Development 135, 1189-1199 (2008) doi:10.1242/dev.007401

Interaction of amyloid precursor protein with contactins and NgCAM in the retinotectal system

Miriam Osterfield, Rikke Egelund, Lauren M. Young and John G. Flanagan*

The amyloid precursor protein (APP) plays a central role in Alzheimer's disease, but its actions in normal development are not well understood. Here, a tagged APP ectodomain was used to identify extracellular binding partners in developing chick brain. Prominent binding sites were seen in the olfactory bulb and on retinal axons growing into the optic tectum. Co-precipitation from these tissues and tandem mass spectrometry led to the identification of two associated proteins: contactin 4 and NgCAM. In vitro binding studies revealed direct interactions among multiple members of the APP and contactin protein families. Levels of the APP processing fragment, CTFα, were modulated by both contactin 4 and NgCAM. In the developing retinotectal system, APP, contactin 4 and NgCAM are expressed in the retina and tectum in suitable locations to interact. Functional assays revealed regulatory effects of both APP and contactin 4 on NgCAM-dependent growth of cultured retinal axons, demonstrating specific functional interactions among these proteins. These studies identify novel binding and functional interactions among proteins of the APP, contactin and L1CAM families, with general implications for mechanisms of APP action in neural development and disease.

KEY WORDS: Amyloid precursor protein, Axon, Contactin, L1CAM, Retina

INTRODUCTION

Amyloid precursor protein (APP) is a transmembrane protein that plays a key role in Alzheimer's disease. This disease is characterized by intraneuronal tangles and extracellular plaques, and the amyloid beta-peptide (Aβ), which is derived from APP upon cleavage by β- and γ-secretases, is the major component of the plaques. Mutations in APP have been linked to familial Alzheimer's disease, and the most widely accepted models of disease etiology propose that Aβ aggregates or oligomers trigger a cascade of events causing damage to neuronal connections and cell death (Selkoe, 1999; Hardy and Selkoe, 2002; Tanzi and Bertram, 2005; Catalano et al., 2006).

The functions of APP in normal physiology and development are not well understood. APP-deficient mice are viable and fertile, but have some abnormalities, including susceptibility to seizures (Steinbach et al., 1998) and a defect in corpus callosum formation (Magara et al., 1999), indicating roles in neural development and function. Triple knockout mice for APP and its two homologs, APLP1 (amyloid precursor like protein 1) and APLP2, exhibit a cortical defect reminiscent of human type 2 lissencephaly, suggesting a role in neuronal migration, and die perinatally, although the exact reasons for this remain mysterious (Herms et al., 2004).

Since its initial identification, APP was thought likely to be a receptor, based on its transmembrane structure (Kang et al., 1987). By analogy to Notch (Mumm and Kopan, 2000), an APP signaling pathway has been proposed where α-secretase cleavage yields the C-terminal CTFα fragment, then γ-cleavage liberates the APP intracellular domain to participate in downstream pathways (Chan and Jan, 1998). Some evidence has accumulated to support this model of signaling (Cao and Sudhof, 2001; Kimberly et al., 2001; Kinoshita et al., 2002), although some aspects remain controversial (Cao and Sudhof, 2004; Hass and Yankner, 2005; Hebert et al., 2006). Several cytoplasmic binding partners for APP have been identified, delineating potential downstream pathways (Kerr and Small, 2005). However, the key signaling mechanisms remain unclear, especially as it is not clear which, if any, of the identified extracellular binding partners for APP might function as a physiological ligand.

In addition to the potential receptor function of APP, its cleaved ectodomain, APPsα, may function as a ligand. Numerous studies have shown that APPsα can modulate cell behaviors including neurite outgrowth, synaptogenesis, neurogenesis and cell survival and proliferation (Mattson, 1997; Turner et al., 2003; Caille et al., 2004). Distinct domains of APPsα have been implicated in these actions, suggesting the existence of more than one receptor. To date, however, no cell-surface receptor capable of mediating APPsα-induced signaling has been identified.

More than a decade of work has led to the identification of a number of extracellular partners that can interact with APP, directly or indirectly. Binding has been reported for extracellular matrix components, including heparan sulfate (Multhaup, 1994; Small et al., 1994), collagen (Beher et al., 1996) and fibulin 1 (Ohsawa et al., 2001); zinc and copper ions (Turner et al., 2003); and the lipoprotein receptors, scavenger receptor A (Santiago-Garcia et al., 2001) and LRP (Kounnas et al., 1995). More recently identified extracellular proteins that can interact with APP include F-spondin (also known as spondin 1) (Ho and Sudhof, 2004; Hoe et al., 2005), *Drosophila* FASII (Ashley et al., 2005), BRI2 (ITM2B) (Fotinopoulou et al., 2005; Matsuda et al., 2005), APLP1, APLP2 and APP itself (Soba et al., 2005), Notch family members (Fassa et al., 2005; Fischer et al., 2005; Oh et al., 2005; Chen et al., 2006), LRRTM3 (Majercak et al., 2006) and NgR (RTN4R) (Park et al., 2006). Some of these proteins can influence candidate downstream signaling pathways or APP processing. However, these interactions have generally not yet been characterized thoroughly with regard to whether they have affinity and specificity in the range of cognate receptor-ligand interactions, involve direct interaction with APP, and whether they can affect cell behavior. There is also generally little evidence regarding potential roles for these interactions in vertebrate neural development.

Department of Cell Biology and Program in Neuroscience, Harvard Medical School, Boston, MA 02115, USA.

*Author for correspondence (e-mail: flanagan@hms.harvard.edu)

Accepted 9 January 2008

Contactins, which are GPI-anchored, and L1CAM family proteins, which are transmembrane proteins, are members of the IgCAM (immunoglobulin-related cell adhesion molecule) superfamily. Contactins and L1CAMs can bind one another, as well as partially overlapping sets of other cell-surface or extracellular-matrix proteins (Sonderegger, 1997). L1CAM and its chicken homolog NgCAM have been widely studied for functions in axon growth, guidance and fasciculation (Hortsch, 1996; Sonderegger, 1997; Maness and Schachner, 2007), as have contactins 1 and 2 (Falk et al., 2002). Contactins 3 (PANG/BIG-1) and 4 (BIG-2) are less well characterized, although both can promote neurite outgrowth (Connelly et al., 1994; Yoshihara et al., 1994; Yoshihara et al., 1995). Like APP, contactin 4 and L1CAM are implicated in neurological disorders. Contactin 4 gene disruption is proposed to cause 3p deletion syndrome, involving mental retardation (Fernandez et al., 2004; Dijkhuizen et al., 2006). Mutations in L1CAM cause CRASH syndrome, which includes mental retardation and corpus callosum hypoplasia (Fransen et al., 1995).

Here, we took the approach of initially testing embryonic chick brain for APP-binding sites, and found particularly prominent binding on retinal axons growing into the optic tectum, a well-characterized model of axon development (McLaughlin and O'Leary, 2005; Flanagan, 2006). We next identified extracellular binding partners and found APP to have a direct high affinity interaction with contactins 3 and 4. APP also associates, directly or indirectly, with tectal NgCAM. APP, contactins and NgCAM are all expressed in the retinotectal system. In functional assays of cultured retinal ganglion cells (RGCs), contactin 4 and APP modulated axon behavior specifically in the context of NgCAM-dependent axon growth, demonstrating functional interactions among these proteins. Our studies of binding, expression and functional effects on cell behavior identify novel interactions of APP, with general implications for development and disease.

MATERIALS AND METHODS

DNA constructs

AP-APPsα contains amino acids 18-612 of APP695; human in Fig. 4, mouse elsewhere. AP-APLP1 encodes residues 34-567 of human APLP1 (NM_005166). N-terminal alkaline phosphatase (AP) fusion constructs were in APtag4 (Flanagan et al., 2000). APP-HA contains full-length human APP695, then an HA tag, in pcDNA3.1 Zeo+. A start codon was placed upstream of APP amino acid 597 for CTFβ, and 613 for CTFα.

NgCAM-Fc encodes residues 1-1134 from pSCT-NgCAM, kindly provided by P. Sonderegger (Buchstaller et al., 1996), in pSecTagIg. Fusions of all six human contactins were full length up to the GPI anchor site, followed by Fc, in IgTag2Eco. Accession numbers and predicted GPI sites are: contactin 1 (NM_001843, 993); 2 (NM_005076, 1012); 3 (NM_020872, 1002); 4 (NM_175607, 1000); 5 (NM_014361, 1072); 6 (NM_014461, 999). Deletion constructs encode the following regions in pSecTagIg: c3Ig1-4 (20-404), c3Ig5-6 (405-597), c3FN (598-1005), c4Ig1-4 (19-404), c4Ig5-6 (405-595), c4FN (596-1000). Contactin 4-AP contains residues 1-1000, then C-terminal AP, in APtag2. Chick contactin 4-myc-His contains residues 1-1005 in pcDNA3.1/myc-His.

Chick in situ probes in pBluescript II SK(–) contain ORF nucleotides: APP (AF289218), 471-1218; NgCAM (Z75013), 2827-3759; contactin 3 (NM_414433), 2465-3277; contactin 4 (XM_414435), 418-1212.

Immunolocalization, RNA and affinity probe in situs

AP fusion proteins were used as in situ probes as described (Flanagan et al., 2000). In situ hybridization used 10-μm sections of E11.5 chick with detection by AP-coupled anti-digoxigenin (Roche) or, for fluorescent in situs, peroxidase-conjugated anti-digoxigenin antibody (Roche) then Alexa Fluor 488 with tyramide amplification (Molecular Probes). Immunolocalization used rabbit anti-GAP43 (Novus Biologicals), or goat anti-APP (44-63) (Calbiochem), then fluorescent secondary antibodies (Molecular Probes).

AP and Fc fusion proteins

Fusion proteins were produced in transiently transfected 293T cells (Flanagan et al., 2000). For axon growth experiments, fusion proteins were produced in Opti-MEM plus ITS-A (Invitrogen), then AP fusions were concentrated 10-fold using Amicon Centricon (Millipore), or Fc fusions were purified with Protein A-Sepharose beads (4 Fast Flow, Amersham).

Protein purifications from brain

For purification of APP-binding proteins, tecta from E12.5 chick embryos (approximately 100 for small-scale experiments, 1200 for large-scale) were treated as follows, at room temperature unless indicated: Hank's buffered salt solution (HBSS) wash; 0.2 mg/ml EZ-link NHS-LC-Biotin (Pierce) in HH (HBSS with 20 mM HEPES, pH 7.0), 45 minutes; 50 mM Tris pH 7.5 in HH, 20 minutes; HBAH (HBSS with 0.5 mg/ml BSA, 0.05% sodium azide and 20 mM HEPES, pH 7.0) wash; AP fusion-containing conditioned media, at least 90 minutes; ice-cold HBAH, six washes; ice-cold HBSS, six washes; 0.5 mg/ml DTSSP (Pierce) in HH, 45 minutes; 50 mM Tris pH 7.5 in HH, 20 minutes; Lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-114 and 0.05% SDS in PBS) plus protease inhibitors (5 mM EDTA plus cOmplete, Roche), 45 minutes, ice. Supernatant was cleared 100,000 ***g***, 4°C, then incubated overnight at 4°C with anti-AP (MIA1801, Seradyn) beads as described (Flanagan et al., 2000). Beads were washed with ice-cold Lysis buffer, then Wash buffer (0.1% Triton X-100 in PBS), eluted with 100 mM glycine, 150 mM NaCl, pH 3.0, then buffered with 100 mM Tris pH 8.0. For SDS-PAGE, proteins were precipitated with 10% TCA. For 2D gels, sample was boiled 15 minutes in 100 mM DTT, then incubated with 9.25 mg/ml iodoacetamide, 15 minutes, before TCA precipitation.

For purification of tectal surface proteins, 800 E12.5 chick tecta were incubated in HBSS (one wash); 1 mg/ml Sulfo-NHS-SS-Biotin (Pierce) in HH, 1 hour; 50 mM Tris pH 7.5 in HH, 20 minutes; then HBSS (several washes). Tectal lysis and clearing were as above, followed by overnight incubation at 4°C with NeutrAvidin beads (Pierce). Beads were washed with the following buffers: Lysis, Wash, High salt wash (1 M NaCl, 20 mM HEPES pH 7.0 and 0.1% Triton X-100), then Wash. The biotinylation reagent was cleaved in 100 mM DTT (65°C, 40 minutes), then protein was TCA precipitated.

The ZOOM IPGRunner system (Invitrogen) was used for 2D gels, using pH 3-10 strips and 4-20% Tris-glycine gels. Sample buffer was from the 2D Insoluble Protein Sample Prep Kit (Pierce) with carrier ampholytes (Invitrogen), 20 mM DTT and 0.02 mg/ml bromophenol blue. Silver staining was as described (Shevchenko et al., 1996). For western blots, biotin was detected with horseradish peroxidase-conjugated NeutrAvidin (Pierce).

Antibodies to test candidate tectal proteins were anti-PSA-NCAM (Amersham), anti-chick NCAM-1 (Amersham), anti-tenascin (Chemicon), anti-neogenin (R&D systems), anti-contactin 1 (BD Biosciences) and three chick NgCAM antibodies: mouse monoclonal H23, kindly provided by J. Sanes (Yamagata et al., 1995) and mouse monoclonal mAb 12-I-4E-311 and rabbit polyclonal rb 4003, both kindly provided by F. Rathjen (Chang et al., 1990).

Binding and APP processing assays

Binding assays using fusion proteins were as described (Flanagan and Cheng, 2000). AP fusions were normalized for AP activity. Fc fusions were quantitated by anti-Fc western with Li-COR Odyssey scanner. For Fig. 5A, relative concentration of Fc fusion proteins, as compared with contactin 3 FN-Fc, were: c3Ig1-4-Fc, 2.7; c3Ig5-6-Fc, 0.2; c3FN-Fc, 1; c4Ig1-4-Fc, 0.1; c4Ig5-6-Fc, 5.5; c4FN-Fc, 1.7. For Fig. 4A, Fc concentrations were normalized.

For APP processing assays, 293T cells were transfected using TransIT-LT1 (Mirus Bio). After 1-2 days, plates were ice chilled, 5 minutes; lysed in 1% Triton X-100, 10 mM Tris pH 8.0, plus 140 mM NaCl, 20 minutes; and cleared lysates were combined with sample buffer. Western blots were probed with anti-HA (monoclonal 3F10, Roche), followed by HRP- or IRdye800-conjugated anti-rat antibody (Rockland).

RNAi experiments

Control virus contains EGFP-F (Clontech) in RCASBP(B) replication-competent retrovirus. For contactin 4 shRNA virus, pGSU6-GFP (Genlantis) was used according to manufacturer's instructions with target sequence

CTACGAGTGTGTCGCTGAA (Reynolds et al., 2004), and the U6 promoter and shRNA sequence were inserted into the control virus downstream of EGFP-F. Because we do not have an antibody to chick contactin 4, knockdown was tested on tagged chick contactin 4-myc-His, co-transfected with contactin 5-Fc control, APtag5 and shRNA or control RCASBP(B) plasmid into 293T cells. Supernatants, normalized for transfection by AP activity, were analyzed by western blot with IRdye800-conjugated anti-myc and IRdye700DX-conjugated anti-human Fc. The shRNA construct, compared with control virus, was confirmed to specifically knockdown protein levels of contactin 4 ($P<0.0001$), and not the contactin 5 internal control (see Fig. S1D in the supplementary material). For outgrowth assays, stage 9-11 chick embryos were electroporated with RCASBP(B) plasmids as described (Schulte et al., 1999). Green fluorescence was used to select highly infected (approximately 50-95%) retinas.

RGC axon growth assays

Stage 27-28 chick retinas were mounted on polycarbonate filter (Sartorius), cut into 300 μm strips, and cultured RGC-side down for 2 days in 47.6% Neurobasal media, 37.5% DMEM/F12, 4.8% FBS, 2.4% chick serum, 1% B27, 0.15% methyl cellulose, 0.14% glucose, 20 mM HEPES pH 7.0, Pen-Strep and 1.76 μg/ml glutamate. Supernatant from one homogenized stage 27-28 chick brain was added per 5 ml media.

Glass coverslips were first coated with 20 μg/ml poly-L-lysine. Where multiple purified proteins (Fc fusions, 20 μg/ml; or laminin, 10 μg /ml, BD Biosciences) were used, they were mixed to coat coverslips simultaneously. For purified proteins plus a supernatant (AP fusion or mock), coating was first with purified proteins, then supernatants. Axons were fluorescently labeled in 33 μM carboxyfluorescein diacetate, succinimidyl ester (Invitrogen), 10 minutes. Since basal growth varied between experiments, most figures show a single experiment, with similar trends being observed in multiple experiments, except Fig. 7K which shows three experiments normalized to the average growth of control axons on NgCAM+APPsα.

RESULTS

Localization of two distinct APP-binding partners in embryonic chick brain

To search for novel extracellular binding partners for APP, we generated an alkaline phosphatase (AP) fusion protein, AP-APPsα, for use as an affinity probe (Flanagan and Cheng, 2000). We found that AP-APPsα binds to embryonic chick brain, with particularly prominent binding to the optic tectum (Fig. 1B). Binding was also seen in the olfactory bulb, as well as other regions (Fig. 1B). Within the tectum, the most prominent AP-APPsα binding appeared to be localized to RGC axons. The staining spread across the tectum with a developmental time course similar to RGC axon ingrowth (Goldberg, 1974), appearing at the anterior end by Hamburger-Hamilton (HH) stage 33 (E8.5 under our conditions), with some labeled axons reaching the posterior tectum by HH 38 (E12.5), and covering the tectum by HH 41 (E15.5) (Fig. 1D-F and data not shown). The staining also had a prominently striated appearance that is characteristic of axons (Fig. 1C). Furthermore, in monocular chick embryos (one naturally occurring, two surgically enucleated), AP-APPsα binding was strongly reduced in the tectal hemisphere contralateral to (and therefore normally innervated by) the missing eye (Fig. 1G).

Two lines of evidence suggested that AP-APPsα was detecting more than one binding partner. First, a nested deletion analysis of APP identified two regions that produced different binding patterns (Fig. 2A). A middle domain (amino acids 199-293) was sufficient for weak binding to RGC axons in the tectum (Fig. 2C), whereas a somewhat longer region (amino acids 199-345) gave stronger RGC binding comparable to full-length AP-APPsα (Fig. 2B; binding pattern 1; green bar in Fig. 2A), but both gave little binding above background to the olfactory bulb (Fig. 2B,C). By contrast, an N-terminal domain of APP (amino acids 18-205) showed particularly strong binding to the olfactory bulb, in addition to widespread binding above background throughout the brain, including the tectum (Fig. 2F; binding pattern 2; blue bar in 2A). Second, treatment of brains with PI-PLC (phosphatidylinositol-specific phospholipase C), which cleaves GPI anchorages, greatly reduced AP-APPsα binding to the olfactory bulb, whereas binding to tecta appeared less affected (Fig. 2D,E), suggesting that the predominant APP binding partner detected in olfactory bulbs is GPI-anchored, whereas the tectum appears to contain a GPI-anchored binding partner and additional non-GPI-anchored partner(s).

The APLP1 ectodomain, fused to an AP tag, was also tested for binding to embryonic chick brain. Binding was widespread, but particularly prominent in the olfactory bulb (Fig. 2G), similar to that observed for the APP N-terminal domain, suggesting that the N-terminal domains of APP and APLP1 might share binding partners (consistent with our subsequent molecular experiments).

APP binds specific contactins

We next developed a protocol to selectively purify extracellular binding partners for APP from sites in developing brain with prominent APP binding. Tecta were surface biotinylated, then incubated in one of three probes (AP-APPsα, AP-APP(199-345) or AP control), washed, incubated in DTSSP (a cleavable, membrane-impermeable crosslinker) then lysed and immunoprecipitated for the AP tag. Subsequent western blots for biotin revealed prominent bands that co-immunoprecipitated with AP-APPsα but not with AP

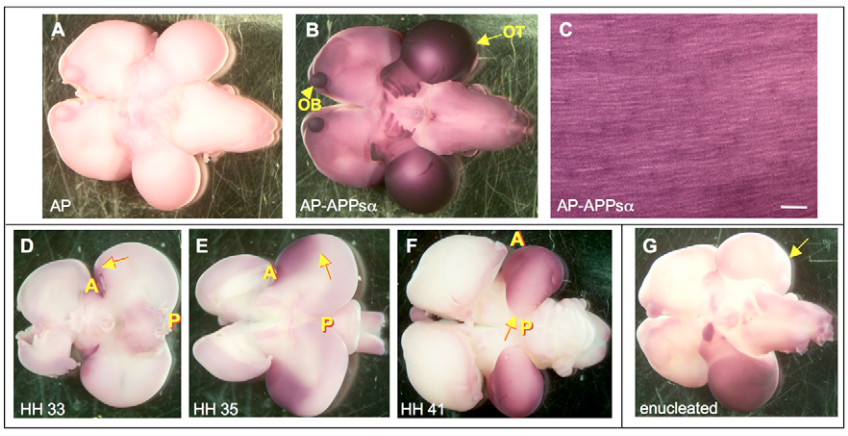


Fig. 1. APP-binding sites in embryonic chick brain. (**A**,**B**) Binding of AP-APPsα versus AP control. Ventral view, E11.5-12.5 brains. Prominent binding in optic tectum (OT, arrow), with additional staining in olfactory bulbs (OB, arrowhead) and other regions. (**C**) AP-APPsα staining of tectum at greater magnification, showing anterior-posterior striation characteristic of axons in the stratum opticum. Anterior-posterior is horizontal. Scale bar: 100 μm. (**D-F**) Time-course of AP-APPsα binding to brains. Dorsal view. Anterior (A) and posterior (P) extremes of tecta. Arrows indicate posterior limit of staining. (**G**) Binding of AP-APPsα to brain from embryo with single enucleated eye. Ventral view. Staining in contralateral tectum (arrow) is reduced to background levels. AP, alkaline phosphatase.

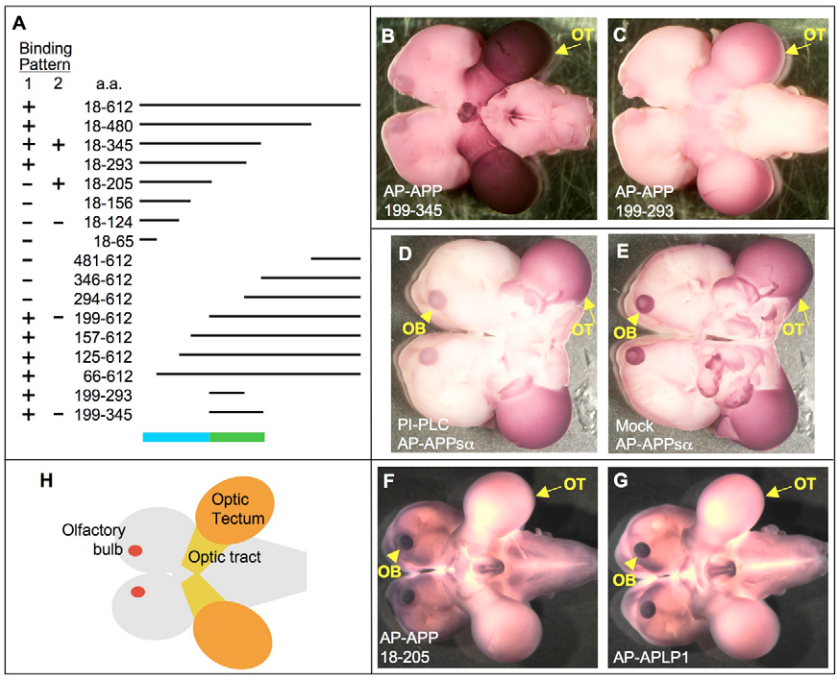


Fig. 2. Two different domains of APP exhibit distinct binding properties. AP in situs on E11.5-12.5 brains. Ventral views. Arrowheads indicate olfactory bulbs. Arrows indicate optic tecta. (**A**) Binding of AP-APP deletion series (numbered according to APP695). Complete AP-APPsα is AP-APP(18-612). Binding pattern 1 refers to prominently striated staining specifically in the anterior tectum at E9.5. Binding pattern 2 refers to widespread staining of the brain, including throughout the tectum, and prominent olfactory bulb staining, at E11.5-12.5. Colored bars indicate regions of APP with strong binding in patterns 1 (green) and 2 (blue). (**B**,**C**) AP-APP(199-345) gave strong tectal staining. AP-APP(199-293) also bound to RGC axons in tecta, though less strongly. (**D**,**E**) PI-PLC or mock treatment of brains, then AP-APPsα staining. PI-PLC treatment eliminated most olfactory bulb staining and some broader staining. (**F**,**G**) AP-APP(18-205) and AP-APLP1 exhibit widespread binding including in the tectum, with particular prominence in olfactory bulb. (**H**) Diagram of chick brain, ventral view.

alone (Fig. 3A). AP-APP(199-345) co-immunoprecipitated the same biotinylated bands, though to a lesser degree (data not shown). Two prominent bands between 120 kDa and 150 kDa were seen in repeated experiments (Fig. 3A, asterisks). Additional, fainter bands at higher molecular weight were sometimes seen.

To identify these bands, we scaled up the above purification. Each co-immunoprecipitation (co-IP) was run on a 2D gel and silver stained (Fig. 3C,D,F); the AP-tagged probes alone were run separately for comparison (Fig. 3G and data not shown). A separate gel with a western blot for biotin was used as an additional guide to determine which region of the silver-stained gels to analyze (Fig. 3B,E). Because the signal was not intense, we pooled the samples from the AP-APPsα co-IP and AP-APP(199-345) co-IP gels, so any proteins identified might interact with AP-APP(199-345), AP-APPsα, or both. Tandem mass spectrometry identified human placental alkaline phosphatase and APP, both presumably derived from the probe, and only one other protein, contactin 4, as a strong match to the sample. Contactin 4 was also among the proteins we identified in an independent approach involving purification and mass spectrometry of GPI-anchored proteins in the olfactory bulb (data not shown).

These mass spectrometry studies suggested that contactin 4 binds APP. However, interactions in the context of an intact cell or tissue can be indirect or non-specific. To verify the interaction directly in a cell-free system, we generated an Fc fusion of contactin 4 and assayed its binding to AP-APPsα. To measure affinity, binding curves were generated by varying the concentration of AP fusion protein. To investigate specificity, we similarly tested the remaining five contactins and another APP family protein, APLP1 (Fig. 4). The results revealed binding of APP to contactins 3 and 4. This fits with the homology relationships, as contactins 3 and 4 are the most closely related pair within the contactin family (Ogawa et al., 1996). APLP1 bound contactins 3 and 4, and also contactin 5. No saturable binding was detected in the other pairs tested, suggesting that if there is an interaction, the affinity is low. The calculated dissociation constant (K_D) for APP and contactin 4 was 17 nM, whereas other K_Ds ranged from 22 to 35 nM (Fig. 4B-F).

To dissect which regions of the molecules were involved, deletion constructs were analyzed. In contactin 3 or 4, the fibronectin domains were found to be sufficient for binding. This is striking, as most other interactions of IgCAMs are mediated by the Ig-like domains. In APP, amino acids 18-205 were sufficient for binding (Fig. 5A,D). The localization of the binding domain to the N-terminal portion of APP, and the observation that contactins 3 and 4 are GPI-anchored proteins, are consistent with these proteins being partly or entirely responsible for what we termed 'binding pattern 2' in embryonic chick brain (see Fig. 2).

APP interacts with NgCAM

Since our PI-PLC experiments (Fig. 2D,E) suggested the presence of at least one non-GPI-anchored APP binding partner in tecta, we generated a list of further candidate APP binding partners by identifying tectal cell-surface proteins of relevant molecular weights. Chick tecta were surface labeled with biotin, biotinylated proteins were purified and separated by SDS-PAGE, and bands corresponding to the molecular weights of the biotinylated bands seen in Fig. 3A were analyzed by tandem mass spectrometry. The sequences of the identified proteins were analyzed for transmembrane domains or signal peptides to confirm likely extracellular expression. The candidate proteins identified in this way were CHL1, contactin 1, NCAM, neogenin, neurofascin, NgCAM, NrCAM, prominin-like 2 and tenascin.

To test these candidate proteins for APP association, we performed a crosslinker-based co-IP as in Fig. 3, then used antibodies against candidate proteins for western blot detection. Of the antibodies tested (see Materials and methods), only two, both directed against NgCAM, recognized bands in immunoprecipitates of AP-APPsα, but not of the AP control (Fig. 5B). The primary band detected by both NgCAM antibodies (Fig. 5B) appears to correspond well to the upper biotinylated band marked in Fig. 3A. Additional specific bands are also likely to be due to NgCAM; species of 80, 136, 190 and 210 kDa have been reported, with the predominant 80 and 136 kDa forms arising by proteolysis (Burgoon et al., 1991), and these are likely to correspond to the predominant

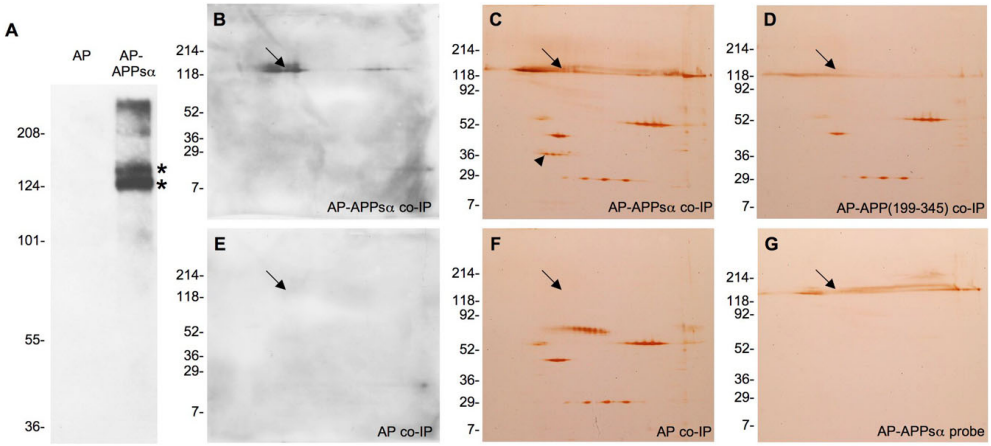


Fig. 3. Crosslinker-based affinity purification of APP-interacting proteins from tecta. AP probes were crosslinked to surface-biotinylated tecta, then immunoprecipitated from lysates to identify associated proteins. Crosslinker was cleaved before gel analysis. (**A**) Western blot using NeutrAvidin-HRP to detect surface-biotinylated co-precipitated proteins. Asterisks mark the two predominant bands. (**B-G**) 2D gels for large-scale co-precipitation. Western blots for biotinylated proteins (B,E) were compared with silver-stained gels (C,D,F,G). Arrows indicate the predominant biotinylated proteins in B and corresponding locations in the silver-stained gels. This region was excised from gels in C and D for tandem mass spectrometry. Arrowhead indicates an additional spot specific for the AP-APPsα co-IP: mass spectrometry identified several proteins, but these lacked obvious signal peptide or transmembrane domains and were not characterized further.

bands detected here (asterisks in Fig. 5B). Similar crosslinker-based affinity purifications using AP-APP(199-345) as a probe revealed that NgCAM does co-precipitate with APP amino acids 199-345 (Fig. 5C), the domain which produces binding pattern 1 (Fig. 2).

Contactin 4 and NgCAM modulate CTFα levels

Proteolytic processing as an early step in putative APP signal transduction has been widely proposed and studied (Chan and Jan, 1998; Cao and Sudhof, 2004; Hass and Yankner, 2005). We therefore examined the effect of contactin 4 and NgCAM on APP processing. In cells transfected with APP-HA, co-expression of contactin 4- or NgCAM-fusion proteins resulted in increased levels of a small HA-tagged fragment (Fig. 5E), identified as APP CTFα (C-terminal fragment α) by apparent molecular weight (Fig. 5F). We also saw increased levels of APP-HA, suggesting that contactin 4 and NgCAM can enhance the stability or expression of full-length APP. The increase in CTFα might be secondary to the increase in full-length APP, but because in some cases the increase in CTFα appeared greater than the increase in full-length APP, regulation of α-site cleavage might also be involved. Although co-transfection of soluble tagged forms of contactin 4 or NgCAM with APP-HA generally increased CTFα levels up to several fold, in a smaller number of experiments, it resulted in an equally dramatic decrease in CTFα levels, often with an accompanying decrease in full-length APP. Although we do not know the reason for these differing results, it might be related to factors such as protein concentration or cellular state, which can affect positive versus negative responses to other extracellular signaling molecules (Song et al., 1998; Hansen et al., 2004). In any case, these experiments demonstrate that the presence of contactin 4 or NgCAM can modulate CTFα levels.

Expression in the visual system

For a binding interaction to have biological significance, the interacting proteins should have in vivo expression patterns that allow them to interact. Since APP binds prominently to retinal axons in the tectum (Fig. 1), we assessed expression of APP, contactins 3 and 4 and NgCAM in the retinotectal system by in situ hybridization in E11.5 chick embryos. In the retina, all four genes were expressed prominently in the RGC layer, which is the source of axons that project to the tectum (Fig. 6A-E,K-N). Contactin 4 was also evident in other retinal layers. In the tectum, all four genes were expressed in multiple layers, including superficial layers through which RGC axons navigate (Fig. 6F-J,O-R). APP protein immunolocalization revealed prominent staining of the layers containing RGC axons in the retina and tectum, as well as staining in other tectal layers (Fig. 6S-Z). Along with previous reports of NgCAM expression (Lemmon and McLoon, 1986; Stoker et al., 1995; Yamagata et al., 1995; Rager et al., 1996) and our results showing that AP-APPsα binds to RGC axons in the tectum (Fig. 1), the expression patterns indicate that these four proteins are suitably placed for interactions involving axon-axon or axon-target contacts in the developing retinotectal system.

Functional interactions in RGC axon outgrowth

Our analyses of binding interactions and expression patterns indicated that APP, NgCAM and contactins might participate in retinal axon development, so we next tested for functional effects on RGC axons. Retinal explants cultured on NgCAM-Fc substrate showed substantial RGC axon outgrowth, as reported previously (Doherty et al., 1995; Morales et al., 1996), whereas no outgrowth was seen on substrate coated with AP-APPsα or contactin 4-Fc (data not shown). Since APPsα was previously reported to promote neurite growth in other cell types (Mattson, 1997), we tested further and found that outgrowth on NgCAM was enhanced by either AP-APPsα (P=0.003) or AP-APP(18-205) (P=0.024) (Fig. 7A-D). Similar results were obtained by quantitating axon number (Fig. 7D-F) or axon extension (see Fig. S1A-C in the supplementary material). We next tested whether this effect of APP is specific for NgCAM, as opposed to a more generalized effect of APP, potentially unrelated to NgCAM. Our results showed that APPsα specifically promoted NgCAM-supported outgrowth, but not outgrowth on other substrates such as laminin (Fig. 7E), demonstrating a specific functional interaction between APP and NgCAM in regulating axon growth.

The observation that the N-terminal domain of APP, which binds contactins 3 and 4, was sufficient to potentiate NgCAM-dependent outgrowth, suggested a model in which axonal contactin 3 or 4 acts as a receptor for APP. This model predicts that soluble contactin 4 could act as a dominant-negative and block signaling through axonally expressed contactin 3 or 4. We therefore tested contactin 4-Fc, and found that it did indeed inhibit outgrowth, reducing levels of outgrowth on AP-APPsα plus NgCAM (P=0.011) to levels seen on

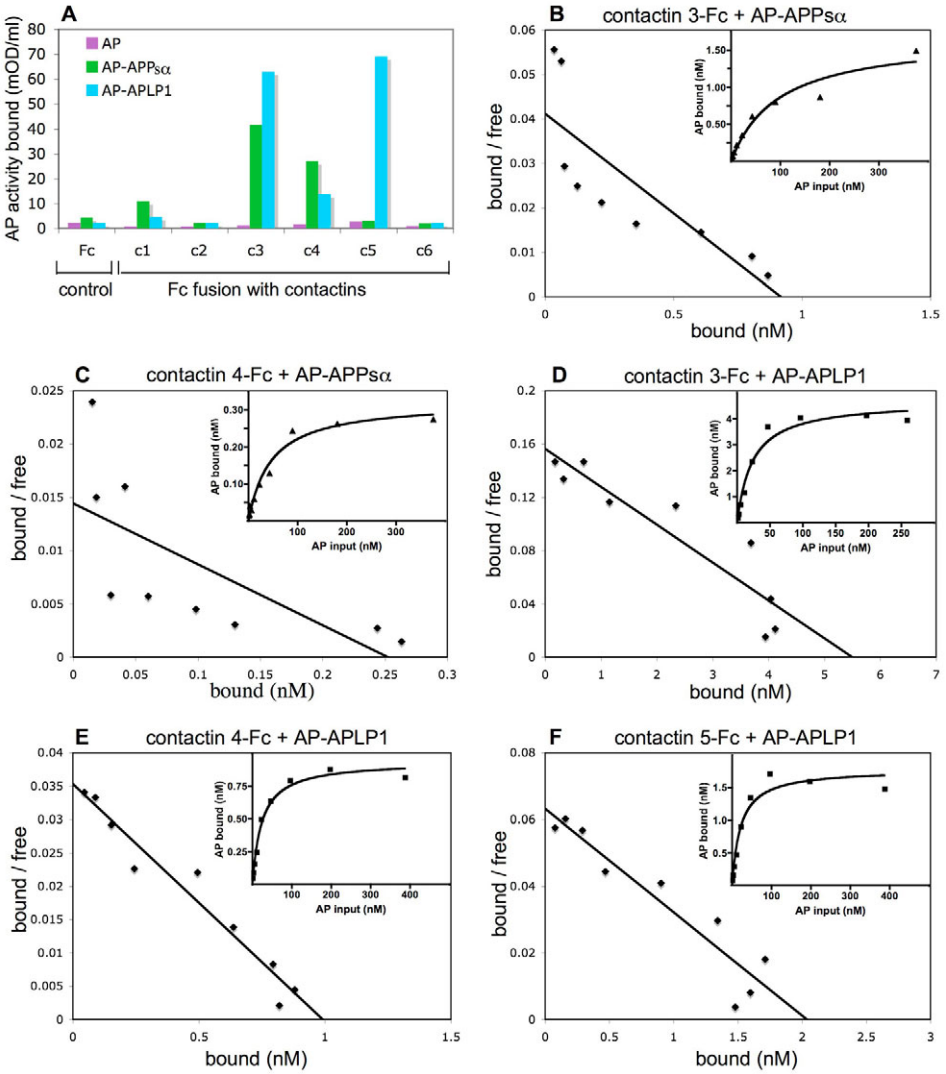


Fig. 4. APP and APLP1 bind to specific contactin family members. (**A**) AP fusion proteins were tested for binding to contactin-Fc fusion proteins immobilized on Protein A beads. AP activity retained after washing beads is shown. APP bound prominently to contactins 3 and 4. APLP1 bound to contactins 3, 4 and 5. Some non-saturable binding indicating weak or non-specific binding was also seen with contactin 1-Fc (data not shown). (**B-F**) Saturation curves (insets) were generated by varying the concentration of AP fusion protein. Lines in corresponding Scatchard plots were generated by least squares fitting. K_Ds: contactin 4-APP, 17 nM; contactin 4-APLP1, 28 nM; contactin 3-APP, 22 nM; contactin 3-APLP1, 35 nM; and contactin 5-APLP1, 32 nM.

NgCAM alone (Fig. 7E). Contactin 4-Fc also reduced growth on NgCAM alone, but, importantly, showed no effect on laminin-dependent growth of RGC axons (Fig. 7E,F), thereby demonstrating specificity. We further tested the model by examining axon outgrowth from retinas infected with virus expressing shRNA that targets contactin 4. Explants expressing shRNA against contactin 4 exhibited less axon growth than control explants, when grown on AP-APPsα plus NgCAM (P=0.021), whereas outgrowth on laminin was not significantly affected (Fig. 7G-K). Thus, data from two independent experimental approaches support a role for contactin 4 in the response by RGCs axons to NgCAM plus AP-APPsα.

DISCUSSION

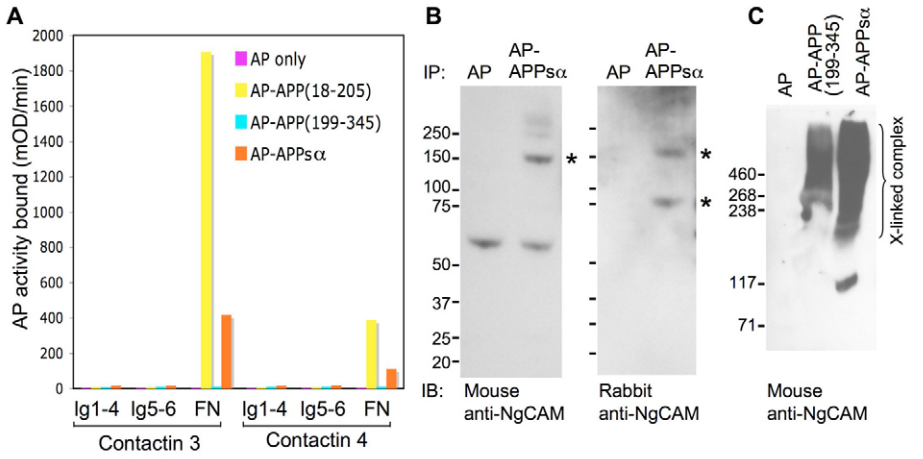
APP has been studied extensively for its role in Alzheimer's disease. Genetic studies show that APP functions in neural development, but specific mechanisms of APP action in vertebrate development have remained unclear. To find developmentally relevant binding partners for APP, we initially undertook a series of biochemical studies in developing chick brain, which revealed interactions of the APP extracellular domain with contactin 3, contactin 4 and NgCAM.

Evidence for a ligand-receptor relationship can be provided by showing a binding interaction, expression in suitable places to interact and a functional interaction. In keeping with this, in addition to the binding interaction demonstrated by our molecular studies, we find that all these molecules are expressed in the developing retinotectal system, and that APPsα and contactin 4 can specifically modulate NgCAM-dependent axon outgrowth, demonstrating functional interactions among these proteins that can induce changes in cellular behavior.

Binding of APP family members to proteins of the contactin and L1CAM families

Our studies show that APP binds both contactin 3 and 4. APLP1 was found to bind contactins 3 and 4, and also contactin 5. These results thus demonstrate a set of promiscuous binding interactions between the APP and contactin families, although binding was not seen in all pairwise combinations, thereby indicating a degree of specificity. Although our functional studies have so far focused on APP and contactin 4, it is likely that interaction among other APP and contactin family members will also have functional consequences.

Binding of APP to contactins 3 and 4 is a direct interaction that can be observed in a cell-free system. On contactin 4, the APP-binding site localizes to the FN-like domains, which could leave the Ig domains of contactin free to interact with other binding partners. On APP, the contactin-binding site is at the N-terminus, involving amino acids 18-205. This corresponds essentially to the E1 domain of APPsα (Daigle and Li, 1993), which has been implicated in many biological processes, including neural stem cell proliferation



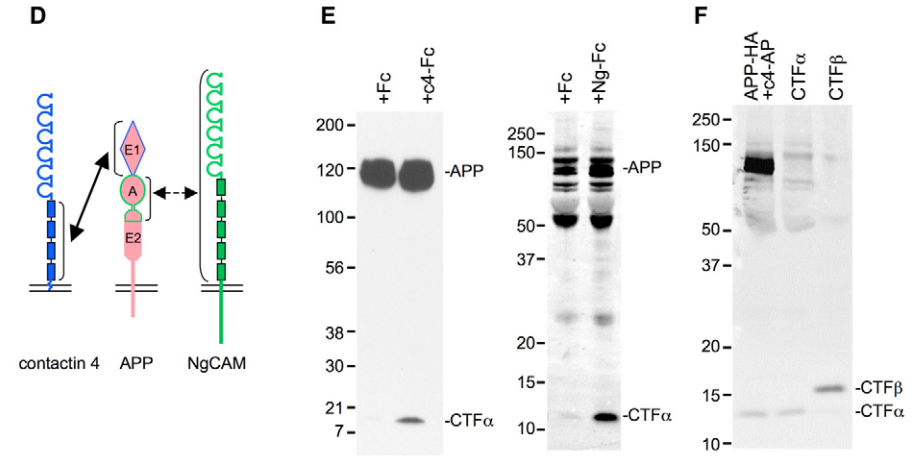


Fig. 5. Characterization of interactions of APP with contactins and NgCAM. (**A**) AP-fused APP domains were tested for binding to Fc-fused contactin 3 or 4 domains [for concentrations, see Materials and methods; for domain selection see Rader et al. (Rader et al., 1996)]. Binding activity localizes to the fibronectin (FN) domains of contactins, and to the N-terminal domain of APP. (**B**) APP association with NgCAM. Western blots with antibodies against NgCAM after immunoprecipitating for AP tag. Asterisks mark major bands specifically co-immunoprecipitated with AP-APPsα, consistent with published molecular weights of NgCAM. (**C**) Amino acids 199-345 of APP are sufficient for association with NgCAM. Non-cleavable crosslinker BS3 used here to examine net molecular weight of immunoprecipitated complexes. Resulting spread of signal might indicate the presence of additional molecules in the crosslinked NgCAM-APP complexes. (**D**) Model for interactions among proteins. N-terminal domain of APP (amino acids 18-205, 'E1') binds directly to fibronectin domains of contactin 4, whereas amino acids 199-345 of APP interact, directly or indirectly, with NgCAM. Amino acids 199-345 of APP encompass the acidic domain (oval, 'A') and the N-terminal portion of the central APP domain, termed E2, which includes the RERMS peptide previously implicated in APPsα function (Reinhard et al., 2005). (**E**) Co-transfection of APP-HA and indicated constructs, followed by anti-HA western blot. Contactin 4-Fc or NgCAM-Fc constructs increased the CTFα level compared with Fc control. (**F**) Co-migration with an artificial CTFα polypeptide confirms the identity of the APP cleavage fragment observed after co-expression with contactin 4-AP.

(Ohsawa et al., 1999), synaptogenesis (Morimoto et al., 1998) and neurite growth (Small et al., 1994; Ohsawa et al., 1997), consistent with the possibility that interactions with contactins could be involved in these biological processes.

APP also associated in our co-precipitation experiments with NgCAM. We have so far not been able to show direct binding in cell-free assays, which could have various interpretations: the association could be indirect, or it might require additional components, or direct binding can occur but was not evident owing to technical aspects of the assay. Interestingly, the interaction with NgCAM does not seem to be mediated by contactins in any simple way, as NgCAM can be crosslinked to AP-APP(199-345), a construct without the binding domain for contactins. The presence of distinct domains in APP that interact with NgCAM versus contactins suggests a model in which the three proteins function together in a three-way complex. Previous studies have shown that L1CAM family members interact with contactin Ig domains (Brummendorf and Rathjen, 1996), so our finding that APP interacts with contactin FN domains could be consistent with simultaneous or cooperative binding in a multimeric complex.

APP processing

APP cleavage can occur along two pathways. In the amyloidogenic pathway, cleavage by β- and γ-secretases generates Aβ, implicated in Alzheimer's disease. In the non-amyloidogenic pathway, cleavage by α-secretase precludes the formation of Aβ, suggesting the potential to protect against Alzheimer's disease. The non-amyloidogenic pathway has also been implicated in biological signaling because it generates the extracellular APPsα fragment that can regulate cell behavior when added to cell cultures, and because α-cleavage has been proposed to generate a signal within the APP-bearing cell. We were therefore interested to find that contactin 4 and NgCAM can alter the level of CTFα, the C-terminal α-cleavage product, by several fold. Although we have not characterized the specific mechanism (regulation of APP cleavage, stability, subcellular localization, etc.), our experiments show effects on the level of CTFα, and any net change in the level of CTFα is a biochemical signaling event that may influence downstream pathways. Further studies would be required to determine the biologically relevant signaling mechanisms, and whether the interactions identified here could modulate Aβ production with therapeutic relevance.

Functional interactions in regulating RGC axon growth

Further evidence for interactions among these proteins comes from our functional studies of RGC axon outgrowth. APPsα specifically potentiated NgCAM-dependent, but not laminin-dependent, axon outgrowth, demonstrating a specific functional interaction between APPsα and NgCAM. Interfering with

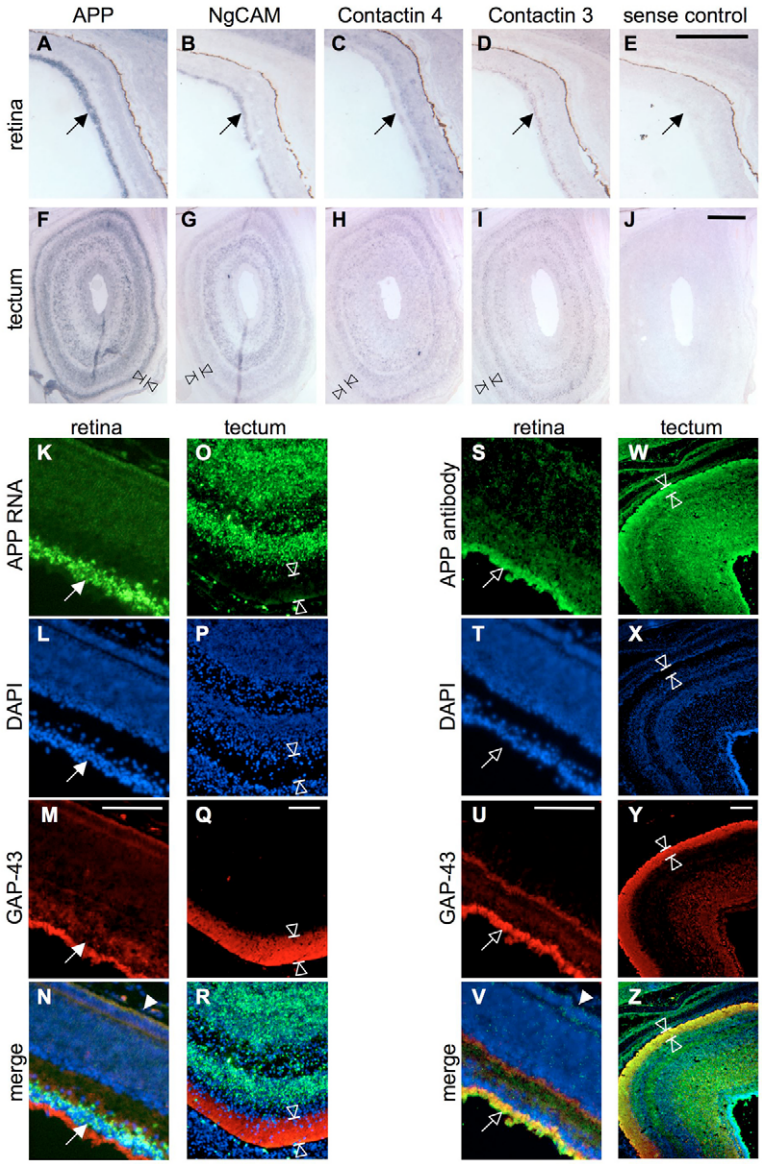


Fig. 6. Expression of APP, NgCAM, contactin 4 and contactin 3 in the developing visual system. RNA in situ hybridization in sagittal sections of E11.5 chick heads. (**A-E**) Retina. Each antisense probe detected RNA expression in the RGC layer (arrow). Pigmented epithelium (dark brown line) bounds the retina opposite the RGC layer. (**F-J**) Each antisense probe detected RNA expression in multiple layers of the tectum. Top, anterior extreme; bottom, posterior extreme; open arrowheads, stratum opticum as identified by GAP43 immunolabeling. (**K-Z**) Co-staining using RNA in situ hybridization (K-R) or immunohistochemistry (S-Z) for APP (green), DAPI nuclear stain (blue) and immunohistochemistry for GAP43 (red). Solid arrow, RGC layer; open arrow, retinal optic fiber layer; solid arrowhead, pigmented epithelium; open arrowheads, tectal stratum opticum as identified by GAP43 immunolabeling. Scale bars: 500 μm in A-J; 100 μm in K-V; 200 μm in W-Z.

contactin 4 by two independent methods, using a putative dominant negative, or using shRNA against contactin 4, inhibited outgrowth on NgCAM and APPsα, but not on laminin, again demonstrating specific functional interactions.

If interactions among APP, NgCAM and contactin 4 are to affect RGC axon behavior in vivo, these genes must be expressed at a relevant time and location. RNA in situ hybridization showed that all three genes are expressed in both the RGC layer of the retina and in the optic tectum at the time of retinotectal development. To investigate functional effects on RGC axons, we used an assay of axon outgrowth, although it is worth noting that molecules with activity in axon outgrowth assays can have biological roles in axon growth, guidance or synaptogenesis. Since none of these molecules was seen in an obvious gradient, they are presumably not acting as positional mapping labels, but they might function in other axon-axon or axon-target interactions that are involved in retinotectal development (McLaughlin and O'Leary, 2005; Flanagan, 2006). Indeed, genetic studies have already shown that the mammalian NgCAM homolog, L1CAM, is required for normal development of the retinotectal map (Demyanenko and Maness, 2003). In view of the interactions seen here, it will ultimately be interesting to further test the roles of the L1CAM, APP and contactin families in vivo.

Since APPsα and NgCAM stimulated RGC axon growth when presented together in trans, and as they can interact physically, a likely model is that they can function as a co-ligand complex, jointly interacting with and activating a receptor. Since the domain of APP that interacts with contactin 4 is sufficient to promote axon growth, because both contactin 4-Fc and RNAi against contactin 4 inhibited growth on NgCAM and APPsα, and because published studies have implicated contactin 2 as a co-receptor in NgCAM-promoted neurite outgrowth (Buchstaller et al., 1996), our data support the model that axonal contactin 4 acts here by mediating a response to molecules on the substratum. Taken together, our functional studies of axon growth in vitro lead to a working model (Fig. 7L) in which contactin 4 would act as an axonal receptor or co-receptor for an APPsα-NgCAM co-ligand complex. However, although our experiments clearly support functional interactions among these proteins, further studies would be required to investigate exactly which cis and trans interactions may occur in a biological context.

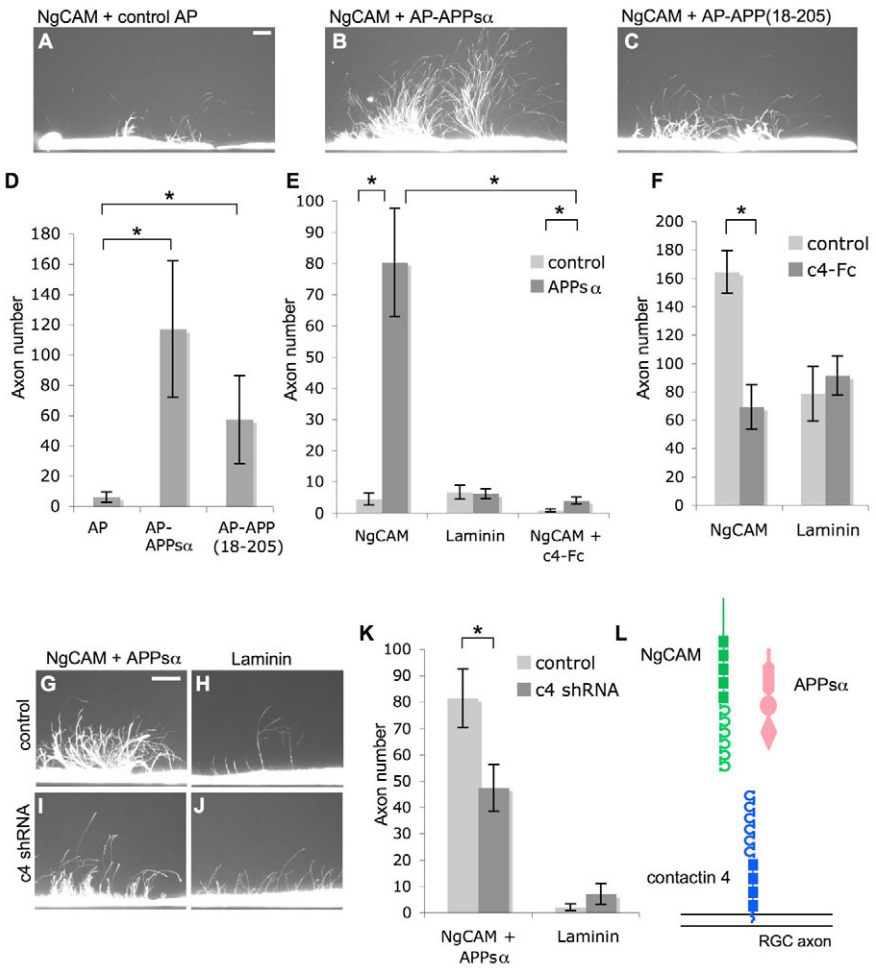


Fig. 7. Interactions among NgCAM, contactin 4 and APPsα in RGC axon outgrowth. Retinal strips cultured on different substrates before axon imaging. (**A-D**) APPsα and APP(18-205) both enhanced outgrowth on NgCAM. AP, *n*=12; other conditions, *n*=6. (**E**) APPsα enhanced NgCAM-dependent, but not laminin-dependent, axon outgrowth. *n*=6. (**F**) Contactin 4-Fc inhibitied NgCAM-dependent, but not laminin-dependent, axon outgrowth. *n*=10. Protein coating was varied in different experiments; the high basal outgrowth here allowed for robust quantitation of the inhibitory effect of contactin 4-Fc. (**G-K**) Contactin 4 shRNA in replication-competent retrovirus inhibited axon outgrowth on NgCAM+AP-APPsα, but not on laminin. Laminin, *n*=11 (shRNA), *n*=11 (control virus); NgCAM+APPsα, *n*=21 (shRNA), *n*=19 (control virus). (**L**) Model of APPsα, NgCAM and contactin 4 function based on the assays of RGC axon outgrowth shown here. Other cis and trans interactions might also occur. See text for details. *, $P<0.025$ by Student's *t*-test. Error bars, s.e.m. Scale bars: 300 μm.

APP has been widely proposed to function as both a receptor and a ligand. In principle, these two functions could involve either the same or different molecular interactions. Our results showing effects of contactin 4 and NgCAM on APP processing could be consistent with a receptor function for APP. Meanwhile, our studies of RGC axon outgrowth support a model in which APPsα and NgCAM can act together as ligands. These models are entirely consistent, assuming formation of a complex that can have multiple signaling outputs. Precedent for such a model comes from signaling complexes, such as ephrins and Eph receptors, which can interact in cis and trans, and signal bidirectionally (Kullander and Klein, 2002; Flanagan, 2006).

APP, contactins and L1CAMs in development and disease

Interestingly, there are a number of biological and pathological processes in which both APP and IgCAMs related to NgCAM or contactins have been implicated. For example, mice with a disrupted APP gene exhibit defects in corpus callosum formation (Magara et al., 1999) reminiscent of the defects seen in patients with CRASH syndrome, which is caused by mutations in L1CAM, the human homolog of NgCAM (Fransen et al., 1995). Overexpression of FASII in *Drosophila* results in an increased number of neuromuscular synaptic boutons, which is dependent on downstream function of the APP homolog APPL (Ashley et al., 2005). FASII is the *Drosophila* homolog of NCAM, which is distinct from, but closely related to, the *Drosophila* homologs of the contactins (CONT) and the L1CAMs (Neuroglian) (Vaughn and Bjorkman, 1996; Hortsch, 2000; Faivre-Sarrailh et al., 2004), so these results might reflect an underlying evolutionary conservation of interactions.

More generally, multiple studies implicate members of the APP, contactin and L1CAM families in the development of neural connectivity in vertebrates. APPsα can affect neurite outgrowth, synaptogenesis, and synaptic plasticity (Mattson, 1997; Turner et al., 2003). Contactins and L1CAMs have been extensively studied for their roles in neurite outgrowth (Sonderegger, 1997; Kamiguchi et al., 1998; Falk et al., 2002), and contactin 1 and L1CAM have been implicated in synapse formation or plasticity (Hoffman, 1998; Murai et al., 2002; Saghatelyan et al., 2004).

A ligand for APP might also be expected to affect progression of Alzheimer's disease, either by regulating functions of APP that control cell behavior, or by modulating the processing of APP to Aβ. Consistent with this, *CNTN4* maps to chromosome 3p26, only 1.1 Mb from the D3S2387 marker, which was reported to have suggestive genetic linkage to Alzheimer's disease (Blacker et al., 2003); intriguingly, the only other identified genes as closely located to this marker are *CNTN6* (encoding contactin 6) and *CHL1* (encoding the L1CAM family protein, close homolog of L1). The work described here identifies molecular and functional interactions of APP with contactin and L1CAM family proteins, which might have general roles in neural development and disease.

We thank Edna Sun, Masaru Nakamoto, Jonaki Sen, Mark Emerson, Sanjiv Harpavat, and Samira Pontes for advice and help with experiments; Ross Tomaino and Steve Gygi for performing and interpreting the mass spectrometry analysis; Peter Sonderegger, Josh Sanes and Fritz Rathjen for antibodies and plasmids; David Van Vactor, Connie Cepko, Alan Tenney, Xinmin Li, David Feldheim, Qiang Lu, Nicolas Preitner, Dan Nowakowski and other members of the Flanagan and Van Vactor laboratories for discussions; and Dennis Selkoe and Rudolph Tanzi for comments on the manuscript. This work was supported by grants EY11559 and HD29417 from the National Institutes of Health (to J.G.F.) and by a postdoctoral fellowship from the Alfred Benzon Foundation, Denmark (to R.E.).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/6/1189/DC1

References

Ashley, J., Packard, M., Ataman, B. and Budnik, V. (2005). Fasciclin II signals new synapse formation through amyloid precursor protein and the scaffolding protein dX11/Mint. *J. Neurosci.* **25**, 5943-5955.

Beher, D., Hesse, L., Masters, C. L. and Multhaup, G. (1996). Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. *J. Biol. Chem.* **271**, 1613-1620.

Blacker, D., Bertram, L., Saunders, A. J., Moscarillo, T. J., Albert, M. S., Wiener, H., Perry, R. T., Collins, J. S., Harrell, L. E., Go, R. C. et al. (2003). Results of a high-resolution genome screen of 437 Alzheimer's disease families. *Hum. Mol. Genet.* **12**, 23-32.

Brummendorf, T. and Rathjen, F. G. (1996). Structure/function relationships of axon-associated adhesion receptors of the immunoglobulin superfamily. *Curr. Opin. Neurobiol.* **6**, 584-593.

Buchstaller, A., Kunz, S., Berger, P., Kunz, B., Ziegler, U., Rader, C. and Sonderegger, P. (1996). Cell adhesion molecules NgCAM and axonin-1 form heterodimers in the neuronal membrane and cooperate in neurite outgrowth promotion. *J. Cell Biol.* **135**, 1593-1607.

Burgoon, M. P., Grumet, M., Mauro, V., Edelman, G. M. and Cunningham, B. A. (1991). Structure of the chicken neuron-glia cell adhesion molecule, Ng-CAM: origin of the polypeptides and relation to the Ig superfamily. *J. Cell Biol.* **112**, 1017-1029.

Caille, I., Allinquant, B., Dupont, E., Bouillot, C., Langer, A., Muller, U. and Prochiantz, A. (2004). Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone. *Development* **131**, 2173-2181.

Cao, X. and Sudhof, T. C. (2001). A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* **293**, 115-120.

Cao, X. and Sudhof, T. C. (2004). Dissection of amyloid-beta precursor protein-dependent transcriptional transactivation. *J. Biol. Chem.* **279**, 24601-24611.

Catalano, S. M., Dodson, E. C., Henze, D. A., Joyce, J. G., Krafft, G. A. and Kinney, G. G. (2006). The role of amyloid-beta derived diffusible ligands (ADDLs) in Alzheimer's disease. *Curr. Top. Med. Chem.* **6**, 597-608.

Chan, Y. M. and Jan, Y. N. (1998). Roles for proteolysis and trafficking in notch maturation and signal transduction. *Cell* **94**, 423-426.

Chang, S., Rathjen, F. G. and Raper, J. A. (1990). Neurite outgrowth promoting activity of G4 and its inhibition by monoclonal antibodies. *J. Neurosci. Res.* **25**, 180-186.

Chen, C. D., Oh, S. Y., Hinman, J. D. and Abraham, C. R. (2006). Visualization of APP dimerization and APP-Notch2 heterodimerization in living cells using bimolecular fluorescence complementation. *J. Neurochem.* **97**, 30-43.

Connelly, M. A., Grady, R. C., Mushinski, J. F. and Marcu, K. B. (1994). PANG, a gene encoding a neuronal glycoprotein, is ectopically activated by intracisternal A-type particle long terminal repeats in murine plasmacytomas. *Proc. Natl. Acad. Sci. USA* **91**, 1337-1341.

Daigle, I. and Li, C. (1993). apl-1, a Caenorhabditis elegans gene encoding a protein related to the human beta-amyloid protein precursor. *Proc. Natl. Acad. Sci. USA* **90**, 12045-12049.

Demyanenko, G. P. and Maness, P. F. (2003). The L1 cell adhesion molecule is essential for topographic mapping of retinal axons. *J. Neurosci.* **23**, 530-538.

Dijkhuizen, T., van Essen, T., van der Vlies, P., Verheij, J. B., Sikkema-Raddatz, B., van der Veen, A. Y., Gerssen-Schoorl, K. B., Buys, C. H. and Kok, K. (2006). FISH and array-CGH analysis of a complex chromosome 3 aberration suggests that loss of CNTN4 and CRBN contributes to mental retardation in 3pter deletions. *Am. J. Med. Genet. A* **140**, 2482-2487.

Doherty, P., Williams, E. and Walsh, F. S. (1995). A soluble chimeric form of the L1 glycoprotein stimulates neurite outgrowth. *Neuron* **14**, 57-66.

Faivre-Sarrailh, C., Banerjee, S., Li, J., Hortsch, M., Laval, M. and Bhat, M. A. (2004). Drosophila contactin, a homolog of vertebrate contactin, is required for septate junction organization and paracellular barrier function. *Development* **131**, 4931-4942.

Falk, J., Bonnon, C., Girault, J. A. and Faivre-Sarrailh, C. (2002). F3/contactin, a neuronal cell adhesion molecule implicated in axogenesis and myelination. *Biol. Cell* **94**, 327-334.

Fassa, A., Mehta, P. and Efthimiopoulos, S. (2005). Notch 1 interacts with the amyloid precursor protein in a Numb-independent manner. *J. Neurosci. Res.* **82**, 214-224.

Fernandez, T., Morgan, T., Davis, N., Klin, A., Morris, A., Farhi, A., Lifton, R. P. and State, M. W. (2004). Disruption of contactin 4 (CNTN4) results in developmental delay and other features of 3p deletion syndrome. *Am. J. Hum. Genet.* **74**, 1286-1293.

Fischer, D. F., van Dijk, R., Sluijs, J. A., Nair, S. M., Racchi, M., Levelt, C. N., van Leeuwen, F. W. and Hol, E. M. (2005). Activation of the Notch pathway in Down syndrome: cross-talk of Notch and APP. *FASEB J.* **19**, 1451-1458.

Flanagan, J. G. (2006). Neural map specification by gradients. *Curr. Opin. Neurobiol.* **16**, 59-66.

Flanagan, J. G. and Cheng, H. J. (2000). Alkaline phosphatase fusion proteins for molecular characterization and cloning of receptors and their ligands. *Meth. Enzymol.* **327**, 198-210.

Flanagan, J. G., Cheng, H. J., Feldheim, D. A., Hattori, M., Lu, Q. and Vanderhaeghen, P. (2000). Alkaline phosphatase fusions of ligands or receptors as in situ probes for staining of cells, tissues, and embryos. *Meth. Enzymol.* **327**, 19-35.

Fotinopoulou, A., Tsachaki, M., Vlavaki, M., Poulopoulos, A., Rostagno, A., Frangione, B., Ghiso, J. and Efthimiopoulos, S. (2005). BRI2 interacts with amyloid precursor protein (APP) and regulates amyloid beta (Abeta) production. *J. Biol. Chem.* **280**, 30768-30772.

Fransen, E., Lemmon, V., Van Camp, G., Vits, L., Coucke, P. and Willems, P. J. (1995). CRASH syndrome: clinical spectrum of corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraparesis and hydrocephalus due to mutations in one single gene, L1. *Eur. J. Hum. Genet.* **3**, 273-284.

Goldberg, S. (1974). Studies on the mechanics of development of the visual pathways in the chick embryo. *Dev. Biol.* **36**, 24-43.

Hansen, M. J., Dallal, G. E. and Flanagan, J. G. (2004). Retinal axon response to ephrin-as shows a graded, concentration-dependent transition from growth promotion to inhibition. *Neuron* **42**, 717-730.

Hardy, J. and Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353-356.

Hass, M. R. and Yankner, B. A. (2005). A gamma-secretase-independent mechanism of signal transduction by the amyloid precursor protein. *J. Biol. Chem.* **280**, 36895-36904.

Hebert, S. S., Serneels, L., Tolia, A., Craessaerts, K., Derks, C., Filippov, M. A., Muller, U. and De Strooper, B. (2006). Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes. *EMBO Rep.* **7**, 739-745.

Herms, J., Anliker, B., Heber, S., Ring, S., Fuhrmann, M., Kretzschmar, H., Sisodia, S. and Muller, U. (2004). Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. *EMBO J.* **23**, 4106-4115.

Ho, A. and Sudhof, T. C. (2004). Binding of F-spondin to amyloid-beta precursor protein: a candidate amyloid-beta precursor protein ligand that modulates amyloid-beta precursor protein cleavage. *Proc. Natl. Acad. Sci. USA* **101**, 2548-2553.

Hoe, H. S., Wessner, D., Beffert, U., Becker, A. G., Matsuoka, Y. and Rebeck, G. W. (2005). F-spondin interaction with the apolipoprotein E receptor ApoEr2 affects processing of amyloid precursor protein. *Mol. Cell. Biol.* **25**, 9259-9268.

Hoffman, K. B. (1998). The relationship between adhesion molecules and neuronal plasticity. *Cell. Mol. Neurobiol.* **18**, 461-475.

Hortsch, M. (1996). The L1 family of neural cell adhesion molecules: old proteins performing new tricks. *Neuron* **17**, 587-593.

Hortsch, M. (2000). Structural and functional evolution of the L1 family: are four adhesion molecules better than one? *Mol. Cell. Neurosci.* **15**, 1-10.

Kamiguchi, H., Hlavin, M. L. and Lemmon, V. (1998). Role of L1 in neural development: what the knockouts tell us. *Mol. Cell. Neurosci.* **12**, 48-55.

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733-736.

Kerr, M. L. and Small, D. H. (2005). Cytoplasmic domain of the beta-amyloid protein precursor of Alzheimer's disease: function, regulation of proteolysis, and implications for drug development. *J. Neurosci. Res.* **80**, 151-159.

Kimberly, W. T., Zheng, J. B., Guenette, S. Y. and Selkoe, D. J. (2001). The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. *J. Biol. Chem.* **276**, 40288-40292.

Kinoshita, A., Whelan, C. M., Smith, C. J., Berezovska, O. and Hyman, B. T. (2002). Direct visualization of the gamma secretase-generated carboxyl-terminal domain of the amyloid precursor protein: association with Fe65 and translocation to the nucleus. *J. Neurochem.* **82**, 839-847.

Kounnas, M. Z., Moir, R. D., Rebeck, G. W., Bush, A. I., Argraves, W. S., Tanzi, R. E., Hyman, B. T. and Strickland, D. K. (1995). LDL receptor-related protein,

a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* **82**, 331-340.
Kullander, K. and Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 475-486.
Lemmon, V. and McLoon, S. C. (1986). The appearance of an L1-like molecule in the chick primary visual pathway. *J. Neurosci.* **6**, 2987-2994.
Magara, F., Muller, U., Li, Z. W., Lipp, H. P., Weissmann, C., Stagljar, M. and Wolfer, D. P. (1999). Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the beta-amyloid-precursor protein. *Proc. Natl. Acad. Sci. USA* **96**, 4656-4661.
Majercak, J., Ray, W. J., Espeseth, A., Simon, A., Shi, X. P., Wolffe, C., Getty, K., Marine, S., Stec, E., Ferrer, M. et al. (2006). LRRTM3 promotes processing of amyloid-precursor protein by BACE1 and is a positional candidate gene for late-onset Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **103**, 17967-17972.
Maness, P. F. and Schachner, M. (2007). Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. *Nat. Neurosci.* **10**, 19-26.
Matsuda, S., Giliberto, L., Matsuda, Y., Davies, P., McGowan, E., Pickford, F., Ghiso, J., Frangione, B. and D'Adamio, L. (2005). The familial dementia BRI2 gene binds the Alzheimer gene amyloid-beta precursor protein and inhibits amyloid-beta production. *J. Biol. Chem.* **280**, 28912-28916.
Mattson, M. P. (1997). Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiol. Rev.* **77**, 1081-1132.
McLaughlin, T. and O'Leary, D. D. (2005). Molecular gradients and development of retinotopic maps. *Annu. Rev. Neurosci.* **28**, 327-355.
Morales, G., Sanchez-Puelles, J. M., Schwarz, U. and de la Rosa, E. J. (1996). Synergistic neurite-outgrowth promoting activity of two related axonal proteins, Bravo/Nr-CAM and G4/Ng-CAM in chicken retinal explants. *Eur. J. Neurosci.* **8**, 1098-1105.
Morimoto, T., Ohsawa, I., Takamura, C., Ishiguro, M. and Kohsaka, S. (1998). Involvement of amyloid precursor protein in functional synapse formation in cultured hippocampal neurons. *J. Neurosci. Res.* **51**, 185-195.
Multhaup, G. (1994). Identification and regulation of the high affinity binding site of the Alzheimer's disease amyloid protein precursor (APP) to glycosaminoglycans. *Biochimie* **76**, 304-311.
Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* **228**, 151-165.
Murai, K. K., Misner, D. and Ranscht, B. (2002). Contactin supports synaptic plasticity associated with hippocampal long-term depression but not potentiation. *Curr. Biol.* **12**, 181-190.
Ogawa, J., Kaneko, H., Masuda, T., Nagata, S., Hosoya, H. and Watanabe, K. (1996). Novel neural adhesion molecules in the Contactin/F3 subgroup of the immunoglobulin superfamily: isolation and characterization of cDNAs from rat brain. *Neurosci. Lett.* **218**, 173-176.
Oh, S. Y., Ellenstein, A., Chen, C. D., Hinman, J. D., Berg, E. A., Costello, C. E., Yamin, R., Neve, R. L. and Abraham, C. R. (2005). Amyloid precursor protein interacts with notch receptors. *J. Neurosci. Res.* **82**, 32-42.
Ohsawa, I., Takamura, C. and Kohsaka, S. (1997). The amino-terminal region of amyloid precursor protein is responsible for neurite outgrowth in rat neocortical explant culture. *Biochem. Biophys. Res. Commun.* **236**, 59-65.
Ohsawa, I., Takamura, C., Morimoto, T., Ishiguro, M. and Kohsaka, S. (1999). Amino-terminal region of secreted form of amyloid precursor protein stimulates proliferation of neural stem cells. *Eur. J. Neurosci.* **11**, 1907-1913.
Ohsawa, I., Takamura, C. and Kohsaka, S. (2001). Fibulin-1 binds the amino-terminal head of beta-amyloid precursor protein and modulates its physiological function. *J. Neurochem.* **76**, 1411-1420.
Park, J. H., Gimbel, D. A., GrandPre, T., Lee, J. K., Kim, J. E., Li, W., Lee, D. H. and Strittmatter, S. M. (2006). Alzheimer precursor protein interaction with the Nogo-66 receptor reduces amyloid-beta plaque deposition. *J. Neurosci.* **26**, 1386-1395.
Rader, C., Kunz, B., Lierheimer, R., Giger, R. J., Berger, P., Tittmann, P., Gross, H. and Sonderegger, P. (1996). Implications for the domain arrangement of axonin-1 derived from the mapping of its NgCAM binding site. *EMBO J.* **15**, 2056-2068.
Rager, G., Morino, P., Schnitzer, J. and Sonderegger, P. (1996). Expression of the axonal cell adhesion molecules axonin-1 and Ng-CAM during the development of the chick retinotectal system. *J. Comp. Neurol.* **365**, 594-609.
Reinhard, C., Hebert, S. S. and De Strooper, B. (2005). The amyloid-beta precursor protein: integrating structure with biological function. *EMBO J.* **24**, 3996-4006.
Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. and Khvorova, A. (2004). Rational siRNA design for RNA interference. *Nat. Biotechnol.* **22**, 326-330.
Saghatelyan, A. K., Nikonenko, A. G., Sun, M., Rolf, B., Putthoff, P., Kutsche, M., Bartsch, U., Dityatev, A. and Schachner, M. (2004). Reduced GABAergic transmission and number of hippocampal perisomatic inhibitory synapses in juvenile mice deficient in the neural cell adhesion molecule L1. *Mol. Cell. Neurosci.* **26**, 191-203.
Santiago-Garcia, J., Mas-Oliva, J., Innerarity, T. L. and Pitas, R. E. (2001). Secreted forms of the amyloid-beta precursor protein are ligands for the class A scavenger receptor. *J. Biol. Chem.* **276**, 30655-30661.
Schulte, D., Furukawa, T., Peters, M. A., Kozak, C. A. and Cepko, C. L. (1999). Misexpression of the Emx-related homeobox genes cVax and mVax2 ventralizes the retina and perturbs the retinotectal map. *Neuron* **24**, 541-553.
Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **399**, A23-A31.
Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850-858.
Small, D. H., Nurcombe, V., Reed, G., Clarris, H., Moir, R., Beyreuther, K. and Masters, C. L. (1994). A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *J. Neurosci.* **14**, 2117-2127.
Soba, P., Eggert, S., Wagner, K., Zentgraf, H., Siehl, K., Kreger, S., Lower, A., Langer, A., Merdes, G., Paro, R. et al. (2005). Homo- and heterodimerization of APP family members promotes intercellular adhesion. *EMBO J.* **24**, 3624-3634.
Sonderegger, P. (1997). Axonin-1 and NgCAM as "recognition" components of the pathway sensor apparatus of growth cones: a synopsis. *Cell Tissue Res.* **290**, 429-439.
Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M. and Poo, M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* **281**, 1515-1518.
Steinbach, J. P., Muller, U., Leist, M., Li, Z. W., Nicotera, P. and Aguzzi, A. (1998). Hypersensitivity to seizures in beta-amyloid precursor protein deficient mice. *Cell Death Differ.* **5**, 858-866.
Stoker, A. W., Gehrig, B., Newton, M. R. and Bay, B. H. (1995). Comparative localisation of CRYP alpha, a CAM-like tyrosine phosphatase, and NgCAM in the developing chick visual system. *Brain Res. Dev. Brain Res.* **90**, 129-140.
Tanzi, R. E. and Bertram, L. (2005). Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell* **120**, 545-555.
Turner, P. R., O'Connor, K., Tate, W. P. and Abraham, W. C. (2003). Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog. Neurobiol.* **70**, 1-32.
Vaughn, D. E. and Bjorkman, P. J. (1996). The (Greek) key to structures of neural adhesion molecules. *Neuron* **16**, 261-273.
Yamagata, M., Herman, J. P. and Sanes, J. R. (1995). Lamina-specific expression of adhesion molecules in developing chick optic tectum. *J. Neurosci.* **15**, 4556-4571.
Yoshihara, Y., Kawasaki, M., Tani, A., Tamada, A., Nagata, S., Kagamiyama, H. and Mori, K. (1994). BIG-1: a new TAG-1/F3-related member of the immunoglobulin superfamily with neurite outgrowth-promoting activity. *Neuron* **13**, 415-426.
Yoshihara, Y., Kawasaki, M., Tamada, A., Nagata, S., Kagamiyama, H. and Mori, K. (1995). Overlapping and differential expression of BIG-2, BIG-1, TAG-1, and F3: four members of an axon-associated cell adhesion molecule subgroup of the immunoglobulin superfamily. *J. Neurobiol.* **28**, 51-69.