2013; Hurst and Fritz, 1996; Prat et al., 2001; Sobue et al., 1999). Therefore, we evaluated if astrocyte-conditioned media was sufficient to induce expression of Dll1 and Dll4 in endothelia. Although there is a trend ($p=0.08$) astrocyte-conditioned media treatment do not alter significantly Dll1 expression in endothelia (Fig. 9A). However, we found that treating bEND.3 endothelia with astrocyte-conditioned media increases Dll4 as detected by Western blot or immunofluorescence (Fig. 9B). These results suggest a reciprocal communication between astrocytes and endothelia. In this reciprocal signaling, astrocytes secrete a signal(s) that induce expression of Dll4 in endothelia; endothelia then use this Notch ligands to increase expression of GLT-1 in astrocytes. Therefore, we tested the requirement of a reciprocal communication between endothelia and astrocytes for endothelia-dependent induction of GLT-1. We grew endothelial cells to confluency, then fixed them with ice cold methanol (MeOH), and let them air dry before plating astrocytes on top. It has been shown that fixation preserves cell membrane structure, but because it kills them, it prevents them from responding to feedback from astrocytes (Stogsdill et al., 2017). We found that only live endothelial cells induce expression of GLT-1 (Fig 10). This result provides additional evidence that astrocytes and endothelia are in continuous communication which allows them to regulate each others proteome.

4. Discussion

In the present study, we demonstrated that co-culturing astrocytes with endothelia increases GLT-1 and GLAST protein and mRNA levels. The γ -secretase inhibitor, DAPT, completely blocked the endothelia-dependent increase in GLT-1 mRNA, but only partially blocked the endothelia-dependent increase in GLT-1 protein. In these same cultures, DAPT completely blocked endothelia-dependent increase in GLAST mRNA and protein. We found that endothelia-conditioned media partially recapitulated the endothelia-dependent increase in GLT-1 protein, but had no effect on GLAST protein. Together, these results suggest that endothelia engage two mechanisms to regulate GLT-1 that partially overlap with the mechanism they use to regulate GLAST. We also found that endothelia activate Hes5, one of two major transcription factors that are activated by Notch (Andersson et al., 2011). By

Western blot and immunofluorescence cytochemistry, we demonstrated that brain endothelial cells express the Notch ligands, Dll1 and Dll4. Using neutralizing antibodies, shRNAs and recombinant proteins, we found evidence that both of these ligands contribute to the endothelia-dependent regulation of GLT-1. We also found that co-culturing endothelia with astrocytes or treating them with astrocyte-conditioned media increased endothelial expression of Dll4. We also found that methanol fixation of endothelia, a treatment that preserves the structural integrity but abolishes the ability of these cells to respond to signals, prevents them from inducing astrocytic expression of GLT-1. Together these results suggest that reciprocal communication between astrocytes and endothelia is required to maintain proper levels of GLT-1 and Delta-like Notch ligands in astrocytes and endothelia, respectively.

4.1 Endothelial cells regulate astrocyte maturation

It has been estimated that astrocyte endfeet cover almost the entire vasculature contacting pericytes or endothelial cells (Kacem et al., 1998; Mathiisen et al., 2010). With this anatomic interaction, it is not surprising that endothelia regulate different aspects of astrocyte biology and vice versa. Recently, Kubotera et al. used laser ablation to remove astrocyte endfeet from blood vessels. They found that astrocytes are capable of positioning other endfeet to surround these blood vessels, suggesting that an active mechanism is responsible for maintaining this astrocyte-blood vessel interaction (Kubotera et al., 2019). Several groups have demonstrated that endothelia regulate astrocyte morphology, differentiation and maturation by activation of LIF and BMP/Smad signaling pathways (Estrada et al., 1990; Imura et al., 2008; Mi et al., 2001; Sakimoto et al., 2012; Yoder, 2002; Zerlin and Goldman, 1997). Here we show that in addition to these pathways, endothelial cells present Delta-like Notch ligands Dll1 and Dll4 to astrocytes to increase expression of GLT-1. GLT-1 expression increases dramatically during synaptogenesis *in vivo* (Furuta et al., 1997; Shibata et al., 1996; Ullensvang et al., 1997); for reviews see (Danbolt, 2001; Sims and Robinson, 1999); this suggests that endothelia may contribute to induction of GLT-1 *in vivo* by a Notch-dependent mechanism.

In the present study, we demonstrated that endothelia regulate GLT-1 expression by two mechanisms: First, by increasing GLT-1 mRNA in a DAPT-sensitive mechanism. Second, by increasing GLT-1 protein by a mechanism that is insensitive to DAPT and not accompanied by an increase in GLT-1 mRNA. This suggests that endothelia may regulate GLT-1 expression by a transcription-independent mechanism. Although still relatively under-explored, there is evidence that several mRNAs, including *Aquaporin4*, *Kir4.1* and *Slc1a2* (GLT-1 mRNA), are trafficked to astrocyte endfeet where translation can be regulated (Boulay et al., 2017). There is also evidence that endothelia can signal to astrocytes to increase expression of aquaporin 4 (Camassa et al., 2015). In future studies, it will be interesting to learn if endothelia provide a signal that regulates local translation of GLT-1 and/or other mRNAs in astrocyte endfeet.

4.2 Brain development: Interaction of astrocytes with endothelia

In rodents, perivascular vessels invade the dorsal telencephalon by E11, concomitant with neurogenesis, growing in a dorsal to ventral gradient dictated by homeobox transcription

factors and vascular endothelial growth factor secreted by neural cells (Vasudevan et al., 2008; Zhao et al., 2015). A primitive blood brain barrier is formed by E15 (Zhao et al., 2015); however, this barrier is permeable (Semple et al., 2013; Stolp et al., 2005) and endothelia do not respond to brain activity with increases in blood flow (Iadecola, 2017). Between E16 and E18 the brain environment changes, favoring a switch from neurogenesis to gliogenesis (Schiweck et al., 2018); however, the first astrocytes are only detected after birth (Sloan and Barres, 2014). By the second and third postnatal weeks, synaptogenesis peaks (Chung et al., 2015; Lee and Chung, 2019) and astrocytes arrive at their final positions (Schiweck et al., 2018). As indicated above, this is associated with a large increase in GLT-1 expression in rodent and human (Bar-Peled et al., 1997; Furuta et al., 1997; Milton et al., 1997; Shibata et al., 1996; Ullensvang et al., 1997). Concomitantly, there is an increase in vascular density (Iadecola, 2017), the blood brain barrier becomes less permeable (Semple et al., 2013; Stolp et al., 2005), and increased neural activity becomes coupled to increases in blood flow (Iadecola, 2017). These studies suggest that astrocytes and endothelia ‘mature’ at about the same time. These mature brain endothelia have a different, narrower morphology and express higher levels of tight junction proteins, efflux transporters, and γ -glutamyl transpeptidase (Abbott, 2002). These properties are not observed in peripheral vasculature (Lee et al., 2019). When brain endothelia from adult animals are grown in culture, they lose some of these properties and switch to low resistance/high permeability. This loss of resistivity and increase in permeability can be prevented by co-culturing these endothelial cells with astrocytes (Rubin et al., 1991). Similarly, when peripheral endothelia are co-cultured with astrocytes, or treated with astrocyte conditioned media, the endothelia assimilate a brain endothelia-like phenotype (Abbott, 2002; Alvarez et al., 2013; Prat et al., 2001). There is also some evidence that astrocytes contribute to endothelial maturation *in vivo*. For example, reducing astroglialogenesis by genetically deleting astrocytic *Orc3*, a protein essential for DNA replication, reduces blood vessel density and the frequency of blood vessel branching (Ma et al., 2012). Together, these studies suggest that although astrocytes do not participate in blood vessel formation, they contribute to their maturation and the maintenance of the blood brain barrier.

It has been shown that deletion of even a single allele of *Dll4* is lethal due to vascular abnormalities (Gale et al., 2004). Inhibition or knock-down of *Dll4* in endothelia results in an increase in angiogenesis (Cristofaro et al., 2013; Leslie et al., 2007), excessive sprouting (Lobov et al., 2007; Pitulescu et al., 2017; Ubezio et al., 2016), and increased vascular permeability (Boardman et al., 2019). Here we have demonstrated for the first time that astrocytes increase expression of the Delta-like Notch ligand *Dll4* in endothelial cells. If they have this same effect *in vivo*, it suggests that astrocytes regulate Notch ligand *Dll4* in endothelia to arrest sprouting and induce maturation. Further studies are required to identify the signal(s) secreted by astrocytes that induce *Dll4* expression in endothelial cells.

There is some evidence that the interactions between astrocytes and endothelia are different in arterial and venous blood vessels. For example, arterial endothelial cells are highly enriched in Notch signaling while none of the Notch components are expressed in venous blood vessels (de la Paz and D’Amore, 2009). Astrocyte endfeet that wrap veins are thinner than those covering arteries and fail to regulate vasodilatation, suggesting that astrocyte endfeet in the arteries have unique properties (Takano et al., 2006). It is tempting to

speculate that astrocytic endfeet that contact arteries and those that contact veins might differentially secrete factors that control blood vessel properties.

4.3 Endothelia and Neurons use partially overlapping mechanisms to increase expression of GLT-1

We and others had shown that co-cultures of astrocytes with neurons have higher levels of GLT-1 and GLAST than astrocyte monocultures (Gegelashvili et al., 1997; Schlag et al., 1998; Swanson et al., 1997; Yang et al., 2009). The effect of neurons is partially reproduced by neuron conditioned media and is mimicked by activation of protein kinase A (Schlag et al., 1998; Swanson et al., 1997). In addition, part of the effect seems to be contact-dependent (Yang et al., 2009). In a recent study, the Hardingham group showed that neurons activate Notch in astrocytes as measured using a RBPJ-luciferase reporter. They also showed that the neuron-dependent increase in GLT-1 is blocked by DAPT (Hasel et al., 2017). This study suggests that neurons also engage a Notch signal to induce astrocyte maturation.

In the present study, we found that the Notch ligands Dll1 and Dll4 contribute to the effect of endothelia. Expression of Dll4 is essentially restricted to endothelial cells (Shutter et al., 2000) and has not been detected in neurons. Dll1 expression is required for maintenance of quiescence in neural stem cells (Kawaguchi et al., 2013) and directs neuroglia fate choice during development (Wiszniak and Schwarz, 2019); however, to the best of our knowledge, there are no reports of Dll1 expression in mature neurons. Therefore, either neurons and endothelia use different Notch ligands to regulate astrocyte maturation or Notch is required for induction of GLT-1 expression, but not for maintenance of GLT-1 expression. This could be addressed in future studies by knocking out expression of these ligands after synaptogenesis. Our current understanding of endothelia- and neuron-dependent regulation of GLT-1 expression comes from *in vitro* studies that lack the 3-dimensional structure observed in the intact brain. In vivo, astrocytes extend processes that cover non-overlapping domains with a ratio of 50 μ m in mice and can contact up to one hundred thousand synapses (Allen and Eroglu, 2017; Bushong et al., 2002). The density of blood vessels is very high in the intact nervous system and had been estimated that in average 16.80 astrocyte processes contact 100 μ m segment of capillary in the somatosensory cortex (McCaslin et al., 2011; Todorov et al., 2020), however, it is not clear if every astrocyte is in contact with blood vessels. Although unexplored at this time, it is possible that varying the proportions of astrocytic contacts with neurons and endothelia contributes to astrocyte heterogeneity.

4.4 Dll1 and Dll4: redundant versus distinct roles

Although Dll1 and Dll4 are 50% identical, they can have redundant or different functions depending on context (Nandagopal et al., 2018; Tveriakhina et al., 2018). In the present study, our observations implicate both Dll1 and Dll4 in endothelia-dependent regulation of GLT-1. We found that an anti-Dll1 neutralizing antibody blocked the effect of endothelia while an anti-Dll4 antibody had no effect. In contrast, shRNAs directed against Dll1 or Dll4 blocked the effect of endothelia. Although the anti-Dll4 antibody has been successfully used to block Dll4 signaling *in vivo* (Oishi et al., 2010), it is possible that the antibody was not active in our studies. If this is the case, these studies would suggest that both Dll1 and Dll4 are required for the effects of endothelia. Although both recombinant Dll1 or Dll4 activated

Hes5 in astrocytes, only Dll4 increased GLT-1 levels. Together these data suggest that both Dll1 and Dll4 are required for induction of GLT-1, but that only Dll4 is sufficient. The fact that Dll1 activates Hes5 to that same extent as that observed with Dll4 suggests that activation of Hes5 may not be sufficient downstream signal to induce GLT-1 transcription.

The activation of Notch downstream transcription factors is known to be complicated by two factors. First, Notch signaling is stoichiometric, does not include an amplification step, and is therefore very sensitive to small changes in the number of Notch ligands or receptors (Andersson et al., 2011). Second, Dll1 and Dll4 are known to differentially activate Hes and Hey. While Dll4 can activate both Hes and Hey, Dll1 preferentially activates the Hes gene family (Nandagopal et al., 2018). The simplest explanation of our data is that reducing Dll1 or Dll4 might reduce the concentration of Delta Notch ligands below a threshold required for Notch activation in astrocytes; in this scenario Dll1 and Dll4 would both be required to activate Notch sufficiently to induce GLT-1. In the present study, we did not evaluate Hey activation, and therefore we cannot rule out Hey participation in endothelia-dependent GLT-1 expression. We examined the *Slc1a2* (GLT-1) promoter for evolutionarily-conserved Hes transcription factor binding sites using the DCODE database as previously described (Ghosh et al., 2011). We and others have found that the proximal 2.5 kb of *Slc1a2* promoter contains evolutionarily-conserved domains that contribute to the regulation of GLT-1 expression (Martinez-Lozada et al., 2016; Su et al., 2003). Within this region we found three putative Hes binding sites, and two additional Hes binding sites in evolutionarily conserved domains in the distal (>2.5kb) region of the promoter. The DCODE database does not contain information about Hey binding sites, so they were not analyzed. In future studies, it will be interesting to determine if Hes and Hey binding sites in *Slc1a2* promoter are required for endothelia-dependent induction of GLT-1.

5. Conclusions

In summary, we have demonstrated that reciprocal communication between astrocytes and endothelia is required to maintain proper levels of the glutamate transporters GLT-1 and GLAST in astrocytes. Our current model indicates that astrocytes secrete an un-identified signal(s) to increase expression of the Delta-like Notch ligand Dll4 in endothelial cells, that endothelia, in turn, use this Notch ligand and Dll1 to activate Notch signaling in astrocytes, and that Notch activation in astrocytes increases GLT-1 and GLAST expression (see Fig 11). These results provide evidence that astrocytes and endothelial cells communicate with each other which allows them to regulate their proteome accordingly.

Acknowledgements:

This work was supported by NIH grant R01 NS092067 to M.B.R. Z.M-L was partially supported by AHA postdoctoral fellowship 17POST33670330. We would like to thank Dr. Judy Grinspan of the Preclinical Models Core of the Institutional Intellectual and Developmental Research Center at CHOP/Penn (U54 HD086984) for providing the A2B5 hybridoma supernatant and Dr. Mary Putt of the Biostatistics and Bioinformatics Core of the Institutional Intellectual and Developmental Research Center at CHOP/Penn (U54 HD086984) for assisting with the statistical analyses. We would also like to thank Elizabeth Krizman and Meredith Lee for their helpful suggestions and editing of this manuscript.

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- Endothelia increase astrocytic expression of GLT-1 by two mechanisms.
- Delta-like Notch ligands, Dll1 and Dll4, contribute to endothelia-dependent regulation of GLT-1.
- Astrocytes secrete a signal that increases expression of Dll1 and Dll4 in endothelia.
- Reciprocal communication is required to induce maturation of astrocytes and endothelia.

Figure 1. Effects of endothelia on astrocytic expression of the glutamate transporters GLT-1 and GLAST.

Rat cortical astrocytes were cultured alone (monocultures) or on top of a monolayer of bEND.3 cells in the presence or absence of the γ -secretase inhibitor, DAPT (10 μ M) for 7-10 days. A) The cultures were harvested for Western blot analysis. GLT-1, GLAST and β -actin were detected on the same membranes. Representative blots and summary of quantification of GLT-1 or GLAST normalized to β -actin are shown.

GLT-1 and GLAST were essentially undetectable in bEND.3 cultures. Data are the mean $\pm$ SEM of 13 independent experiments. ****p < 0.0001, indicate comparisons to astrocyte monocultures (control). #####p < 0.00001 for indicated comparisons. B) mRNA was reversed transcribed and qPCR was used to measure *Slc1a2* (GLT-1), *Slc1a3* (GLAST) and *Gapdh*

mRNAs. Summary of quantification of *Slc1a2* or *Slc1a3* normalized to *Gapdh* are shown. Data are the mean $\pm$ SEM of 4 independent experiments. ** $p < 0.01$ and **** $p < 0.0001$, indicate comparisons to astrocyte monocultures (control). #### $p < 0.0001$ for indicated comparisons. Distribution of GLT-1 (Panel C) or GLAST (Panel D) was examined in rat astrocytes, bEND.3 cells, and in co-cultures using confocal imaging. Anti-CD31 and anti-GFAP antibodies were used as markers of endothelial cells and astrocytes, respectively. Nuclei were counter-stained with 4-,6-diamidino-2-phenylindole (DAPI). No staining was observed when the primary antibodies were omitted (data not shown). The magnification is the same in the first four columns (scale bar 75 μ m). The fifth column is a 2X optical zoom of the yellow-outlined portion of the fourth column (scale bar 50 μ m). When astrocyte monocultures were analyzed, fields were chosen using the GFAP channel. In astrocyte-endothelia co-cultures fields were chosen using the CD31 channel to blind the analyzer. In no case, was GLT-1 or GLAST observed in CD31⁺ cells (bEND.3 cells) and both were consistently higher in astrocytes when they were near CD31⁺ cells. Data are representative of three independent experiments.

Figure 2. Effects of endothelia conditioned media on astrocytic expression of the glutamate transporters GLT-1 and GLAST.

Rat cortical astrocytes were cultured alone, on top of a monolayer of bEND.3 cells, or treated with endothelia conditioned media (bEND.3 Cond Media) for 7 to 10 days. The cultures were harvested for Western blot analysis. GLT-1, GLAST, and β -actin were detected on the same membranes. Representative blots and summary of quantification of GLT-1 or GLAST normalized to β -actin are shown. Data are the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared to astrocyte monocultures (control), ## $p < 0.01$ for indicated comparison.

Figure 3. Effect of endothelia on astrocytic Hes5 activity.

Rat cortical astrocytes were grown to 70-80% confluency and then transfected with a mixture of a Hes5 promoter firefly luciferase reporter and a thymidine kinase (Tk) promoter renilla luciferase reporter. Astrocytes transfected with a Notch intracellular domain construct (NICD) in addition to the luciferase reporters were used as a positive control. Astrocytes were maintained as monocultures or bEND.3 cells were added on top in the presence or absence of DAPT (10 μ M). Seven days later, cells were harvested for analysis of luciferase activities using dual-luciferase reporter assay. Data are the mean $\pm$ SEM of 6 independent

experiments. *** $p < 0.001$, **** $p < 0.0001$, compared to astrocyte monocultures (control); ## $p < 0.01$ for indicated comparison.

Figure 4. Expression of Notch ligands by glia, neurons and endothelia cells of the cerebral cortex. RNA-seq transcriptome data from the Barres group showing expression of the four Notch ligands capable of Notch activation, Dll1, Dll4, Jag1, and Jag2 in mouse astrocytes (Ast), neurons, oligodendrocyte precursor cells (OPC), newly formed oligodendrocytes (Immature oligos), myelinated oligodendrocytes, microglia, and endothelia cells (Zhang et al., 2014). FPKM stands for fragments per kilobase of transcript per million mapped reads. Endothelia is highlighted in red for clarity.

Figure 5. Effect of inhibition of Notch ligands on bEND.3-dependent induction of GLT-1.

A) Confluent bEND.3 cells were incubated with medium containing 5 μ g/mL of anti-Dll1, anti-Dll4, anti-Jag1, anti-Jag2, or an IgG of the same isotype for one hour prior to the introduction of astrocytes. The cultures were maintained for 7-10 days with complete media changes every 3-4 days. With every media change antibody was added at a final concentration of 5 μ g/mL. The γ -secretase inhibitor DAPT (10 μ M) was used as a positive control of Notch inhibition. GLT-1 and β -actin were detected on the same membranes. Representative blot and summary of quantification of GLT-1 normalized to β -actin are shown. This Western blot was cut to keep the layout of the figure similar to that shown in Fig 1. Data are the mean $\pm$ SEM of 6 independent experiments **** $p < 0.0001$ indicates comparison to astrocyte monocultures (control); \$\$ $p < 0.01$, ##### $p < 0.0001$ for indicated comparisons. B) bEND.3 cells were infected with lentiviral vectors that contained shRNA directed against Dll1, Dll4, or a scrambled shRNA control (shScr), 2-3 days after plating. The media was replaced with fresh media 24 hours later, and the next day astrocytes were layered on top of transduced bEND.3 cells. Cells were harvested 7-10 later and GLT-1 and β -actin were detected on the same membranes. Representative blots and summary of quantification of GLT-1 normalized to β -actin are shown. Data are the mean $\pm$ SEM of 5 independent experiments. *** $p < 0.001$, **** $p < 0.0001$ indicates comparison to astrocyte monocultures, ##### $p < 0.0001$ for indicated comparison, \$ $p < 0.05$ indicate comparisons to shScr.

Figure 6. Effects of recombinant-Notch ligands on astrocytic expression of the glutamate transporter GLT-1 and on the activity of Hes5.

Tissue culture plates were coated with 5µg/mL of recombinant Notch ligands fused at the C-terminus to the Fc portion of human IgG1; the Fc portion of the same isotype was used as a control (Rc-Fc). Astrocytes were layered on top of the recombinant Notch ligands or a confluent monolayer of bEND.3 cells and maintained for 7-10 days. A) GLT-1 and β -actin were detected on the same membranes. A representative blot (all lanes from the same blot) and summary of quantification of GLT-1 normalized to β -actin are shown. Data are the mean $\pm$ SEM of 6 independent experiments. **** $p < 0.0001$ indicates comparison to astrocyte monocultures, ## $p < 0.01$ indicates comparison to control Rc-Fc. B) Astrocytes were plated by themselves or on top of the recombinant Notch ligands. Two days later astrocytes were transfected with a mixture of a Hes5 promoter firefly luciferase reporter and a thymidine kinase promoter renilla luciferase reporter. Where indicated, bEND.3 cells were plated on top of astrocytes two days post-transfection. Cells were harvested after 7 to 10 days, and luciferase activities were analyzed using dual-luciferase reporter assay. Data are the mean $\pm$ SEM of 5 independent experiments. *** $p < 0.001$ indicates comparison to astrocyte monocultures, ## $p < 0.01$ indicate comparisons to control Rc-Fc.

Figure 7. Expression of Delta like Notch ligands in endothelia cells.

bEND.3 cells (panel A) or primary cultures of rat brain endothelia cells (RBEC) (panel B) were cultured for 7-10 days. The cultures were harvested for Western blot analysis. Dll1, Dll4 and β -actin were detected. Representative blots of 6 independent experiments for bEND.3 and of 2 independent experiments for RBEC are shown. Although several cross-reactive bands were detected with antibodies directed against Dll1 or Dll4, we detected a band that corresponds to the predicted molecular weight of Dll1 and Dll4 (74 and 78kDa respectively).

Figure 8. Effects of astrocytes on endothelial expression of Delta-like Notch ligands 1 and 4 (Dll1 and Dll4).

bEND.3 were cultured alone or underneath astrocytes. Expression of Dll1 (Panel A) or Dll4 (Panel B) was examined in rat astrocytes, bEND.3 cells, and co-cultures using confocal imaging. Anti-CD31 and anti-GFAP antibodies were used as markers of endothelial cells and astrocytes, respectively. Nuclei were counter-stained with DAPI. No staining was observed when the primary antibodies were omitted (data not shown). The magnification is the same in the first four columns (scale bar 75 μ m). The fifth column is a 2X optical zoom of the yellow-outlined portion of the fourth column (scale bar 50 μ m). When endothelia

monocultures and astrocyte-endothelia co-cultures were compared, fields were chosen using the CD31 channel to blind the analyzer. Dll1 and Dll4 immunoreactivity were consistently higher in CD31+ cells (bEND.3) when they were co-cultured with GFAP⁺ cells (astrocytes). Data are representative of 3 independent experiments.

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Figure 9. Effects of astrocyte conditioned media on expression of Dll1 and Dll4.

bEND.3 cells were cultured with or without astrocyte conditioned media (ACM). Cultures were harvested 7 to 10 days later and Dll1 (Panel A), Dll4 (Panel C) and β -actin were measured by Western blot. Representative blots and summary of quantification of Dll1 or Dll4 normalized to β -actin are shown. Data are the mean $\pm$ SEM of 5 independent experiments ** $p < 0.01$ indicates comparison to bEND.3 monocultures. Panel B and D: Expression of Dll1 (B) or Dll4 (D) was examined in bEND.3 cells with or without ACM treatment using confocal imaging. Anti-CD31 antibodies were used as marker of endothelial

cells. Nuclei were counter-stained with DAPI. No staining was observed when the primary antibodies were omitted (data not shown). The magnification is the same in the first three columns (scale bar 75 μm). The fourth column is a 2X optical zoom of the yellow-outlined portion of the third column (scale bar 50 μm). Fields were chosen using the CD31 channel to blind the analyzer. Dll4 immunoreactivity was consistently higher in CD31⁺ cells (bEND.3) when they were treated with astrocyte conditioned media. Data are representative of 3 independent experiments.

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Figure 10. Effects of methanol-fixed endothelia on astrocytic expression of the glutamate transporter GLT-1.

bEND.3 cells were grown to confluency, fixed with ice-cold methanol for 5 or 15 minutes, then air-dried before layering astrocytes on top. Astrocytes were grown in the presence of live or fixed endothelia for 7-10 days. The cultures were then harvested for Western blot analysis. GLT-1 and β -actin were detected on the same membranes. Representative blots and summary of quantification of GLT-1 normalized to β -actin are shown. Data are the mean $\pm$ SEM of 5 independent experiments. *** $p < 0.001$ indicates comparison to astrocyte monocultures.

Figure 11. Current Model: Reciprocal communication between astrocytes and endothelia is required for astrocytic glutamate transporter expression.

Astrocytes secrete an unidentified soluble signal to endothelia that triggers increased expression of the delta like Notch ligand Dll4. Endothelia then use Dll4, and Dll1, to activate Notch signaling in astrocytes. Notch activation results in increased levels of GLT-1 and GLAST mRNA and protein. In addition to Dll1 and Dll4, endothelia secrete an unidentified signal that increases GLT-1, with no effect on GLAST.