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ORIGINAL ARTICLE

A Contactin–Receptor-Like Protein Tyrosine Phosphatase β Complex Mediates Adhesive Communication Between Astroglial Cells and Gonadotrophin-Releasing Hormone Neurones

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Although it is well established that gonadotrophin-releasing hormone (GnRH) neurones and astrocytes maintain an intimate contact throughout development and adult life, the cell-surface molecules that may contribute to this adhesiveness remain largely unknown. In the peripheral nervous system, the glycosylphosphatidyl inositol (GPI)-anchored protein contactin is a cell-surface neuronal protein required for axonal-glial adhesiveness. A glial transmembrane protein recognised by neuronal contactin is receptor-like protein tyrosine phosphatase β (RPTPβ), a phosphatase with structural similarities to cell adhesion molecules. In the present study, we show that contactin, and its preferred *in cis* partner Caspr1, are expressed in GnRH neurones. We also show that the RPTPβ mRNA predominantly expressed in hypothalamic astrocytes encodes an RPTPβ isoform (short RPTPβ) that uses its carbonic anhydrase (CAH) extracellular subdomain to interact with neuronal contactin. Immunoreactive contactin is most abundant in GnRH nerve terminals projecting to both the organum vasculosum of the lamina terminalis and median eminence, implying GnRH axons as an important site of contactin-dependent cell adhesiveness. GT1-7 immortalised GnRH neurones adhere to the CAH domain of RPTPβ, and this adhesiveness is blocked when contactin GPI anchoring is disrupted or contactin binding capacity is immunoneutralised, suggesting that astrocytic RPTPβ interacts with neuronal contactin to mediate glial–GnRH neurone adhesiveness. Because the abundance of short RPTPβ mRNA increases in the female mouse hypothalamus (but not in the cerebral cortex) before puberty, it appears that an increased interaction between GnRH axons and astrocytes mediated by RPTPβ–contactin is a dynamic mechanism of neurone–glia communication during female sexual development.

Key words: adhesion molecules, hypothalamus, GnRH neurones, astrocytes.

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Female sexual maturation and the initiation of reproductive cyclicity are events governed by a network of hypothalamic neurones secreting the neuropeptide gonadotrophin-releasing hormone (GnRH). In turn, the secretory activity of GnRH neurones is co-ordinately regulated by changes in transsynaptic inhibitory and excitatory inputs, as well as by astroglial influences involving growth factor-dependent pathways (1). Glial cells facilitate GnRH secretion by activating specific glia-to-glia and glia-to-neurone signalling pathways, and via plastic rearrangements (2, 3).

Although these changes in glial-neuronal architecture are reversible and subjected to a regulatory control exerted by gonadal steroids (3–7), little is known about the cell surface molecules mediating the adhesive interaction between glial cells and GnRH neurones. A recent study (8) demonstrated the presence of three

multigene families of adhesion/signalling molecules with complementary functions in both the prepubertal female monkey hypothalamus and the GnRH-secreting cell line GT1-7. In addition to a large family of synaptic specifiers (neurexins) and one of synaptic adhesion molecules (protocadherins), the developing hypothalamus also expresses three of the four known members of the contactin-associated protein (Caspr) gene family (8). Caspr1 mRNA is the family member most abundantly expressed in the hypothalamus of the monkey (8). Caspr1 is a transmembrane protein that binds *in cis* (i.e. via lateral interactions in the same neuronal plasma membrane) with the glycosylphosphatidyl inositol (GPI)-anchored axonal surface glycoprotein contactin (also known as F3 and F11) (9). The contactin–Caspr1 complex has been shown to recognise a glial membrane-associated protein known as receptor-like protein tyrosine phosphatase β (RPTPβ) (10) via binding of RPTPβ to contactin (11). This interaction results in neurone–glia adhesion, neurite growth, and bidirectional neurone–glia communication (12, 13). In the peripheral nervous system, the contactin–Caspr1 complex is targeted to the paranodal junctions of myelinated fibres via interaction with RPTPβ (14), a localisation that appears to be required for appropriate saltatory conduction.

RPTPβ expression is restricted to the nervous system, and preferentially localised to glial progenitor cells, radial glia and astrocytes (12, 15, 16). There are four isoforms of RPTPβ resulting from alternative splicing: two transmembrane receptor forms (one long and one short) and two secreted forms containing only the extracellular region of the receptor (17–19). These secreted forms are known as phosphacan and phosphacan short isoform, respectively. The long and short receptor forms differ by the presence of a stretch of 860 amino acids in the extracellular portion of the long form. This 860 amino acid insert, also present in phosphacan, contains glycosaminoglycan side chains, which appear to confer to these proteins inhibitory and/or repulsive effects on cell adhesion (20).

The extracellular domain of the short RPTPβ form consists of a region with sequence homology to the enzyme carbonic anhydrase (CAH) followed by a fibronectin type III repeat and a long cysteine-free spacer domain. This spacer domain is connected through the transmembrane region to two intracellular phosphatase domains (12). The CAH domain of the short RPTPβ form mediates glial adhesion to neurones expressing contactin (10).

Although formation of a contactin–RPTPβ adhesive complex may only be important for the function of myelinated axons, the abundance of RPTPβ (15, 21) and contactin (22, 23) throughout the brain implies that the complex may also be important for neurone–glia communication of a wider spectrum of neuronal populations in the central nervous system. Furthermore, the abundance of RPTPβ mRNA in the basal forebrain, and in particular in the periventricular region of the hypothalamus (21), along with the abundance of Caspr1, one of the contactin partners, in the hypothalamus of prepubertal female monkeys (8), suggests that the neuroendocrine brain may be an important site of RPTPβ/contactin-mediated neurone–astroglia communication. Our results identify GnRH neurones as a neuronal population of the hypothalamus that utilises this cell–cell communication complex for adherence to glial cells.

Materials and methods

Animals

For all experiments, except those involving single cell reverse transcription-polymerase chain reaction (scRT-PCR), we used mice of the FvB strain (FVB/NTAC, Taconic, Hudson, NY, USA). The animals were housed under a 14 : 10 h light/dark cycle (lights on 05.00 h) and temperature (23–25 °C) and were given *ad libitum* access to tap water and food. Animals younger than 21 days of age were housed with their mothers. For scRT-PCR experiments, we used transgenic female mice expressing green fluorescent protein (EGFP) under the control of the GnRH promoter (EGFP-GnRH) (24). The animals were maintained under controlled temperature and a 12 : 12 h light dark cycle (lights on 06.00 h) and given free access to food and water. They were group-housed until surgery at which time they were housed individually. Adult females were ovariectomised under ketamine/xylazine (ketamine, 1 mg/10 g; xylazine 0.2 mg/10 g i.p.) anaesthesia and implanted with an oil or oestrogen capsule for 6 days, at which time they were injected at 09.00 h with oil (100 μl) or 2 μg (in 100 μl) oestradiol benzoate (EB) as previously described (25). The animals were sacrificed at 09.00–10.00 h the following day. All animal procedures were performed in accordance with institutional guidelines based on National Institutes of Health standards, and were performed with institutional Animal Care and Use Committee approval at both the Oregon National Primate Research Centre (ONPRC) and Oregon Health and Science University.

Cell culture

Astrocytes were isolated from the hypothalamus of 1–2-day-old FvB mice and cultured as described previously (26, 27). After a growth period of 8–10 days in 75-cm culture flasks containing Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% calf serum, the astrocytes were isolated from oligodendrocytes and other contaminant cells by overnight shaking at 250 r.p.m. and were replated on either 15-cm dishes (for immunoblot analysis) or six-well plates (for RT-PCR experiments). After reaching 80–90% confluency, the medium was replaced with a serum free, astrocyte-defined medium consisting of DMEM devoid of phenol red, supplemented with 2 mM L-glutamine, 15 mM HEPES, 100 μM putrescine and 5 μg/ml insulin (27). The cells were used for the experiments 48 h later. Thereafter, the astrocytes were washed in phosphate-buffered saline (PBS) and frozen on dry ice before RNA or protein extraction.

The immortalised GnRH-secreting GT1-7 cells (28) (passage number 17–20) were cultured in DMEM medium supplemented with 10% foetal calf serum in six-well plates. After reaching 80% confluency, the cells were washed with PBS and frozen on dry ice before RNA extraction. When used for adhesion assays, the cells were plated at low density (10 000–50 000 cells/well, 12-well plates) onto coverslips coated with different adhesive substrates and examined 20 h later for attachment and neurite extension (see below).

RT-PCR

This procedure was used to detect RPTPβ, contactin and Caspr1 mRNAs in primary hypothalamic astrocytes and GT1-7 cells. Five-hundred ng of total RNA were reverse transcribed in a volume of 20 μl, using 4 U of Omniscript reverse transcriptase (Qiagen, Valencia, CA, USA), 20 U of RNase inhibitor (Promega, Madison, WI, USA) and 25 pmol of an oligo-dT (12–18) primer. After 1 h of incubation at 37 °C, the reaction was terminated by heating at 92 °C for 5 min. To amplify an RPTPβ DNA fragment, we first used primers targeting the intracellular domain-encoding region of the receptor. Accordingly, these primers recognise the mRNAs encoding both the long and the

short transmembrane RPTPβ forms (12). The sense primer (5′-AAGTGGGCCCATTTCCATGAC-3′) corresponded to nucleotides (nt) 4239–4259 in rat RPTPβ (NCBI accession no. U09357). The antisense primer (5′-TGCCTTGCCCTGACCCACTCT-3′) was complementary to nt 4739–4759. To detect the long RPTPβ mRNA form, we used a sense primer (5′-ACGGGTGTAACTAGGGTGGATA-3′) corresponding to a region in the intervening sequence (nt 4585–4606); the antisense primer (5′-TCTAAATAAAAGTGAGCCGTCTGG-3′) was complementary to a region in the intracellular domain (nt 5106–5129). The sense primer used to detect the short RPTPβ mRNA form (5′-ACTGGCTTGAATCCCGGTAGAGAC-3′) corresponds to nt 1426–1449 in the spacer region; the antisense primer (5′-TTGGAAAGATGGGTGTTGGTGGTG-3′) is complementary to nt 5159–5182 in the intracellular domain. In the absence of the RPTPβ mRNA intervening sequence (2560 nt), these primers amplify a 1198-bp DNA fragment. We used the reported rat sequence to design the above described primers because a complete mouse RPTPβ mRNA sequence was unavailable at the time. Subsequently, the predicted mouse sequence became available (XM_988353), allowing us to verify both the sequence of the primers and the identity of the amplified fragments. A fragment of cyclophilin mRNA, which is constitutively expressed in brain and other tissues, was simultaneously amplified to correct for procedural variability of the PCR reaction, using a sense primer (5′-CAAGACTGAATGGCTGGATGG-3′) corresponding to nt 392–412 in mouse cyclophilin mRNA (NM_008907), and an antisense primer (5′-ACTTGAAGGGGAATGAGGAAAATA-3′) complementary to nt 662–685.

To amplify a mouse contactin DNA fragment, we used a sense primer (5′-AACAATTTTGGTTACATA-3′) corresponding to nt 2467–2484 in mouse contactin mRNA (NM_007727) and an antisense primer (5′-GACTTCTATGGAGTGTTT-3′) complementary to nt 3130–3147. Caspr1 was amplified with a sense primer (5′-GTCTCTTTTCGTTTCCGCACC-3′) corresponding to nt 1297–1317 in mouse Caspr1 mRNA (NM_016782) and an antisense primer (5′-CAGCTCCATGCAGCCATGGAA-3′) complementary to nt 1651–1671.

Cell harvesting of EGFP-GnRH neurones and scRT-PCR

Diagonal band-preoptic area (POA) slices (300 μm) were cut on a vibratome and placed in an auxiliary chamber containing oxygenated artificial cerebrospinal fluid (aCSF). The slices are allowed to recover for 1–2 h in the chamber before dispersion. A discrete region of the diagonal band-rostral POA was microdissected and incubated in 10 ml aCSF (124 mM NaCl, 5 mM KCl, 2.6 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM Hepes, 10 mM D-glucose, in RNase-free water (Pyrogard-D filtered water, Millipore, Billerica, MA, USA), pH 7.3, 300 mOsm) containing 1 mg/ml protease for 17 min at 37 °C. The tissue was then washed four times in low calcium CSF (0.1 mM $CaCl_2$) and two times in aCSF. The cells were isolated by trituration with flame-polished Pasteur pipettes. The cells were dispersed onto a 35-mm glass bottom Petri dish (MatTek Corp., Ashland, MA, USA), and were visualised under a Nikon inverted microscope equipped with fluorescence illumination (Nikon Corp., Tokyo, Japan). The fluorescence cells and adjacent cells were patched and then harvested into the patch pipette by applying negative pressure. Samples of aCSF in the vicinity of the cells were also harvested. The contents of the pipette were expelled into a siliconised microcentrifuge tube containing 0.5 μl 10 × buffer (100 mM Tris-HCl, 500 mM KCl, 1% Triton-X), 15 U RNasin, 0.5 μl 100 mM DTT and diethylpyrocarbonate (DEPC)-treated water in a 5 μl volume. The harvested cells and aCSF samples were reverse-transcribed as described previously (29, 30). Briefly, the harvested cell solution and 25 ng of hypothalamic total RNA were denatured for 5 min at 65 °C, then cooled on ice for 5 min. Single-stranded cDNA was synthesised from cellular mRNA by adding 50 U MuLV reverse transcriptase, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 μM dNTPs, 15 U RNasin, 10 mM DTT and 100 ng random hexamers in a total of 15 μl DEPC-treated water (Ambion, Austin, TX, USA) for a final volume of 20 μl. Cells and tissue RNA used as negative controls were processed as described above but without reverse transcriptase. The reaction mixtures were incubated at 42 °C for 60 min, denatured at 99 °C for 5 min and cooled on ice for 5 min.

To amplify segments from contactin and Caspr1 mRNAs from EGFP-GnRH neurones, we used the following primers: contactin (261 bp product), sense primer (5′-GCGCCAGCCCATTTCCAGTTTAC-3′) corresponding to nt 452–474 in mouse contactin mRNA, and antisense primer (5′-CATGCCCTTCCCTTCCTTCACCTT-3′) complementary to nt 688–711; Caspr1 (104 bp product), sense primer (5′-GGGGCTCCTGACCTTTGTAGACCA-3′) corresponding to nt 2373–2396 in mouse Caspr1 mRNA, and an antisense primer (5′-AGCGCAGGGCCTCAGGAAGAACT-3′) complementary to nt 2453–2476. To confirm that the harvested fluorescent cells were indeed GnRH neurones, we PCR-amplified 239 bp GnRH cDNA from each cell, using a forward primer corresponding to nt 21–40 in GnRH mRNA (NM_008145) and an antisense primer complementary to nt 240–259.

PCR was performed using 3 μl of cDNA template from each RT reaction in a 30 μl PCR volume containing: 3 μl 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM dNTPs, 2 U Taq DNA polymerase (Promega), 0.22 μg TaqStart Antibody (Clontech, Oxford, UK) and 0.33 μM each of forward and reverse oligonucleotides. Taq DNA polymerase and TaqStart Antibody were combined and incubated at room temperature for 5 min then the remainder of the reaction contents was added to the tube. 50 cycles of amplification were performed using an MJ Research PTC-100 thermocycler (MJ Research, Inc., Waltham, Massachusetts, USA) in 0.5 ml thin walled PCR tubes according to one of the following protocols: 2 min at 94 °C; 50 cycles of 30 s at 94 °C; 1 min at 60 °C (GnRH) or 61 °C (contactin1, Caspr1); and 1 min at 72 °C, with a final 5-min extension at 72 °C. Ten μl of the PCR products were visualised with ethidium bromide on a 2% agarose gel. The scRT-PCR products for GnRH, contactin1 and caspr1 were confirmed by sequencing. In addition to the controls described above, aCSF harvested in the vicinity of the dispersed cells and water blanks were used as a control in the RT-PCR.

Western blots

Astrocytes cultures were lysed with 500 μl of freshly prepared lysis buffer (25 mM Tris, pH 7.4, 50 mM β-glycerophosphate, 1% Triton X-100, 1.5 mM ethyleneguaninetetraacetic acid, 0.5 mM ethylenediaminetetraacetic acid, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and pepstatin A, 10 μg/ml aprotinin, and 100 μg/ml phenylmethanesulphonyl fluoride). Protein concentrations were estimated using the Bradford method (Bio-Rad, Hercules, CA, USA). Samples were boiled for 5 min in sample buffer and then fractionated in an 8–16% precast SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA). After electrophoresis at 130 V for 2 h, the proteins were transferred for 4 h at 4 °C onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 3% nonfat milk for 1 h. A mouse monoclonal antibody against the C terminal region of RPTPβ (Becton Dickinson Transduction laboratories, Franklin Lakes, NJ, USA) was used at a 1 : 250 dilution (overnight at 4 °C) followed by an anti-mouse HRP antibody (1 h at room temperature, 1 : 1000; Invitrogen). The signal was developed by enhanced chemiluminescence using the Western lightning chemiluminescence substrate (PerkinElmer Life Sciences, Boston, MA, USA).

Fc fusion protein production and detection

A plasmid (pCDM8) encoding the CAH domain of RPTPβ fused to the hinge region of human immunoglobulin (Ig)G1-Fc (10) was used to determine the ability of GT1-7 cells to adhere to this domain *in vitro*. A plasmid (pCMV-VIgL-C) encoding amino acids 1–48 of rat neurexin 1α (including the signal peptide and 18 amino acids of the NH_2-terminal) fused to the hinge region

of human IgG1-Fc (IgC-Nrx) was used as a control (31). We refer to this construct as ΔNrx-Fc. Each plasmid (9 μg) was transiently transfected into COS-7 cells (4.5×10^6 cells/15 cm dish) using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Eight hours later, the culture medium was replaced with AIM-V serum-free medium (Invitrogen) and the cells were grown for 10 days. The medium was replaced every 2 days, supernatants were collected, spun down and a protease inhibitor cocktail (Sigma Chemicals, St Louis, MO, USA) was added before freezing for later purification. The secreted fusion proteins were purified using protein A-sepharose columns. Each column (Poly Prep Chromatography Columns, Bio-Rad) contained 1 ml of protein A slurry (Sigma). The fusion proteins were eluted from the column with three consecutive 0.5 ml washes of glycine 0.1 M, pH 2.8. The collected fractions were neutralised to pH 7.4 with 1/15 vol. of Hepes 1 M, pH 8, pooled and dialysed overnight against three changes of ten volumes of PBS. The proteins were then concentrated using an Amicon Ultra-15 column (Millipore), size-fractionated on a 10% Tris-glycine gel (Invitrogen), and their purity was evaluated by silver staining (Silver Snap, Pierce, Rockford, IL, USA), followed by western blotting. To perform this procedure, the sized-fractionated proteins were transferred to polyvinylidene difluoride membranes as indicated above. After a blocking step in 5% milk (1 h at room temperature), the Fc component of each protein was detected using goat polyclonal antibodies to human Fc coupled to horse radish peroxidase (1 : 1000, Sigma). A standard curve containing 32–500 ng of purified human IgG (Sigma) per lane was constructed to more precisely determine the amount of fusion protein to be used for coating. Films were digitalised using a Hewlett-Packard SC-Scanjet 8300 (Hewlett-Packard, Palo Alto, CA, USA) and densitometric analysis was performed using AlphaEaseFC software (Alpha Innotec Corporation, Randburg, South Africa).

Coating with fusion proteins

Round glass coverslips (Nalgene, Rochester, NY, USA) were treated overnight with concentrated nitric acid, washed for 2–3 h with distilled water, and coated with two different fusion proteins: CAH-Fc or ΔNrx-Fc. The adhesiveness of the fusion proteins to coverslips was enhanced by first coating the coverslips with protein A (MP Biomedicals Inc, Solon, OH, USA; 1 μg/ml, 1 h at room temperature). After washing with PBS buffer to remove excess of protein A, the coverslips were coated with the different fusion proteins for 4 h at room temperature. To test the efficiency of this procedure, some coverslips were coated with CAH-Fc (80 μg/ml) as indicated above, washed three times with PBS, collecting each wash and also scraping the adherent material into sample buffer. The CAH-Fc content of each fraction was then analysed for Fc content using the Fc antibody described above.

Quantification of cell adhesiveness and neurite outgrowth

To determine whether GT1-7 cells adhere to CAH, the functional adhesive unit of RPTPβ, and extend neurites following adhesion, the coverslips were coated with protein A, followed by CAH-Fc (80 μg/ml) or ΔNrx-Fc (80 μg/ml) as described above. Six coverslips were used for each condition. After rinsing the coverslips three times with PBS, they were placed in 12-well plates, and 50 000 GT1-7 cells (at passage 21) were plated onto each well. Twenty-hours later, the cells were stained with a highly lipophilic cell membrane-labelling fluorescent dye (DiO, Invitrogen) for 25 min at 37 °C, washed twice with normal medium for 20 min and fixed in 4% paraformaldehyde, PBS pH 7.4 (20 min at room temperature) before staining the cell nuclei with Hoechst 33258 (Invitrogen, 0.1 μg/ml for 1 min). Three fields per specimen, uniformly spaced with a random start, were imaged using a 10 × NA0.32 PlApo objective on a Zeiss Axioscope (Zeiss, Thornwood, NY, USA) with a Coolsnap CCD camera (Roper Scientific, Tucson, AZ, USA). Images were analysed using MetaMorph (Molecular Devices, Sunnyvale, CA, USA). Hoechst-stained nuclei were counted manually on rectangular regions (0.52 mm^2) using stereology counting rules (i.e. only cells completely included into the rectangle or touching the top and right borders were counted). 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) $L = \pi/2 \times (q \times D)$, where q is the number of intersections and D is the distance between lines, arbitrarily chosen to be 72 μm. The average length of neurites per cell was calculated by dividing the total length by the number of cells.

Treatment of GT1-7 cells with PI-phospholipase C (PI-PLC) before adhesion assay

Because contactin is a GPI-anchored cell-surface protein, it can be removed from the cell membrane by digesting the GPI anchor with PI-PLC. To obtain GT1-7 cells lacking contactin, GT1-7 cells were first cultured in a 75-ml flask and then were dissociated using an enzyme-free, dissociation buffer containing chelating agents in a Ca^{2+} and Mg^{2+}-free PBS (Invitrogen) to preserve the integrity of the cell membrane. Before plating, the cells were incubated in the absence or presence of PI-PLC (1 U/ml, Boehringer Mannheim, San Francisco, CA, USA) for 1 h at 37 °C. Thereafter, 10 000 cells were plated on coverslips coated with protein A alone or protein A/CAH-Fc. After 20 h, the unattached cells were removed by gentle wash and the adherent cells were stained with DiO, and fixed as described above. Cell adhesiveness and neurite outgrowth was quantified in each condition as outlined above.

Immunoneutralisation of contactin

Coverslips were coated with protein A/ΔNrx-Fc (control) or protein A/CAH-Fc as described above. To block contactin interactions with CAH-Fc, 5 μg/ml of anti-human goat polyclonal contactin antibodies (R&D Systems, Minneapolis, MN, USA) were added to the culture medium at the time of plating the GT1-7 cells. Control wells were incubated with 5 μg/ml of a purified goat IgG (R&D Systems) that has the same isotype as the anti-contactin IgG. Quantification of the number of cells adhered and the extent of neurite outgrowth were carried out as above after 20 h of culture.

Immunohistofluorescence-confocal microscopy

To detect the presence of contactin in GnRH neurones, we used 30-μm frozen sections obtained from the brain of 28–30-day-old female mice fixed by transcardiac perfusion of 4% paraformaldehyde- borate, pH 9.5, and prepared as described in detail elsewhere (33). The sections were incubated with the same goat polyclonal contactin antibodies used for immunoneutralisation (1 μg/ml) and mouse monoclonal antibodies against GnRH (1 : 3000) (34). The contactin reaction was developed with biotinylated donkey anti-goat immunoglobulin (1 : 250) followed by Alexa 488-Streptavidin (Invitrogen; 1 : 500); the GnRH reaction was developed with Alexa 594 chicken antimouse IgG (1 : 500). Cell nuclei were stained with Hoechst 33258, as indicated above.

Fluorescent images were acquired with either a Leica TCS SP confocal microscope (Leica Microsystems, Wetzlar, Germany) (27, 35), or a Marianas digital imaging workstation (Intelligent Imaging Innovations, Denver, CO, USA) with a ×40 C-apochromat NA1.2 objective. The Marianas workstation is equipped with a Zeiss Axiovert 200 M microscope and a motorised stage (API, Eugene, OR, USA). To define the colocalisation of GnRH and contactin immunoreactivity, GnRH axonal terminals of the organum vasculosum of the lamina terminalis (OVLT) and median eminence were imaged using a ×63, PlApo NA1.4 objective and a Coolsnap HQ (Roper Scientific, Tucson,

AZ, USA) camera. Two-colour, three-dimensional stacks of images 250 nm apart were deconvoluted using Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO, USA).

Combined immunohistochemistry/*in situ* hybridisation (IHC/ISH)

To determine whether short RPTPβ mRNA is expressed in astrocytes of the female mouse hypothalamus, we used a combined IHC/ISH procedure, described in detail previously (33, 36). Glial fibrilliary acidic protein (GFAP) was detected on floating 30-μm frozen sections using a monoclonal antibody (1 : 5000; Sigma), and developing the reaction with 3,3′-diaminobenzydine hydrochloride. After completing the immunohistochemical reaction, the sections were mounted on glass slides and dried overnight under vacuum prior to hybridisation with a mouse RPTPβ ^{35}S-UTP-labelled cRNA probe that specifically recognises the short form of RPTPβ mRNA (see below). All reagents used for the immunohistochemical procedure were prepared in DEPC-treated water. The hybridisation procedure was performed as recommended by Simmons *et al.* (37), and as described previously (38, 39). Control sections were incubated with a sense probe transcribed from the same plasmid, but linearised on the 3′ end to transcribe the coding strand of the cDNA template. Following an overnight hybridisation at 55 °C, the slides were washed and processed for cRNA detection (38, 39). After dehydration, the slides were dipped in NTB-2 emulsion, and were exposed to the emulsion for 3 weeks at 4 °C. At this time the slides were developed, counterstained with 0.1% methyl green, quickly dehydrated in ascending concentrations of alcohol, and coverslipped for microscopic examination.

The mouse RPTPβ cRNA probe used was prepared by *in vitro* transcription of a cDNA template generated by RT-PCR amplification of astrocytic total RNA. The forward primer (5′-ATGCCCCAGAGGATTCAG-3′) corresponds to nt 2015–36 in the rat RPTPβ sequence (NCBI accession no. U09357) and the reverse primer (5′-TACGAGACTYCATGGCTACTATT-3′) is complementary to nt 4960–4981. Because the short RPTPβ mRNA form lacks 2560 nt of intervening sequence, these primers generated a 408-bp fragment, which was cloned into the pGEM-T vector (Promega).

Real time-PCR

The changes in abundance of short RPTPβ mRNA that occur during prepubertal development in the POA, medial basal hypothalamus (MBH) and cerebral cortex (CTX) of female mice were measured by real-time PCR, as previously described (40, 41). Total RNA was extracted from the POA at postnatal (PN) days 0, 5, 10, 15, 20, 25 and 30, and from the MBH and CTX (PN days 10 and 30). The RNA was treated with DNAse (2 U, 30 min at 37 °C; Ambion) to remove contaminant DNA. After reverse transcribing 200 ng total RNA, aliquots of each reaction were diluted 1 : 25 before using 2 μl for real-time PCR. Each sample was run in triplicate along with a relative and an absolute standard curve. A relative standard curve was generated by diluting a pool of experimental samples 1 : 10 to 1 : 10000 times, and served to estimate the content of 18S ribosomal RNA of each sample. Absolute standard curves were constructed by using serial dilutions (1 : 10) of sense short RPTPβ mRNA (2 ng to 2 ag), transcribed from a 91-bp RPTPβ cDNA template described below, which was cloned into the plasmid pGEM-T (Promega). The threshold cycle number (C_T) from each sample was referred to this curve to estimate the corresponding RNA content, and each RNA value was then normalised for procedural losses by using the 18S ribosomal RNA values estimated from the relative standard curve. The C_T was the fractional cycle number at which the fluorescence accumulated to a level ten times one SD from basal values. The primers and fluorescent probe used to detect 18S ribosomal RNA were purchased as a kit (TaqMan ribosomal RNA control reagent kit, Perkin Elmer/Applied Biosystems). The primer sequences (Invitrogen) for short RPTPβ mRNA derived from the mouse sequence cloned above were: sense (5′-CTCCATTCGCAGACGACTCA-3′) corresponding to nt 2358–2378 in rat RPTPβ mRNA, and antisense (5′-CAGACT CCAACCCCTCAGCTA-3′) complementary to nt 4988–5008. Because the short RPTPβ mRNA form lacks 2560 nt of intervening sequence, these primers amplify a 91-bp DNA fragment. The internal fluorescent oligodeoxynucleotide probe (Applied Biosystems) had a sequence (5′-TACGAGACTCATG-GCTACTATTACTGGCCTCATTG-3′) and was covalently linked to the fluorescent dye, FAM, at the 5′-end, and to the quencher dye, TAMRA, at the 3′-end. The real-time PCR was performed in a 10 μl volume containing 10 nM of the 18S forward and reverse primers, 250 nM of the 18S Taqman probe, 300 nM of the short RPTPβ primers, 250 nM of the RTPPβ Taqman probe, 5 μl of the PCR master mix and 2 μl of reverse transcription. The real-time PCR programme consisted of an initial annealing period of 2 min at 50 °C followed by 10 min of denaturing at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Statistical analysis

The differences observed between different treatments or ages were analysed by one-way ANOVA followed by the Student–Neuman–Keuls multiple comparison test for unequal replications. Data are expressed as mean $\pm$ SEM. $P < 0.05$ was considered statistically significant.

Results

The mRNAs encoding RPTPβ, contactin and Caspr1 are differentially expressed in astrocytes and GnRH neurones

Detection of these mRNAs by RT-PCR showed that RPTPβ mRNA is abundant in cultured hypothalamic astrocytes but is not present in immortalised GnRH neurones. By contrast, contactin (the RPTPβ binding partner) and Caspr1 (a preferred contactin interacting protein) are only expressed in GT1-7 cells (Fig. 1A). Western blot analysis of proteins extracted from cultured hypothalamic astrocytes, using an antibody that binds specifically to the cytoplasmic domain of RPTPβ, showed a predominant species of approximately 220 kDa (Fig. 1B), which corresponds to the size of the short receptor isoform (16, 18).

PCR amplification with primers that specifically differentiate the long- from the short-RPTPβ form (Fig. 1C) demonstrated that, in agreement with the western blot results, the short form is the most abundant species expressed in hypothalamic astrocytes (Fig. 1D).

Hypothalamic astrocytes in situ contain abundant levels of short RPTPβ mRNA

Consistent with the abundance of short RPTPβ mRNA detected by PCR in cultured astrocytes, *in situ* hybridisation studies showed that hypothalamic astrocytes (identified in the intact hypothalamus by their content of immunoreactive GFAP) are rich in short RPTPβ mRNA (Fig. 2). Most GFAP-positive cells in the medial basal hypothalamus, including the median eminence (Fig. 2A), also appear to contain short RPTPβ mRNA transcripts (Fig. 2B). This localisation illustrated in a higher magnification view of the arcuate nucleus (Fig. 2C), is also apparent in other regions of the brain, as shown by

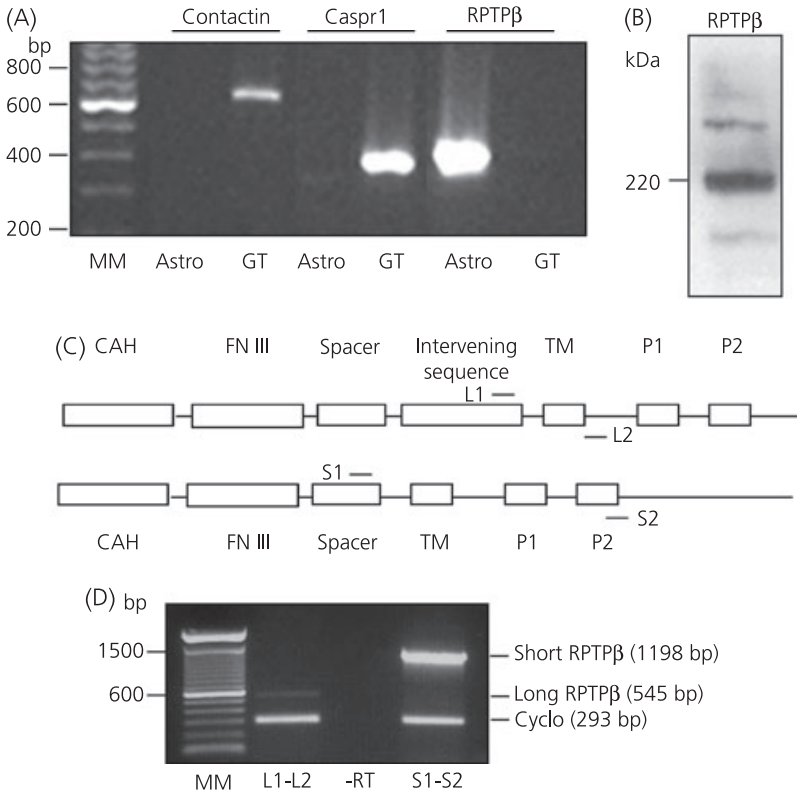

Fig. 1. The mRNAs encoding receptor-like protein tyrosine phosphatase β (RPTPβ), contactin, and Caspr1, a cell surface protein shown to be a preferred contactin partner, are differentially expressed in GT1-7 neurones and hypothalamic astrocytes. (A) Immortalised gonadotrophin-releasing hormone neurones, GT1-7 cells (GT), express contactin mRNA in addition to Caspr1 mRNA whereas hypothalamic astrocytes (astro) in primary culture express only RPTPβ mRNA. (B) Western blot showing that a ~220 kDa protein species corresponding to the short form of RPTPβ is the most abundant RPTPβ isoform expressed in cultured hypothalamic astrocytes. Membrane-bound RPTPβ protein isoforms were identified with an antibody that recognises the common C-terminal phosphatase domain of the receptor. (C) Schematic representation of the short and the long forms of RPTPβ resulting from alternative splicing of the primary RPTPβ mRNA transcript. Both forms share a common N-terminal sequence that contains the carbonic anhydrase (CAH), fibronectin type III (FN III) and spacer domains. The long receptor form contains in addition a long intervening sequence. Both receptors possess a transmembrane domain (TM) and two cytoplasmic tyrosine phosphatase domains (P1 and P2). L1 and L2 indicate the approximate position of the primers used to detect the mRNA encoding the long receptor form; S1 and S2 indicate the position of the primers identifying the short RPTPβ mRNA form. (D) The short (S1–S2) RPTPβ mRNA form is the most abundant RPTPβ mRNA species present in mouse hypothalamic astrocytes in primary culture. MM, 100 bp DNA ladder; cyclo, cyclophilin mRNA, a constitutively expressed mRNA used to control for reverse transcription-polymerase chain reaction procedural variability.

the abundance of RPTPβ mRNA in astrocytes of the piriform cortex (Fig. 2D).

Contactin is abundantly expressed in GnRH nerve terminals

To determine whether contactin is expressed in GnRH neurones of the postnatal female mouse hypothalamus we used double immunohistofluorescence-confocal microscopy. Contactin staining (green colour) was remarkably intense in the POA at the level of the OVLT (Fig. 3B,C). Immediately adjacent to the OVLT, there was extensive colocalisation with GnRH nerve terminals (red colour) (Fig. 3A–C). A single confocal plane of an optical 0.5 μm section illustrates this colocalisation (Fig. 3D, arrows; GnRH neuronal cell body denoted by arrowhead). A higher magnification image iteratively deconvoluted to more accurately visualise the nerve fibres reveal an abundance of contactin along GnRH nerve terminals reaching the OVLT (Fig. 3E). A rendering of confocal stacks of the median eminence deconvoluted using Volocity 3.6 software (Improvision Inc., Lexington, MA, USA) demonstrates contactin immunoreactivity in GnRH nerve terminals projecting to the external layer of the median eminence, where the axons reach the portal vasculature (Fig. 3F, arrows). Contactin does not appear to be present in GnRH perikarya (Fig. 3G, arrows), which instead are seen surrounded by contactin immunoreactive neuronal processes coursing in the immediate vicinity.

GnRH neurones in situ contain contactin and Caspr1 mRNAs

The above immunohistofluorescence studies suggest, but do not prove, that contactin is expressed in GnRH neurones. To determine whether this is indeed the case, we used transgenic mice in which GnRH neurones are tagged with EGFP. The neurones were identified by their EGFP fluorescence, and their cellular content was aspirated (as outlined in the Materials and Methods section) for detection of

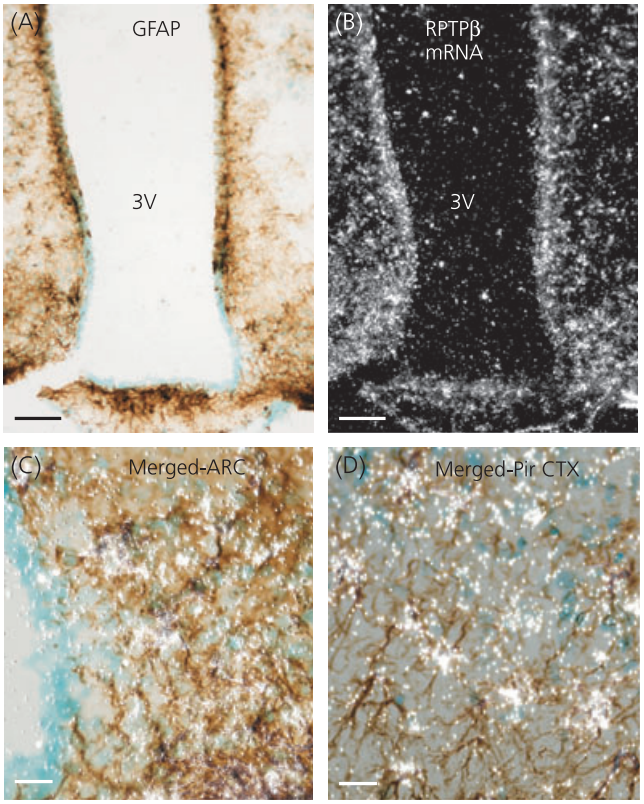

Fig. 2. Detection of short receptor-like protein tyrosine phosphatase β (RPTPβ) mRNA by *in situ* hybridisation in hypothalamic astrocytes identified by their content of immunoreactive glial fibrilliary acidic protein (GFAP). (A) Low magnification view of the medial basal hypothalamus depicting that GFAP-positive astrocytes (brown staining) are more abundant near the ventricular wall and the median eminence. (B) Same section depicted in (A), but showing the presence of short RPTPβ mRNA transcripts (white grains) in the same general areas occupied by astrocytes. (C) Higher magnification view illustrating the abundance of short RPTPβ mRNA transcripts (white grains) in astrocytes (brown staining) of the arcuate nucleus. (D) Astrocytes of the piriform cortex are also rich in short RPTPβ mRNA. (A,B) Scale bars = 100 μm; (C,D) scale bars = 20 μm. 3V, Third ventricle; ARC, arcuate nucleus; Pir, piriform cortex; CTX, cerebral cortex.

contactin mRNA, using the technique of scRT-PCR. We also performed scRT-PCR assays to determine whether GnRH neurones expressing contactin mRNA contain Caspr1 mRNA as well. To carry out these reactions, we used primers and PCR conditions able to detect each mRNA in as little as 1–10 fg of total RNA extracted from the MBH (Fig. 4A,B). The possibility of a major influence exerted by gonadal steroids on the expression of contactin and/or Caspr1 mRNA in GnRH neurones was considered by using ovariectomised and ovariectomised-EB treated mice.

All of the EGFP cells (n = 45) harvested from ovariectomised oil (n = 3) and EB-treated (n = 4) mice were found to express GnRH mRNA, whereas adjacent non-EGFP cells (n = 8) were negative. The GnRH-positive cells were further analysed for contactin and Caspr1. Overall, 66.7% of the GnRH-positive cells expressed contactin (n = 45), 58.8% expressed Caspr1 (n = 34) and 47.1% of GnRH neurones coexpressed contactin and Caspr1 (n = 34) (Fig. 4c). The analysis of 17 individual GnRH neurones from ovariectomised oil and 17 neurones from EB-treated mice revealed no obvious differences in the number of neurones expressing contactin and Caspr1 in the two groups of animals. However, a larger number of cells would be needed for quantification.

RPTPβ induces GT1-7 cells adhesiveness

Because the CAH domain of RPTPβ is required for the contactin-mediated adhesiveness of glial cells to neurones (10), we used a chimeric protein containing the CAH domain of RPTPβ fused to the hinge region of human IgG1-Fc (10). A fusion protein consisting of a truncated, adhesive-inert, form of rat neurexin 1α fused to the hinge region of human IgG1-Fc (31) and termed ΔNrx-Fc, was used as a control. Figure 5(A) shows a silver-stained gel demonstrating that the predominant species of each of these two proteins purified from the supernatant of COS-7 cells correspond in size to the protein species detected by western blots using antibodies against human Fc (Fig. 5B). Although the CAH-Fc preparation showed a contaminant protein of approximately 55 kDa (Fig. 5A), this protein was not recognised by the antibodies against human Fc (Fig. 5B). Different amounts of purified human IgG analysed by western blot yielded a linear relationship between the amount of IgG loaded/well and the resulting densitometric signal (Fig. 5C). Consequently, we used this curve to confirm the concentration of fusion protein to be used for the adhesion assays. During the initial characterisation of the adhesiveness test, it became evident that little fusion protein remained attached to the glass coverslips after coating. However, after precoating the coverslips with protein A, most of the fusion protein remained attached to the glass, with some protein being lost after a first wash, and very little to none lost after the second and third wash, respectively (Fig. 5D).

Using this system, we quantified the adhesiveness of GT1-7 cells to a CAH-Fc substrate, and the ability of the cells to extend neurites on this substrate. In addition to ΔNrx-Fc, we also used protein A as a control (see below). CAH significantly increased (more than three-fold, $P < 0.01$) the number of adherent GT1-7 cells per field in comparison to protein A (Fig. 6A). The average length of neurites was, however, similar in both groups (not shown). Because the CAH domain of RPTPβ has been shown to interact with contactin in a model of neurone-glia adhesiveness (10), we sought to determine whether GnRH neurones also utilise contactin to adhere RPTPβ-expressing astrocytes. When GT1-7 cells were preincubated with PI-PLC, which digests the GPI anchor dissociating GPI-attached proteins (like contactin) from the cell surface, GT1-7 cell adhesion to CAH-Fc was abolished (Fig. 6A), indicating that the attachment of GnRH neurones to CAH requires neuronal GPI-anchored proteins.

To determine whether contactin is a major GPI-anchored protein involved in this interaction, we seeded GT1-7 cells on the CAH-Fc substrate, supplementing the culture medium with neutralising antibodies to contactin. The culture medium of control cells was supplemented with a goat IgG isoform (gIgG) of the same isotype as that recognizing contactin. As shown in Fig. 6(B), the number of GT1-7 cells attached to CAH-Fc in the presence of gIgG was significantly ($P < 0.001$) greater than that of cells seeded on ΔNrx-Fc

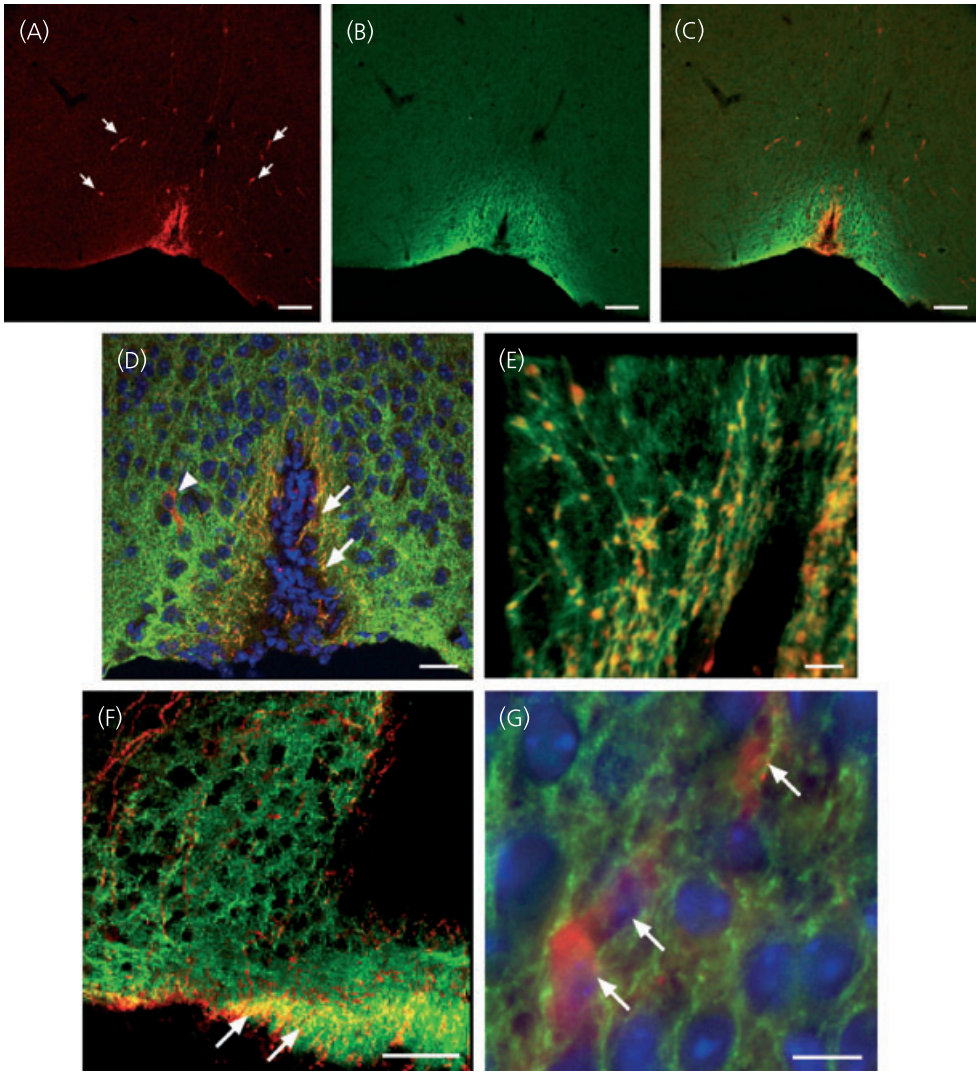

Fig. 3. Contactin immunoreactivity is abundant in gonadotrophin-releasing hormone neurone (GnRH) nerve terminals ending in both the organum vasculosum of the lamina terminalis (OVLT) and median eminence of the female mouse hypothalamus. (A) Projection of confocal stacks showing GnRH neurones (red, examples denoted by arrows) and GnRH nerve terminals (red) reaching the OVLT in the preoptic area (POA) region of juvenile, 28-day-old female mice. (B) Contactin (green) is abundant throughout the medio-ventral region of the POA. (C) Merged image showing colocalisation of GnRH and contactin (red–yellowish colour) in GnRH nerve terminals of the OVLT. (D) Single confocal plane showing the presence of contactin immunoreactivity (green colour) in GnRH terminals (red colour) reaching the OVLT (arrows). The arrowhead points to a GnRH cell body. (E) High magnification image of deconvoluted confocal stacks showing the presence of contactin along GnRH nerve terminals projecting to the OVLT. (F) Deconvoluted confocal stacks showing the presence of contactin in GnRH nerve endings (red) reaching the external layer of the median eminence. Colocalisation is seen as a yellowish colour (arrows). (G) Single confocal plane showing the apparent presence of contactin immunoreactivity in fibres surrounding GnRH neuronal perikarya, but not in the GnRH cell bodies themselves (arrows). Cell nuclei stained with Hoechst are seen in blue. (A–C) Scale bars = 100 μm; (D) scale bar = 20 μm; (E,G) scale bars = 10 μm; (F) scale bar = 50 μm.

substrate. By marked contrast, the adhesion of cells to CAH-Fc was obliterated by the contactin antibodies (Fig. 6B). The effectiveness of these antibodies to prevent the adhesion of GT1-7 cells to CAH-Fc indicates that the contaminant found in the CAH-Fc preparation is either irrelevant to cell attachment or is a CAH fragment dissociated from the fusion protein. These findings suggest that contactin is the main neuronal partner recognised by the CAH domain of glial RPTPβ to establish an adhesive relationship with GnRH neurones. Figure 6(C–E) illustrates the adhesion of GT1-7 cells to the control ΔNrx-Fc substrate (Fig. 6C), to CAH-Fc in the presence of control goat IgG (Fig. 6D), and to CAH in the presence of neutralisng antibodies to contactin (Fig. 6E). No differences in neurite extension were observed between the three groups, and this similarity is shown in the insets to Fig. 6(C–E).

Expression of the short RPTPβ mRNA form increases in the POA and MBH, but not the CTX of female mice during prepubertal development

To determine whether the expression of the short RPTPβ mRNA form changes during postnatal development, we measured short RPTPβ mRNA in the POA by real-time PCR at several intervals after

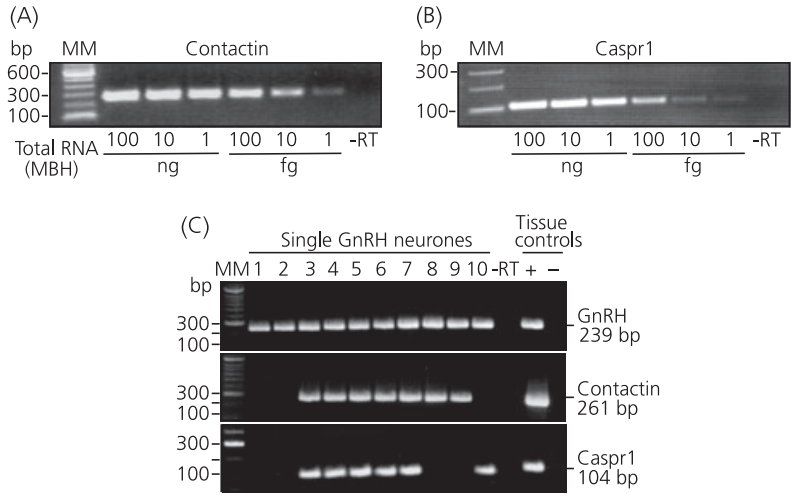

Fig. 4. Gonadotrophin-releasing hormone (GnRH) neurones *in situ* contain contactin and Caspr1 mRNAs. (A) Detection of contactin mRNA by reverse transcription-polymerase chain reaction (RT-PCR) using different amounts (1 fg to 100 ng) of total RNA extracted from the female mouse medial basal hypothalamus (MBH). (B) Detection of Caspr1 mRNA from the same RNA samples. (C) Single cell RT-PCR identification of contactin and Caspr1 transcripts in GnRH neurones. A representative gel illustrating that GnRH neurones express the mRNAs for contactin and Caspr1 (also confirmed by sequencing). As a negative control, a cell reacted without RT (–RT) did not express any of the transcripts. POA positive (+, with RT) and negative (–, without RT) tissue controls were also included. Additional controls included artificial cerebrospinal fluid from the dispersed cellular milieu and water blanks, all of which were negative after RT-PCR (data not shown). MM, Molecular markers.

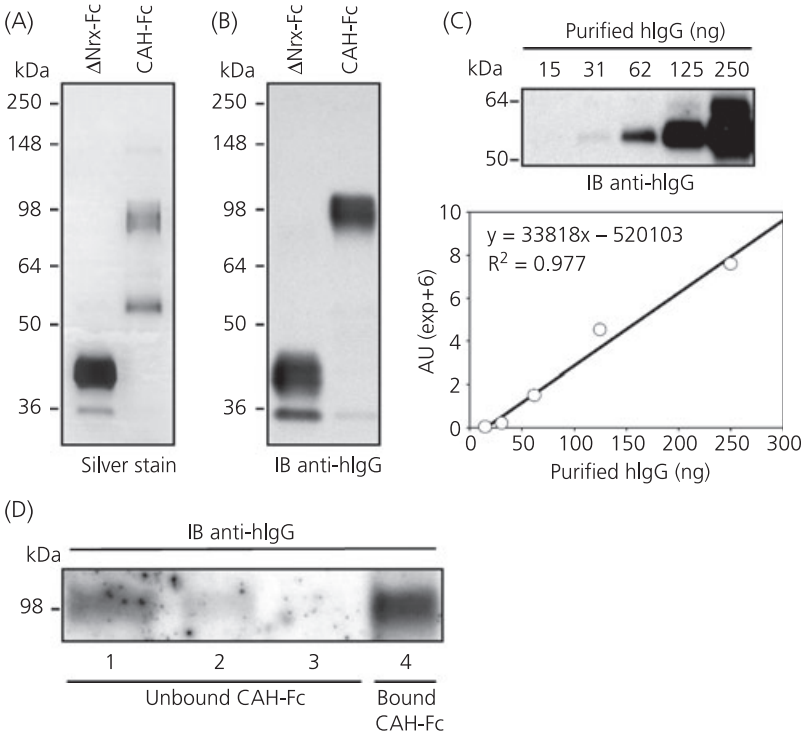

Fig. 5. Production of immunoglobulin (Ig)G fusion proteins in COS-7 cells. Fusion proteins containing the carbonic CAH-Fc domain of receptor-like protein tyrosine phosphatase β, or a mutated, adhesive-inert, extracellular domain of neurexin 1β used as a control protein fused to the human IgG1-Fc domain (ΔNrx-Fc) were purified, size-fractionated on SDS gels and immunoblotted with antibodies against human Fc. (A) Silver staining of the purified fusion proteins. (B) Western blot of the same proteins. (C) IgG standard curve generated by immunoblotting different amounts of purified human IgG with antihuman Fc antibodies. (D) CAH-Fc adhesiveness to glass coverslips. Coverslips were coated with protein A (1 μg/ml, 1 h at room temperature) before being coated with CAH-Fc (80 μg/ml). The coverslips were washed three times with phosphate-buffered saline (washes 1–3) and then scrapped into Laemmli sample buffer (bound CAH-Fc line 4). Each wash and the protein remaining on the coverslips after the washes were analysed for CAH-Fc content by western blot using the antihuman Fc antibody indicated above. IB, Immunoblot.

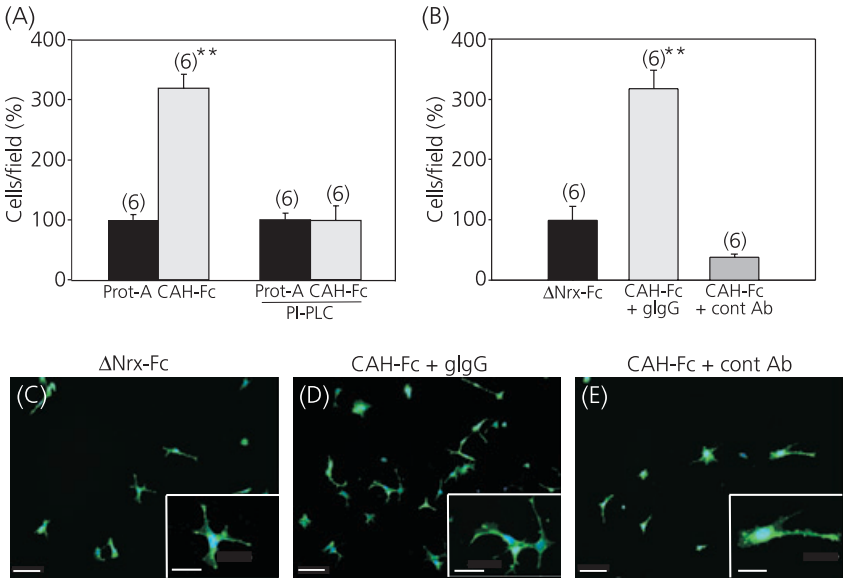

Fig. 6. GT1-7 cells adhere to a substrate containing the carbonic anhydrase (CAH) domain of receptor-like protein tyrosine phosphatase β (CAH-Fc), and this adhesiveness is blocked by either enzymatic removal of the glycosylphosphatidyl inositol (GPI) anchor or by immunoneutralisation of neuronal contactin. All measurements were carried out 20 h after plating. (A) Treatment of GT1-7 cells with phosphatidyl inositol-phospholipase C to dissociate GPI-anchored proteins from the cell surface, abolishes cell adhesion to the CAH-Fc substrate. The number of cells adhered per field is expressed as percent of cells adhered under control (Prot-A) conditions. (B) Immunoneutralisation of contactin with goat anti-contactin antibodies (Cont Ab, 5 μg/ml added to the culture medium at the time of plating) abolished GT1-7 adhesiveness to CAH-Fc. Control cells were exposed to a goat immunoglobulin (Ig)G (gIgG) of the same isotype as that recognizing contactin. The number of cells adhered per field is expressed as percent of the cell number detected when cells are plated onto the control substrate ΔNrx-Fc. **$P < 0.001$ versus control. Bars indicate the mean ± SEM and numbers on top of bars are number of coverslips analysed for each condition. Values from each coverslip derive from three fields examined per coverslip. (C) Low magnification image of GT1-7 cells grown on coverslips coated with the control substrate ΔNrx-Fc. (D) Cells seeded on CAH-Fc and exposed to control goat antibodies (gIgG). (E) Cells seeded on CAH-Fc and treated with contactin goat polyclonal antibodies (Cont-Ab). (Insets in C–E) higher magnification images showing that neurite length was not affected by adhesion of the cells to CAH-Fc or exposure to contactin antibodies. Nuclear Hoechst staining was used to identify adherent cells, and DiO staining to visualise neurites. Scale bars = 100 μm; scale bars in insets = 20 μm.

birth. Although short RPTPβ mRNA levels decreased during the first 10 days of postnatal life, they increase markedly ($P < 0.01$) thereafter, reaching maximum levels during the period preceding the onset of puberty in this strain of mice (days 25–30) (Fig. 7A). In our laboratory, FvB mice ovulate for the first time at 31 days of age (42).

To determine whether these changes also occur in the MBH, we performed a similar PCR analysis using tissues from 10- and 30-day-old female mice. These ages were selected because they more faithfully represent the postnatal changes in short RPTPβ mRNA detected in the POA. As seen in Fig. 7(B), the MBH exhibited changes in short RPTPβ mRNA similar to those detected in the POA. By contrast, no such changes were detected in the CTX (Fig. 7B), indicating that a prepubertal increase in short RPTPβ mRNA abundance is specific to the neuroendocrine brain.

Discussion

The present study identifies one of the cell–cell communication complexes mediating glial cell adhesiveness to GnRH neurones in the female mouse hypothalamus. Our results show that the astroglial transmembrane receptor protein RPTPβ recognises the cell surface axonal protein contactin via the CAH domain of the receptor, resulting in GnRH neuronal adhesiveness.

The results also show that contactin is abundant in GnRH nerve terminals reaching the OVLT in the POA and the median eminence, suggesting that these hypothalamic regions represent major sites of RPTPβ/contactin-mediated interactions between GnRH neurones and astrocytes. This conclusion is in keeping with earlier findings showing that glial apposition to GnRH neurones is more prevalent in GnRH axonal terminal fields (such as the OVLT and median eminence) than at the level of the neuronal perikarya (3, 7, 43–46).

Although the results obtained with GT1-7 cells need to be interpreted with caution, because these cells have some embryonic characteristics, and thus may not faithfully reflect the physiology of fully differentiated GnRH cells, the adhesiveness of GT1-7 cells to the CAH domain of RPTPβ gives rise to some reasonable interpretations. For example, RPTPβ may play a role in anchoring glial cells to GnRH neuronal axons, both along the trajectory of the axons to the OVLT and median eminence, the two main terminal fields of GnRH neurones, and within these regions themselves. This function is not only suggested by the abundance of RPTPβ mRNA we observed in the medial basal hypothalamus, but also by the high level of RPTPβ expression displayed by radial glial and glial cells along fibre tracks in the developing brain (15) and in regions of axonal sprouting following brain injury (21). The elevated RPTPβ mRNA levels observed in the hypothalamus shortly after birth reinforces this notion, because, at least in rats, this is a time when the

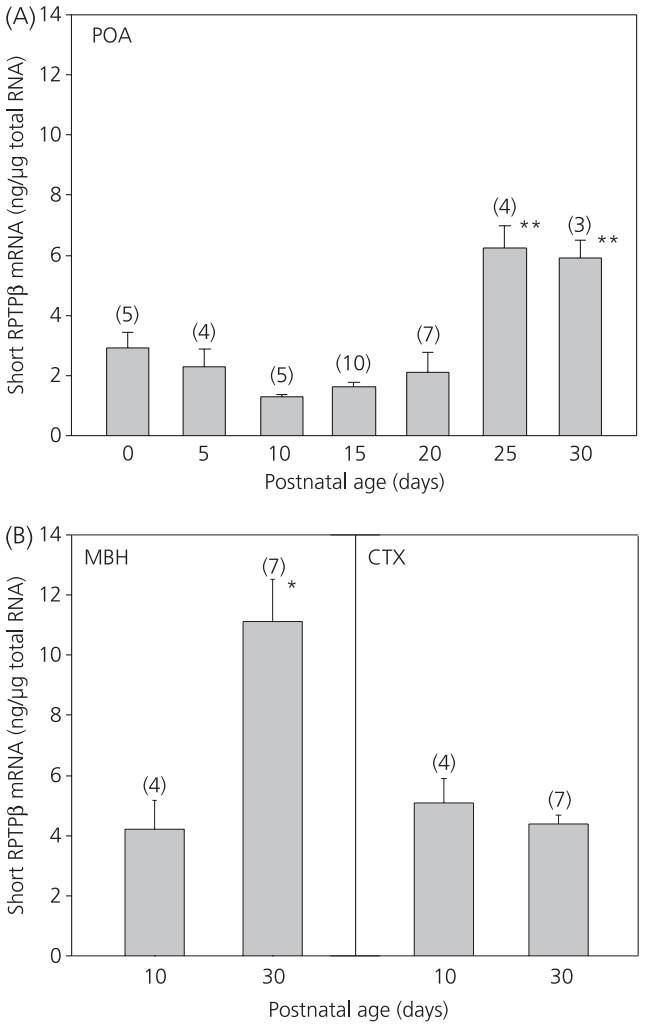

Fig. 7. Postnatal changes in short receptor-like protein tyrosine phosphatase β (RPTPβ) mRNA expression in (A) the female mouse preoptic area (POA), and (B) medial basal hypothalamus (MBH) and cerebral cortex (CTX). Short RPTPβ mRNA abundance was measured by real-time polymerase chain reaction. Bars are means and vertical bars are the SEM. Numbers on top of bars indicate number of mice per group. **P < 0.001 and *P < 0.01 versus values at postnatal day 10.

OVLT and median eminence become firmly innervated by GnRH axons (47–49).

Previous studies have shown that neuronal adhesiveness cannot be used to predict neurite growth (50), and have suggested that neurite outgrowth induced by cell adhesion molecules may result from the activation of a different set of specific signalling pathways (51). By showing that GT1-7 cell adhesiveness to the CAH domain of RPTPβ does not immediately facilitate neurite extension, the results of the present study are consistent with this view. Nevertheless, cell adhesion alone can activate bidirectional signalling pathways not necessarily related to neurite growth, depending on the adhesive proteins involved. In the case of contactin, this membrane-anchored protein can effect intracellular signalling on the neuronal side via interactions with associated proteins containing intracellular signalling motifs (10, 52, 53). Because most GnRH neurones expressing contactin mRNA also express the Caspr1 gene, our results suggest that Caspr1 is a major contactin partner in GnRH neurones. The cytoplasmic domain of Caspr1 contains a proline-rich sequence with a canonical SH3 domain that associates with at least four SH domain-containing proteins, including Src, Fyn, p85 and PLCγ (10, 54). The RPTPβ–contactin complex has also been shown to recruit in *cis* the cell adhesion molecule Nr-CAM to promote neuronal attachment (53). This system may also operate in GnRH neurones, especially in those cells that express contactin in the absence of Caspr1. Given the ability of cyclic AMP to increase GnRH release by activating voltage-gated Na^+ channels (55), it is interesting to note that contactin associates with the β_1 subunit of Na^+ channels to increase the channel function (22), and that this effect involves an interaction with RPTPβ (56).

On the glial side, RPTPβ has been shown to interact with cytoskeletal proteins involved in the regulation of cellular plasticity, including PSD95 (57) and β-catenin (58). MAGI-3, a PDZ domain-containing scaffolding protein localised in astrocytes to focal adhesion sites and regions of the cell membrane enriched in E-cadherin, has been shown to interact with the cytoplasmic domain of RPTPβ (59), suggesting that MAGI-3 is a scaffolding protein that links RPTPβ to its substrates at the astrocytic membrane.

The implications of these interactions for the understanding of astroglial–GnRH neuronal plasticity are vast because they may provide an understanding of the molecular mechanisms underlying the changes in glial apposition to GnRH nerve endings observed during the oestrous cycle and in response to gonadal steroids (44–46). The striking increase in RPTPβ mRNA levels observed during prepubertal development of the POA and MBH of female mice, and the regional selectivity of this change, raise the tantalising possibility that changes in astroglial–GnRH neurone plasticity determined by enhanced RPTPβ/contactin-dependent bidirectional signalling are intrinsic components of glial–neuronal communication underlying the initiation of female reproductive capacity. It is also likely that these interactions do not work in a vacuum but, instead, are functionally integrated to other molecular complexes involved in the adhesive communication of glial cells with GnRH neurones. One of such systems is the recently described SynCAM-dependent glia–neurone communication pathway, which mediates astroglial–GnRH neurone adhesiveness via homophilic SynCAM interactions (60).

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