Supplemental Information

The synaptic cell adhesion molecule, SynCAM1, mediates astrocyte-to-astrocyte and astrocyte-to-GnRH neuron adhesiveness in the mouse hypothalamus

Ursula S. Sandau, Alison E. Mungenast, Jack McCarthy, Thomas Biederer, Gabriel Corfas, and Sergio R. Ojeda

Supplemental Material and Methods

Cell culture

Astrocytes were isolated from the hypothalamus of neonatal WT and transgenic mice, and cultured as previously described (1,2). After a growth period of 8-10 days in 75 cm^2 culture flasks containing DMEM/F12 medium supplemented with 10% donor calf serum (DCS; HyClone, Logan, UT), the astrocytes were isolated from contaminant cells by shaking at 250 rpm for 48-72 hours, prior to being replated for protein extraction, RNA extraction, cell adhesion or immunohistofluorescence experiments. GT1-7 cells (3) were cultured at 37°C in 5% CO_2 in DMEM containing 10% fetal calf serum (FCS) as described (4), and used for different experiments upon reaching 80-90% confluency.

Antibodies

Rabbit polyclonal antibodies against SynCAM were generated by Sigma-Genosys (St. Louis, MO), and were identical as those described by Biederer et al. (5), i.e. they were directed against the sequence CNNSEEKKEYFI corresponding to the C-terminal amino acids of SynCAM1 plus an N-terminal cysteine for coupling. Because these pleio-SynCAM antibodies recognize conserved C-terminal sequences in SynCAM1, 2 and 3 (6), we also used a SynCAM1-specific chicken monoclonal antibody raised against the extracellular domain of SynCAM1 (MBL Laboratories, Nagoya, Japan; clone 3E1). The

other antibodies used in these studies were: mouse monoclonal anti GFAP (Sigma), goat polyclonal anti EGFP antibodies (Abcam, Cambridge, MA), mouse monoclonal anti-GnRH HFU 4H3 (provided by Dr. Henryk Urbanski) (7), goat polyclonal anti-human IgG, Fc portion (Sigma-Aldrich, St Louis, MO), and mouse monoclonal antibody 5A5 anti-PSA (generously supplied by Dr. Urs Rutishauser, Memorial Sloan-Kettering Cancer Center, NY) (8).

Immunohistofluorescence

SynCAM staining was performed on GT1-7 cells and astrocytes cultured as described above, but plated on poly-L-lysine-coated coverslips. In both cases, the coverslips were placed into 6 well culture dishes. The cells were seeded at 5 x 10^4 cells/per coverslip and grown to 50-60% confluency in DMEM / 10% fetal calf serum (FCS) for GT1-7 cells and DMEM/F12 with 10% donor calf serum (DCS) for astrocytes. The cells were then washed in PBS, fixed with 95% methanol- 5% glacial acetic acid for 10 min at -20°C, rinsed in PBS, and stored in this buffer at 4°C until processed for immunohistofluorescence. SynCAM was identified with pleio-SynCAM antibodies (1:1,000 dilution) and the reaction was developed to a green color using biotinylated donkey antirabbit immunoglobulins (1:250, Jackson ImmunoResearch, West Grove, PA) followed by Alexa 488-conjugated Streptavidin (1:800, Molecular Probes-Invitrogen, San Diego, CA). GnRH was detected in GT1-7 cells with monoclonal antibody HFU 4H3 (7) diluted 1:3,000, and the reaction was developed with Alexa 594 chicken antimouse IgG (Molecular Probes-Invitrogen; 1:500). GFAP was identified in astrocytes with monoclonal antibodies (Sigma; 1:5,000) and the reaction was developed with Alexa-594 chicken antimouse gamma globulin (1:500, Jackson ImmunoResearch). For staining of brain tissue, we used brains derived from mice expressing EGFP in GnRH neurons (9). Tissue blocks including the POA and MBH were fixed in 95% methanol- 5% glacial acetic acid for 20 min at -20°C, cryoprotected in 20% sucrose-PBS overnight at 4^{o}C, and stored at -85^{o}C until sectioned. Fourteen μm cryostat sections were post-fixed in methanol-acetic acid for 20 min at -20^{o}C before the immunohistochemical procedure**.** GnRH neurons and their processes

were identified with goat polyclonal antibodies to EGFP (1:1000, Abcam, Cambridge, MA) and the immunoreaction was developed to a green color with Alexa 488 donkey antigoat gamma globulin. Astrocytes were stained with GFAP antibodies as outlined above. The SynCAM immunoreaction was developed to a red color with Streptavidin Alexa 568 (1:500). Fluorescent images were acquired with either a Leica TCS SP confocal microscope as described (2,10), or a Mariannas™ imaging workstation (Intelligent Imaging Innovations, Denver, CO). Slidebook 4.1 was used to construct a montage of large fields of view.

Protein Extraction

Protein lysates from cell cultures were obtained using a protocol to maximize membrane proteins (11). Briefly, cultured cells were grown to 80-90% confluency, rinsed with PBS, and snap-frozen on dry ice. Proteins were then extracted with a lysis buffer (25 mM Tris pH7.4, 150 mM NaCl, 1% Triton X100, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM 1 mM Na_3VO_4, 10 µg/ml Leupeptin, 10 µg/ml aprotinin, 100 µg/ml PMSF, and 1x HALT Phosphatase Inhibitor [Thermo Scientific, Rockford, IL]). The lysates were homogenized by passage through a 27g syringe 4-6 times and insoluble proteins were pelleted by centrifugation. Proteins were measured using a Bradford assay (BioRad, Hercules, CA).

SynCAM polysialylation

To determine if SynCAM in hypothalamic astrocytes is a target for polysialylation, immunoprecipitation assays with pleio-SynCAM antibodies were conducted. Upon reached 80-90% confluency, astrocytes were washed with cold PBS and the proteins were extracted as before. Cell lysates (1 mg) were incubated overnight at 4°C with 4 µg of the pleio-SynCAM antibody. Thereafter, the SynCAM-antibody complexes were incubated with a slurry of Protein A Sepharose (60 µl sepharose beads / 750 µl immunoreaction for 2 hours at 4°C). The sepharose beads were collected by centrifugation, washed twice with lysis buffer, resuspended in 2X SDS sample buffer (SDS, β-mercaptoethanol, bromophenol blue, glycerol), and boiled for 5 minutes. After boiling, the supernatants were subjected to

SDS-PAGE as described (2,12). Western blotting to detect polysialic acid (PSA) was performed using the mouse monoclonal antibody 5A5 (1:1,000) at 4°C overnight. The membrane was stripped (62.5 mM Tris HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol, 30 min at 60 4°C) and reprobed with pleio-SynCAM antibodies (1:1,000) to confirm successful SynCAM immunoprecipitation. The blots were developed as described above, and as reported (12,13).

RNA extraction and RT-PCR detection of SynCAM1 mRNAs

Cultured astrocytes and GT1-7 cells were rinsed with PBS and snap-frozen on dry ice before RNA extraction. Cells were homogenized (100 mg/ml) in TriReagent solution (MRC, Cincinnati, OH), and the aqueous and organic phases were separated by the addition of 0.1vol bromo-chloropropane (BCP, Sigma) followed by centrifugation at 4°C. RNA was precipitated from the aqueous phase with 1 volume of isopropanol followed by centrifugation at 13,000 rpm for 30 minutes at 4°C. RNA was resuspended in DEP-treated H2O and treated with Ambion's DNA-free DNAse I (2U per reaction, Ambion, Austin, TX) for 30 minutes at 37°C. RNA concentrations were determined spectrophotometrically, and 500 ng of RNA from each sample were run on denaturing agarose gels to confirm RNA integrity. The 4 known alternatively spliced mRNA species encoding membrane-spanning SynCAM1 isoforms were detected in hypothalamic astrocytes and GT1-7 cells using 500 ng of total RNA and random hexamer primers for reverse transcription. For the PCR reaction we used SynCAM1 primers (forward 5' cgggaaggggatgcatttgagtta 3' and reverse 5' tgctcatcattctgggccgctatt 3'; GenBank Accession number AF539424) , and the following conditions: an initial activation step of 5 min at 94°C, followed by 35 cycles of denaturing at 94°C for 30 sec, primer annealing at 52°C for 30 sec and PCR product extension at 72°C for 1 min; with a final extension at 72°C for 10 min. The samples were loaded in a 2.5% agarose gel and the PCR products were visualized by ethidium bromide staining. They were also cloned into the pGEM-T vector (Promega, Madison WI), sequenced on an ABI 3100 Genetic Analyzer DNA sequencer using M13 forward and reverse primers, and the sequences were analyzed using DNAStar software (DNASTAR, Inc. Madison, WI).

Cell adhesion to SynCAM1 substrates

These adhesion assays were performed as described (14). Round glass coverslips were cleaned overnight in concentrated nitric acid and washed in distilled water. Three small circles were drawn on each coverslip in different colors, coverslips were sterilized, and 3 µl of fusion protein (80ng/µl) was added to the circles. Coverslips were placed in a 24-well culture plate and allowed to dry for 4 hours at room temperature. They were rinse twice in PBS, and the adherence of the fusion proteins to the coverslips was confirmed by western blotting. To do this, the circles containing the fusion proteins were rinsed twice with 40 µl of PBS, and then 50 µl of 1X sample buffer was added and the coverslips were scraped. Ten microliters of 5X sample buffer were added to the two 40 µl PBS rinse samples. All three samples were boiled and subjected to SDS-PAGE followed by western blotting with the anti-human Fc antibody. The presence of fusion proteins in the scraped sample, but not in the two rinses, confirmed the adherence of these proteins to the glass coverslip (data not shown).

Fifty thousand GT1-7 cells or hypothalamic astrocytes (WT or GFAP-DNerbB4) were added to each well coated with fusion proteins and allowed to settle for 20 hours in serum-free medium. Cells were then stained with the fluorescent membrane dye DiO (1:200, Molecular Probes Invitrogen, San Diego, CA) for 20 minutes at 37°C, fixed with 4% paraformaldehyde PBS pH 7.4 for 20 minutes at room temperature, and stained with the fluorescent nuclear dye Hoechst (1:10,000, Molecular Probes) for 1 minute at room temperature. Coverslips were rinsed in PBS and mounted onto Superfrost microscope slides in aqueous media. Five frames per circle were captured using the 10xNA0.32 PlApo objective of a Zeiss Axioscope (Zeiss, Thornwood, NY) equipped with an Olympus DP 71 camera (Olympus America Inc., Center Valley, PA). Cells were counted using stereology counting rules (i.e. only cells completely included into the rectangle or touching the top and right borders were counted) with the ImageJ software package equipped with the cell-counter plugin (NIH) and averaged as number of cells / field. Cell adhesion to SynCAM1-Fc and ΔNRX-Fc fusion proteins were compared by plating the cells onto two

coverslips each containing one of the fusion proteins, along with a third coverslip containing purified culture medium proteins (CMP), i.e. proteins purified from culture medium of cells tranfected with a control plasmid (5). Total length of neurites was estimated by counting the intersections between DiO-labelled neurites with a grid of parallel and equidistant lines using Buffon's formula (32) L ¼ p/2 · (q · D), where q is the number of intersections and D is the distance between lines, arbitrarily chosen to be 72 lm. The average length of neurites per cell was calculated by dividing the total length by the number of cells.

Adhesion of GT1-7 neurons to hypothalamic astrocytes

SynCAM1-mediated adhesion between hypothalamic astrocytes and GT1-7 neurons was estimated by seeding enhanced green fluorescent protein (EGFP) - expressing GT1-7 cells onto a monolayer of astrocytes seeded on glass coverslips, supplementing the medium with either soluble SynCAM1-Fc or ΔECD-Fc. GT1-7 neurons expressing enhanced green fluorescent protein (EGFP) were generated by infecting the cells with a lentivirus (LV)-EGFP construct, as previously described (15,16). Briefly, the cells (passage 18) were exposed for 24 h to LV-EGFP at a ratio of 5 transducing units (TU) of virus per cell, in DMEM plus 10% FCS. The infected cells (EGFP-GT1-7) were then expanded and used at passage 22 for adhesion experiments. To conduct these experiments, 3 x 10^6 WT astrocytes were plated onto 12 mm poly-L-lysine coated glass coverslips placed into 12 well culture dishes. The cells were grown to 80-90% confluency in DMEM/F12 plus 10% DCS. At this time, they were overlaid with a suspension of EGFP-GT1-7 neurons (5 x 10^5 cells/ml) in 1 ml medium containing 5% DCS, 5% FCS, and either soluble SynCAM1-Fc or ΔECD-Fc (at 5 μg/ ml each). The co-cultures were incubated at 37°C for 18 hours, rinsed with cold PBS, and fixed for 20 min in 4% paraformaldehyde, PBS pH 7.4. After performing immunohistofluorescence for GFAP to visualize the astrocyte monolayer, the coverslips were mounted onto Superfrost microscope slides in aqueous media for imaging analysis. Six fields per specimen, uniformly spaced with a random start, were imaged as indicated above in the methods for Cell

adhesion to SynCAM1 substrates. The total number of EGFP-GT1-7 neurons per frame was manually counted using stereology counting rules (i.e. only cell completely included into the field or touching the top and right borders were counted). The average of the six frames was calculated for each coverslip with a total of five coverslips for each experimental group. The number of GT1-7 cells adhered per field in the presence of soluble SynCAM1-Fc was expressed as a percent change from the control (ΔECD-Fc) group.

References

1. **Ma YJ, Berg-von der Emde K, Moholt-Siebert M, Hill DF, Ojeda SR** 1994 Region-specific regulation of transforming growth factor α (TGFα) gene expression in astrocytes of the neuroendocrine brain. J Neurosci 14:5644-5651

2. **Ma YJ, Hill DF, Creswick KE, Costa ME, Ojeda SR** 1999 Neuregulins signaling via a glial erbB2/erbB4 receptor complex contribute to the neuroendocrine control of mammalian sexual development. J Neurosci 19:9913-9927

3. **Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI** 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5:1-10

4. **Voigt P, Ma YJ, Gonzalez D, Fahrenbach WH, Wetsel WC, Berg-von der Emde K, Hill DF, Taylor KG, Costa ME, Seidah NG, Ojeda SR** 1996 Neural and glial-mediated effects of growth factors acting via tyrosine kinase receptors on LHRH neurons. Endocrinology 137:2593-2605

5. **Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET, Südhof TC** 2002 SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science 297:1525-1531

6. **Fogel AI, Akins MR, Krupp AJ, Stagi M, Stein V, Biederer T** 2007 SynCAMs organize nascent synapses through heterophilic adhesion. J Neurosci 27:12516-12530

7. **Urbanski HF** 1991 Monoclonal antibodies to luteinizing hormone-releasing hormone: Production, characterization, and immunocytochemical application. Biol Reprod 44:681-686

8. **Acheson A, Sunshine JL, Rutishauser U** 1991 NCAM polysialic acid can regulate both cell-cell and cell-substrate interactions. J Cell Biol 114:143-153

9. **Suter KJ, Song WJ, Sampson TL, Wuarin J-P, Saunders JT, Dudek FE, Moenter SM** 2000 Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: Characterization of whole-cell electrophysiological properties and morphology. Endocrinology 141:412-419

10. **DeFazio RA, Heger S, Ojeda SR, Moenter SM** 2002 Activation of A-type γ-aminobutyric acid receptors excites gonadotropin-releasing hormone neurons. Mol Endocrinol 16:2872-2891

11. **Bowers G, Reardon D, Hewitt T, Dent P, Mikkelsen RB, Valerie K, Lammering G, Amir C, Schmidt-Ullrich RK** 2001 The relative role of ErbB1-4 receptor tyrosine kinases in radiation signal transduction responses of human carcinoma cells. Oncogene 20:1388-1397

12. **Rio C, Rieff HI, Qi P, Corfas G** 1997 Neuregulin and erbB receptors play a critical role in neuronal migration. Neuron 19:39-50

13. **Prevot V, Cornea A, Mungenast A, Smiley G, Ojeda SR** 2003 Activation of erbB-1 signaling in tanycytes of the median eminence stimulates transforming growth factor β_1 release via prostaglandin E2 production and induces cell plasticity. J Neuroscience 23:10622-10632

14. **Peles E, Nativ M, Campbell PL, Sakurai T, Martinez R, Lev S, Clary DO, Schilling J, Barnea G, Plowman GD, Grumet M, Schlessinger J** 1995 The carbonic anhydrase domain of receptor tyrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin. Cell 82:251-260

15. **Garcia-Rudaz C, Luna F, Tapia V, Kerr B, Colgin L, Galimi F, Dissen GA, Rawlings ND, Ojeda SR** 2007 Fxna, a novel gene differentially expressed in the rat ovary at the time of folliculogenesis, is required for normal ovarian histogenesis. Development 134:945-957

16. **Heger S, Mastronardi C, Dissen GA, Lomniczi A, Cabrera R, Roth CL, Jung H, Galimi F, Sippell W, Ojeda SR** 2007 Enhanced at puberty 1 (EAP1) is a new transcriptional regulator of the female neuroendocrine reproductive axis. J Clin Invest 117:2145-2154