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Dephrin, a transmembrane ephrin with a unique structure, prevents interneuronal axons from exiting the *Drosophila* embryonic CNS

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SUMMARY

Ephrin/Eph signalling is crucial for axonal pathfinding in vertebrates and invertebrates. We identified the *Drosophila* ephrin orthologue, Dephrin, and describe for the first time the role of ephrin/Eph signalling in the embryonic central nervous system (CNS). Dephrin is a transmembrane ephrin with a unique N terminus and an ephrinB-like cytoplasmic tail. Dephrin binds and interacts with DEph, the *Drosophila* Eph-like receptor, and Dephrin and DEph are confined to different neuronal compartments. Loss of Dephrin or DEph causes the abberant exit of interneuronal axons from the CNS, whereas ectopic expression of Dephrin halts axonal growth. We propose that the longitudinal tracts in the *Drosophila* CNS are moulded by a repulsive outer border of Dephrin expression.

Key words: CNS, *Drosophila melanogaster,* Ephrin, Axonal pathfinding

INTRODUCTION

Identification of the cues and signalling mechanisms that guide outgrowing axons towards their targets is a longstanding and central goal of developmental neurobiology. During the last decade it has become clear that the mechanisms involved in axon guidance are widely conserved between organisms (reviewed by Arendt and Nubler-Jung, 1999; Chisholm and Tessier-Lavigne, 1999). During the development of the spinal cord in vertebrates or the ventral nerve cord in *Drosophila*, interneurones form two major axon tracts to reach their targets. Most interneurones project across the midline of the developing CNS to form commissural tracts. After the axons have crossed the midline, they turn and join the longitudinal tracts that run from anterior to posterior, and in parallel to the midline. Commissure formation depends on two conserved signalling pathways: axons are attracted towards the midline by proteins of the Netrin family (reviewed by Tessier-Lavigne and Goodman, 1996), and are repelled from the midline by the Slit proteins (reviewed by Chien, 1998; Flanagan and Van Vactor, 1998). Both classes of molecules are secreted by cells at the midline. The decision of axons to cross or not to cross depends on the balance between these two opposing signals (Stein and Tessier-Lavigne, 2001).

Less is known about the formation of the longitudinal tracts. The extension of longitudinal axons in the vertebrate spinal cord seems to be defined by two barriers. An inner border defined by the chemorepellent Slit, B class ephrin and semaphorins and an outer border defined by chemorepellents of the Semaphorin family, B class ephrins and BMPs. These repellents squeeze axons out of the cortical layer and force them to grow along a narrow, chemorepellent-free corridor (Augsburger et al., 1999; Imondi and Kaprielian, 2001; Imondi et al., 2000; Zou et al., 2000). In contrast to the vertebrate spinal cord, it is believed that in *Drosophila* longitudinal axons are kept inside the CNS by attractive fasciculation cues. When axons reach a specific distance from the ventral midline, the repulsive activity of Slit decreases and axons can fasciculate with existing pathways to turn and grow in parallel to the midline (Rajagopalan et al., 2000). The pre-existence of 'labelled pathways' (Raper et al., 1983) inside more mature connectives clearly favours this model for guidance of axons during late embryogenesis.

In *Drosophila*, these 'labelled pathways' are laid out during germband retraction, about 9 hours after fertilisation. In response to repulsion by Slit (Brose et al., 1999; Kidd et al., 1999), the first neurones to extend their axons project away from the midline. When the growth cones reach the outer border of the CNS, they turn to the anterior or posterior [figure 8A,B in this report and Jacobs and Goodman (Jacobs and Goodman, 1989)]. The behaviour of these growth cones suggest that, as in vertebrates, the connectives are initially moulded by two repulsive forces: an inner, medial border defined by Slit and an outer, lateral border defined by a second, unknown repellent.

In addition to Slits and Semaphorins, a third major class of axonal repellents is conserved throughout the animal kingdom, the ephrin family (reviewed by Holder and Klein, 1999; Mellitzer et al., 2000). Ephrins are ligands of the Eph receptors, the largest family of receptor tyrosine kinases in vertebrates.

Ligands and receptors are grouped into an A and a B class. Ligands of the A class are tethered to the cell membrane by a GPI anchor. B class ligands have a transmembrane domain and a short cytoplasmic tail.

Ephrin/Eph signalling is important for a diverse array of developmental processes (reviewed by Holder and Klein, 1999) such as topographic mapping of retinal axons onto the tectum in chick embryos (Cheng et al., 1995; Drescher et al., 1995), synaptic remodelling in the adult brain (Gao et al., 1998) and vasculogenesis (Gerety et al., 1999). Ephrin/Eph signalling is cell contact mediated and depends on the clustering of receptors and their ligands (reviewed by Holder and Klein, 1999; Mellitzer et al., 2000; Wilkinson, 2001). Multimerisation activates the kinase activity of the receptor and leads to the phosphorylation of tyrosine, serine and threonine residues in its cytoplasmic tail. The phosphorylated residues permit the binding of a battery of downstream effectors. Eph receptor activation can trigger the depolymerisation of actin in growth cones and can modify integrin based cell adhesion.

Interaction between ephrins and Eph receptors can also activate the ligand (Brueckner et al., 1997; Holland et al., 1996). Tyrosine residues in the cytoplasmic tail of B class ephrins become phosphorylated upon binding to Eph receptors. Cell culture experiments suggest that B class ephrins are clustered into membrane microdomains (rafts) and signal back to the ligand-expressing cell by recruitment of PDZ binding proteins, serine/threonine kinases (Bruckner et al., 1999) and SH2/SH3 adaptor proteins (Cowan and Henkemeyer, 2001). Such bidirectional signalling is important for the formation of the corpus callosum in the vertebrate brain (Kullander et al., 2001; Orioli et al., 1996), to prevent the formation of gap junctions (Mellitzer et al., 1999) and to preclude cell intermingling between rhombomeres in the hindbrain (Xu et al., 1999). The targets and components of the pathway triggered by ephrin activation are not clearly defined yet, but modification of cytoskeletal components and cell adhesion seems to be the main output (Cowan and Henkemeyer, 2001).

The role of ephrins in axon repulsion in vertebrates (reviewed by Wilkinson, 2001) and the identification of an Eph-like receptor (Scully et al., 1999) expressed on the longitudinal axons in *Drosophila* embryos, prompted us to search for an ephrin orthologue in *Drosophila*. We have identified a single *Drosophila* ephrin orthologue, Dephrin. The homology of Dephrin to other ephrins is restricted to the ephrin domain and the most C-terminal amino acids, which form a 'B-like' cytoplasmic tail. Our structural analysis confirms that Dephrin is a transmembrane protein with a cytoplasmic tail. In contrast to all other ephrins, Dephrin has no obvious signal peptide and is cleaved at the N terminus. Dephrin is broadly expressed in neurones and localised on neuronal cell bodies but absent from axons. Conversely, DEph, the *Drosophila* Eph-like kinase, is localised on all interneuronal axons and is absent from cell bodies. We show that inactivation of Dephrin and DEph by RNAi results in the fusion or loss of commissures and breaks in the connectives. Analysis of single axons shows that the loss of Dephrin or DEph causes the aberrant exit of interneurones from the CNS. Ectopic expression of Dephrin in single glial cells or in all midline cells prevents axon extension. We show that these phenotypes rely on Dephrin/DEph interaction. DEphrin binds to DEph in cell culture and repulsion by Dephrin can be overcome by lowering the level of DEph expression. Our results indicate that signalling between Dephrin and DEph creates repulsive barriers that border the commissures and connectives of the embryonic CNS.

MATERIALS AND METHODS

Fly lines

Oregon P embryos served as wild-type controls and were used for dsRNA injections. Transgenic flies were generated by DNA injection into *yw; P(ry, Δ2-3), Sb/TM6, Ubx* embryos (Robertson et al., 1988) as described previously (Brand and Perrimon, 1993). The following GAL4 and UAS lines were used: *sim-GAL4* (Scholz et al., 1997), $GAL4^{24B}$ (Brand et al., 1993), $GAL4^{1580}$ (Hidalgo et al., 1995), $GAL4^{CY27}$ (E. L. Dormand and A. H. B., unpublished), *UAS-Dephrin* (line 31.19) and *UAS-tauGFP6* (line 12/2/3) (Kaltschmidt et al., 2000). $GAL4^{CY27}$ drives expression primarily in MP2 neurones. In stage 17 midline neurones and neurones of the lateral CNS are added to the expression pattern.

Molecular biology

RNA was isolated from embryos of all stages according to the method of Brown and Kafatos (Brown and Kafatos, 1988). Isolated RNA was reverse transcribed using the SMART RACE cDNA Amplification kit (Clontech). The pool of synthesized first strand cDNAs served as templates for 5′ RACE PCR using two different *Dephrin*-specific primers and a commercially available 5′ RACE primer (Clontech). PCR products were cloned into the pCR 2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and sequenced.

Genomic sequence was obtained by *Eco*RI restriction and subsequent ligation of genomic DNA isolated from adult flies. The circularized genomic DNA fragments served as templates for inverse PCR using *Dephrin*-specific primers. PCR products were cloned into the pCR2.1-TOPO vector and sequenced. We also performed PCR on genomic P1 clones (DS04877, DS00309 kindly provided by Steve Russell) to which *Dephrin* has been mapped by hybridisation to P1 blotted filters (Research Genetics).

All constructs were generated by PCR amplification using suitable 5′ and 3′ primers with added restriction sites. The PCR fragments were sequenced and cloned into the pUAST or pWR-Pubq (a kind gift from Nick Brown). For a detailed protocol see http://www.elc.cam.ac.uk/~brandlab/index.html.

ESTs

The following ESTs from the BDGP are derived from the *Dephrin* RNA and contain the start codon: LD01709, LD11081, LD17721, LD01229 and LD11109. LD11109, LD01229 were sequenced. GH24276, GH24311 are partial cDNAs starting 724 bp downstream of the first methionine and include the 3′UTR. The 3′UTR of GH24276, GH24311 and LD01229 are identical. LD11109 has a 300 bp shorter 3′UTR.

Cell culture

Drosophila S2 cells were grown in Schneiders medium with 10% heat-inactivated fetal bovine serum (FBS) added. The calcium phosphate method was used for transformation. To express the various UAS constructs we co-transfected the UAS vectors with a plasmid carrying the alcohol dehydrogenase promoter in front of GAL4. 10 μg of each plasmid were added to 600 μl transformation mix. Cells were kept in the transformation mix for 16-20 hours at room temperature (RT), washed twice with Schneiders medium with 10% FBS and plated back into the six-well plates used for transformation. Cells were harvested after 3 days at 28°C.

For antibody stainings and binding assays, cells were plated into a small silicone rubber ring onto the surface of a clean coverslip. Cells

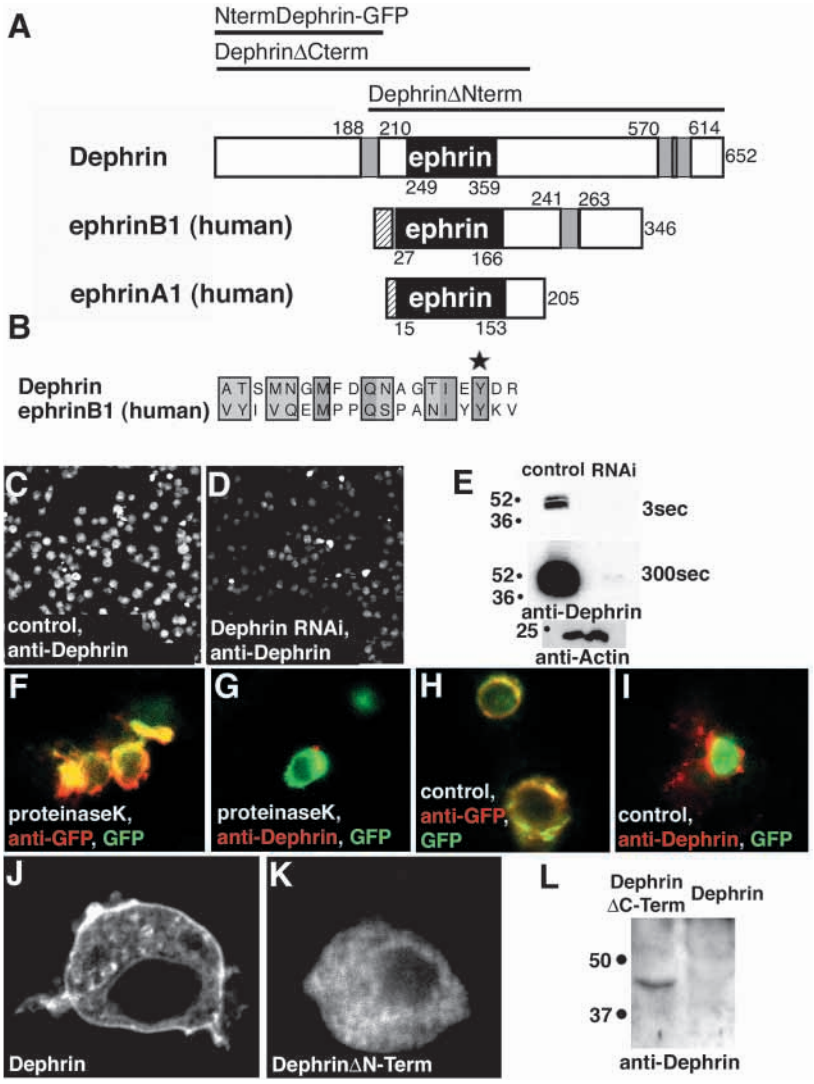

Fig. 1. Dephrin is a membrane protein with a cytoplasmic tail similar to B class ephrins. (A) Structural comparison between Dephrin, a vertebrate B class and an A class ephrin. The C terminus of Dephrin includes two predicted transmembrane domains (grey box). In contrast to all other ephrins, Dephrin also has a predicted transmembrane domain in front of its ephrin domain (black box) and no obvious signal sequence (hatched box in ephrin B1 and A1). Numbers are amino acid positions. Lines at the top denote different parts of Dephrin included in the indicated constructs. (B) ClustalW alignment of the most C-terminal amino acids of Dephrin and a B class ephrin. The tyrosine residue at the –3 position from the C terminus (star) is conserved in Dephrin. (C,D) The ephrin domain of Dephrin is extracellular. (C) Antisera against the ephrin domain of Dephrin bind to the surface of non-permeabilised *Drosophila* S2 cells in vivo. (D) Treatment of S2 cells with doublestranded (ds)Dephrin RNA greatly diminishes the antibody signal. (E) Incubation of S2 cells with dsDephrin RNA significantly reduces the expression of Dephrin. Anti-Dephrin recognizes two strong bands in lysates from untreated S2 cells (control). In dsDephrin RNA treated cells (RNAi), the bands are nearly absent. Protein mass in kDa ($\times 10^3$) on the left, exposure time on the right. Actin served as loading control. (F-I) The C terminus of Dephrin is protected from proteinase digestion. Proteinase K is not able to digest the C-terminal GFP tag in Dephrin-GFP-expressing embryonic clones (F, red) but the proteinase destroys the extracellular ephrin domain (G, red). Without proteinase incubation, anti-GFP (H, red) and anti-Dephrin (I, red) bind to their antigen at the membrane. Clones in F-I were stained without detergent. Dephrin-GFP fluoresces strongly only in the cytoplasm. (J,K) The N terminus of Dephrin (aa 1-202) is necessary for membrane localisation of the protein. In S2 cells transfected with full length Dephrin (Dephrin), the protein is localized at the membrane and in cytoplasmic vesicles (J). Expression of an N-terminal truncated form of Dephrin (DephrinΔN-Term) in S2 cells results in a diffuse cytoplasmic distribution of the protein (K). The high level expression of DephrinΔN-Term obscures endogenous DEphrin at the membrane. (L) The C terminus of Dephrin (aa418-aa652) serves as membrane anchor. S2 cells were transfected with a C-terminal truncation of Dephrin (DephrinΔC-Term) or full length Dephrin (Dephrin). Only cells expressing DephrinΔC-Term showed a strong accumulation of the protein in the medium.

were washed three times with PBS, incubated with DEphex-GFP medium (30 minutes at RT) or anti-Dephrin (1 hour at RT), washed six times with PBS and fixed with 20% formaldehyde in PBS (10 minutes). To detect DEphex-GFP, cells were incubated with α-GFP primary antibody in PBS (1 hour at RT), washed ten times with PBS and incubated with secondary antibody in PBS (1 hour at RT). To detect anti-Dephrin, the cells were washed 10 times with PBS and incubated with the secondary antibody in PBS (1 hour at RT). For all other antibody stainings cells were permeabilised by addition of 0.3% Triton X-100 to PBS (PBT).

Protein biochemistry and antibody generation

Protein was isolated by homogenisation of embryos of all stages in protein sample buffer [62.5 mM Tris (pH 6.8), 50% glycerol, 1% SDS, 0.02% Bromophenol Blue] or by dissolving cell pellets in cell lysis buffer [50 mM Tris (pH 7.8), 150 mM NaCl, 1% IGEPAL]. SDS-PAGE and western blots were performed according to standard procedures.

We generated a polyclonal antiserum against the extracellular domain of Dephrin (aa 242-423). The *Eco*RI fragment from EST GH24276 was cloned into the pRSET C expression vector (Invitrogen). Protein expression was induced in C41 cells (Miroux and Walker, 1996) by addition of 0.2 mM IPTG and the bacteria were incubated for 8 hours at 37°C. Protein was prepared under denaturing conditions (8 M urea) using standard protocols (QIAexpressionist). Rabbits were immunised according to the manufacturer's protocol (abcam).

Immunohistochemistry and in situ hybridisation

Immunohistochemistry was performed as previously described (Bossing et al., 1996). The following primary antibodies were used: mAb BP102, 1:50 (kindly provided by N. Patel) (Seeger et al., 1993); anti-DEK, 1:10 (Scully et al., 1999); anti-Fasciclin II, 1:5 (kindly provided by C. S. Goodman); anti-futsch (22C10), 1:10 (kindly provided by S. Benzer) (Fujita et al., 1982); anti-GFP, 1:2000 (abcam); anti-wrapper, 1:10 (Noordermeer et al., 1998) and preadsorbed anti-Dephrin antiserum, 1:1000. Secondary antibodies conjugated to alkaline phosphatase, biotin, HRP (Jackson

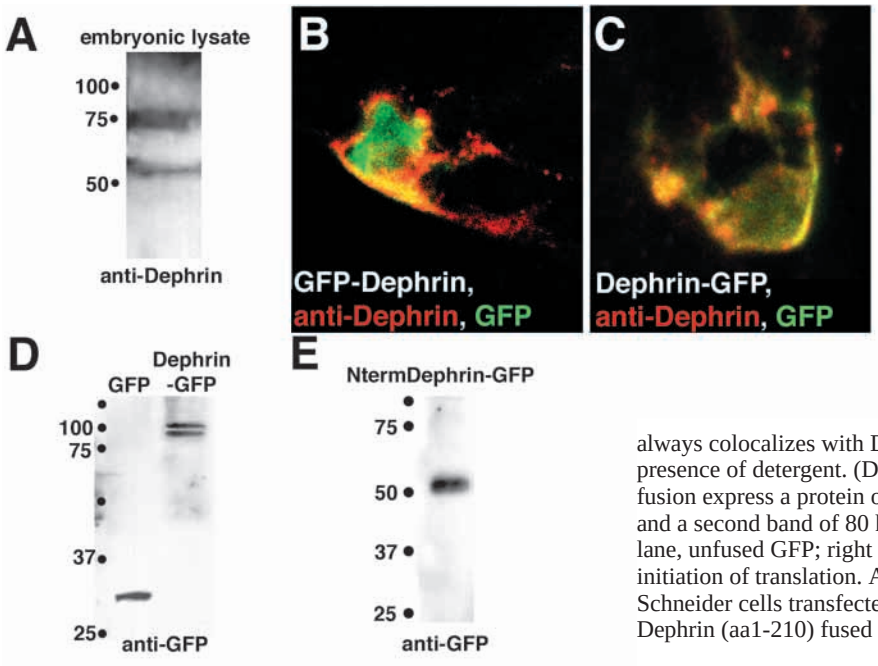

Fig. 2. Dephrin is cleaved at the N terminus and shows no alternative translation. **(A)** Western blots of embryonic lysate reveal a band around 75 kDa, the predicted size of Dephrin and a band at 52 kDa. (B,C) Mosaic clones were generated by injection of a UAS plasmid carrying a fusion of GFP to the N terminus (B, GFP-Dephrin) or the C terminus (C, Dephrin-GFP) of Dephrin. In embryonic cells expressing GFP-Dephrin, the GFP (green) is mainly localised in the cytoplasm, whereas Dephrin (red) accumulates at the membrane (B). The different subcellular localisation of the two parts of the fusion protein indicates a cleavage at the N terminus. In cells expressing Dephrin-GFP (C), the GFP always colocalizes with Dephrin (yellow). Clones were stained in the presence of detergent. (D) Schneider cells transfected with a Dephrin-GFP fusion express a protein of 100 kDa (75 kDa of Dephrin + 27 kDa of GFP) and a second band of 80 kDa (52 kDa of Dephrin + 27 kDa of GFP). Left lane, unfused GFP; right lane, Dephrin-GFP (E) Dephrin shows no alternative initiation of translation. Anti-GFP reveals only one band in extracts from Schneider cells transfected with a plasmid encoding the N terminus of Dephrin (aa1-210) fused to GFP.

Laboratories), Alexa 488, or Alexa 568 (Molecular Probes) were used at a dilution of 1:250 to 1:1000. All antibodies were diluted in PBST (PBS, 0.3% Triton X-100, 20% newborn calf serum). Biotin-coupled antibody reactions were enhanced using the Vectastain ABC Kit (Vector labs).

For in situ hybridisation a digoxigenin-labelled single-stranded DNA probe was generated by PCR (Patel and Goodman, 1992). Post hybridisation washes were carried out according to the method of Lekven et al. (Lekven et al., 1998).

All embryos were mounted as flat preparations in 90% glycerol in PBS. Images were collected on a Zeiss axiophot (DIC optics) or with a BioRad MRC 1024 confocal scanhead mounted on a Nikon E800 microscope. Images were assembled in Adobe Photoshop 6.

RNA interference

dsRNA was generated by PCR amplification of specific regions of *DEph*, *Dephrin*, *GFP* or *CFP* using 5′ and 3′ primers that contain a T7 consensus site and gene-specific sequences. The PCR fragments were gel purified and used as templates for in vitro transcription by T7 polymerase (Ambion; 6 hours at 37°C). The length of dsDephrin is 563 bp (+745 to +1308) comprising the ephrin domain. dsDEph is 1396 bp long (–72 to + 1324) including most of the extracellular domain. dsGFP is 417 bp (+170 to +587) and dsCFP is 718 bp (+1 to +718) long. The concentration of injected dsRNAs was 2.0 mg/ml. Injection of dsGFP at a concentration of 1.5 mg/ml into *sca-GAL4; UAS-GFP* embryos eliminates GFP expression completely.

Embryos were manually dechorionated about 2 hour after fertilisation, glued to a coverslip, desiccated at room temperature (23°C) for 4-6 minutes and covered with halocarbon oil (Voltalef 10s). Embryos were injected laterally at the syncytial blastoderm stage and allowed to develop at 18°C overnight, followed by 25°C until late embryogenesis (stage 16/17). The coverslip was covered with PBT and the halocarbon oil removed by a stream of PBT. The embryos were fixed with 8% formaldehyde in PBT for 20 minutes on a shaker, washed three times in PBS and the vitelline membrane was removed manually. After three washes in methanol, the embryos were rehydrated in PBT and incubated in primary antibody.

For RNAi treatment of S2 cells the protocol of Clemens et al. (Clemens et al., 2000) was slightly modified. The cell culture medium (cell density between 6×10^6 to 10^7) was removed by centrifugation (2000 ***g***, 2 minutes) and the cell pellet was resuspended in 1 ml of DES medium (Invitrogen) with 30 μg of dsRNA added. Cells were plated in six-well plates (Nunclon). After 2 hours of incubation at RT, 2 ml of Schneiders medium with 10% FBS were added. Cells were allowed to grow at 28°C for 3 days.

Mosaic expression and proteinase K treatment

UAS-Dephrin and UAS-tau-mGFP6 (Kaltschmidt et al., 2000) plasmids were purified (Qiagen) and dissolved in H_2O. DNA (100-500 μg/ml) was injected laterally into GAL4-expressing embryos at the syncytial blastoderm stage. The site and frequency of expression depends on the GAL4 driver. More than 70% of the injected embryos usually show expression. Expression can first be detected about 3 hours after the onset of GAL4 expression (25°C). The strength of mosaic expression varies between cells but is significantly stronger than in stable transformants crossed to the same GAL4 drivers. DNA injection and immunohistochemistry were carried out as described for RNAi.

We generated clones of Dephrin-GFP-expressing cells by injection of the pWR-Pubq-Dephrin-GFP plasmid into the syncytial blastoderm of wild-type embryos. Embryos were injected laterally to restrict expression to ectodermal and neuronal cells. Embryos with clones were flat prepped in PBS and incubated for 45 seconds with proteinase K (50 μg/ml; Roche). Proteinase K digestion was stopped by incubation in glycine (2 mg/ml) twice for 2 minutes, followed by several washes in PBS and fixation in 3.7% formaldehyde, PBS for 20 minutes. After additional washes the flat preparations were stained for 2 hours with primary antibodies and 1 hour with secondary antibodies. All antibodies were diluted in PBS. We established the experimental parameters for proteinase K digests of living embryos by using an antiserum against an extracellular antigen (anti-Robo1) (Kidd et al., 1999) and an antiserum against Discs large 1 (Woods and Bryant, 1991), an internal antigen at the cell cortex (data not shown).

Single cell labelling

In vivo labelling of single cells with DiI and photoconversion was performed as described previously (Bossing and Technau, 1994; Bossing et al., 1996).

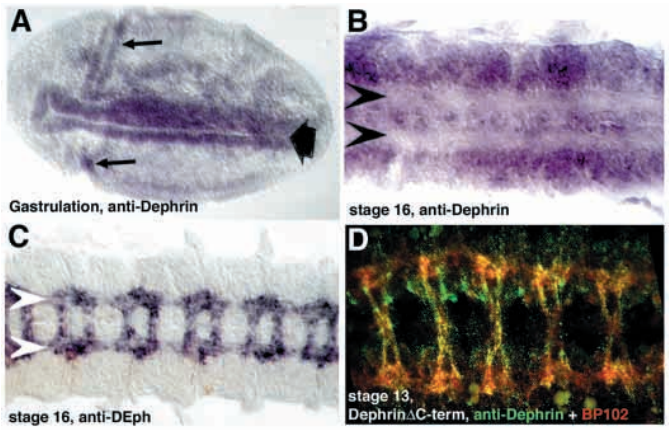

Fig. 3. Dephrin is expressed during gastrulation and in the embryonic CNS. (A) During gastrulation, Dephrin expression concentrates at the invaginating cephalic furrows (thin arrows) and at the invaginating mesoderm (thick arrow). (B) After germband retraction, expression of Dephrin is restricted to neuronal cell bodies. No protein is detectable on axonal tracts (arrowheads). (C) Expression of DEph, the Eph-like kinase in *Drosophila*, is restricted to axons and absent from the cell bodies. (D) Expression in muscles (Gal4 line 24B) of the secreted DephrinΔC-Term, which has an intact receptor binding domain, shows an accumulation of the truncated form (green, α-Dephrin) along axons (red, BP102). Horizontal views; anterior to the left.

Bioinformatics

For homology searches we used FlyBLAST (www.fruitfly.org) and NCBI-Blast (www.ncbi.nlm.nih.gov). The Dephrin structure was analysed using MacVector 7.0 (Oxford Molecular), SMART (www.embl.de), PredictProtein (www.embl.de), prosite pattern search (ca.expasy.ch) and SignalP V1.1 (www.genome.cbs.dtu.dk) (Nielsen et al., 1997).

RESULTS

Dephrin shows homology to the cytoplasmic tail of B class ephrins

We identified an ephrin orthologue in *Drosophila* that we call Dephrin (GenBank accession number AF216287). The overall similarity of Dephrin to other members of the ephrin family is low (8% similarity to human ephrinB1, 7% similarity to the *C. elegans* ephrin vab-2 and only 3% to human ephrinA1). However, Dephrin shows significant homology in its central domain (black, Fig. 1A) to the extracellular domain of ephrins. The ephrin domain in Dephrin is as homologous to A ephrins (41% to human ephrin A1) as to B ephrins (42% to human ephrin B1) with a slightly higher homology to ephrins from *C. elegans* (46% to vab-2).

The Berkeley *Drosophila* Genome Project (BDGP) identified two ESTs with significant homology to the extracellular domain of vertebrate ephrins (see Materials and Methods). Neither EST encodes a potential start codon. We recovered a complete cDNA by 5′ RACE. The 5′ end of this cDNA was compared to the BDGP data set and five additional ESTs were recovered. Two of these were sequenced and shown to be identical to the ORF derived by 5′ RACE. In total, our sequence for *Dephrin* is compiled from four ESTs provided by the BDGP and three full length cDNAs generated by independent PCR reactions using an embryonic cDNA pool as a template. There is no evidence for alternate transcripts other than an alternative polyA site in one of the four ESTs, which shortens the common 3′ untranslated region by about 300 bp.

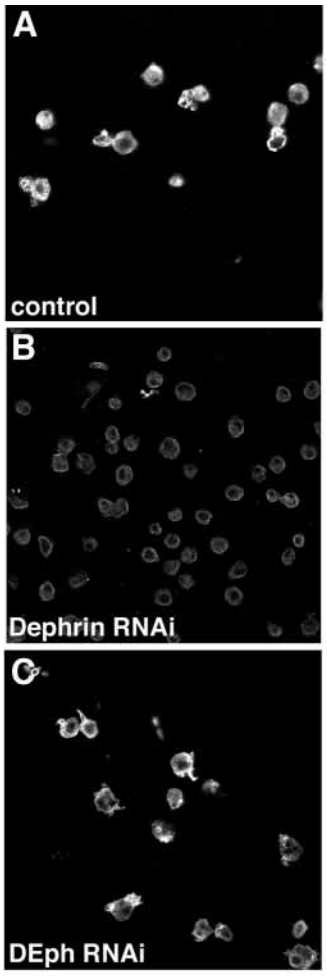

Fig. 4. DEph binds to Dephrin. (A) *Drosophila* S2 cells were incubated with medium derived from cell cultures expressing the extracellular part of DEph fused to GFP at the C terminus (DEphex-GFP). Owing to the endogenous expression of Dephrin, DEphex-GFP in the medium binds to the surface of untreated S2 cells (control) and the cells can be stained with α-GFP. (B) Lowering the expression of Dephrin by incubating S2 cells with dsDephrin RNA (Dephrin RNAi) abolishes the binding of DEphex-GFP, confirming the specificity of the binding. (C) Control incubation of S2 Cells with dsDEph RNA (DEph RNAi) does not interfere with the binding of DEphex-GFP.

In situ hybridisation to salivary glands maps *Dephrin* to position 102C on the fourth chromosome. The gene comprises four exons and three introns. The ephrin domain is encoded by the second exon and the beginning of the third exon.

Dephrin encodes a predicted protein of 652 amino acids. Homology to other ephrins is restricted to the ephrin domain and the C terminus. In vertebrates the most C-terminal sequence differs between A and B class ephrins. A-class ephrins end with a hydrophobic stretch of amino acids. B-class ephrins have a highly conserved and hydrophilic C terminus encoding at least five tyrosines that are phosphorylated upon interaction with Eph receptors in cell culture (Brueckner et al., 1997). Ephrin B1 in the chicken retina is primarily phosphorylated in vivo at the tyrosine residue at position –3 from the C terminus (Kalo et al., 2001). The predicted cytoplasmic tail of Dephrin is hydrophilic and shows sequence homology to B class ephrins (Fig. 1B). In addition, the tyrosine at position –3 from the C terminus is conserved (star, Fig. 1B).

The C terminus of the cytoplasmic tail of B class ephrins and most Eph receptors forms a PDZ binding domain (reviewed by Mellitzer et al., 2000). A PDZ binding consensus

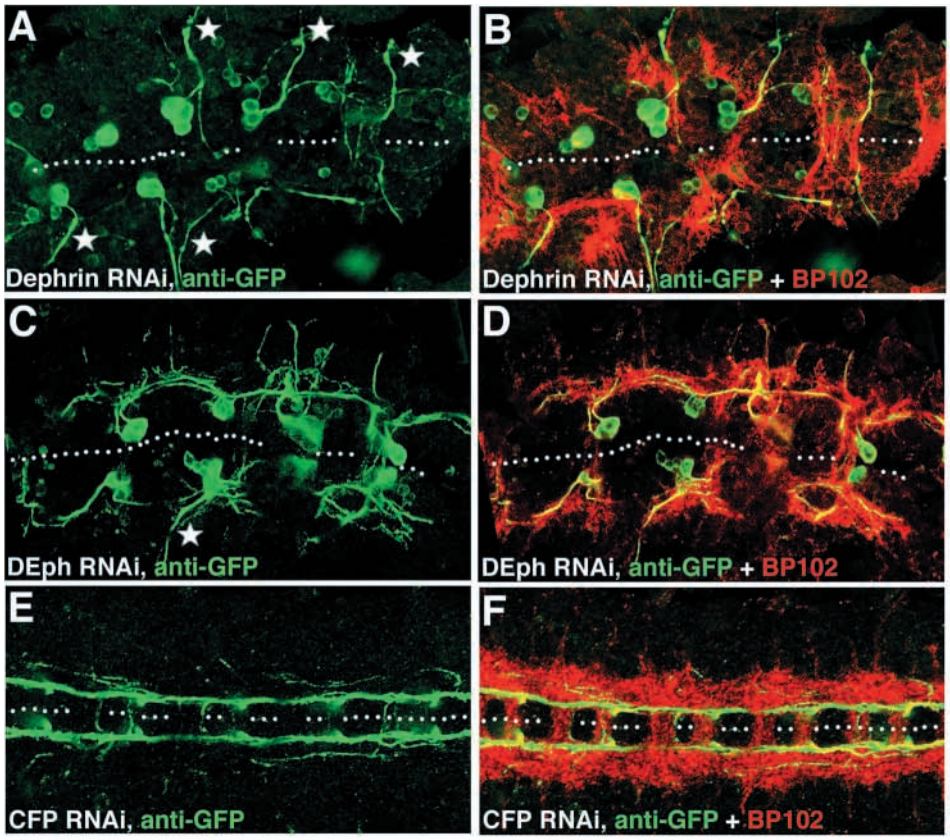

Fig. 5. Loss of Dephrin results in the abnormal exit of interneuronal axons from the CNS. We used a transformant line expressing tau-GFP to primarily label the projections of two interneurones (MP2 neurones, Gal4 line CY27). The axons of these neurones form a fascicle that extends in parallel on both sides of the ventral midline (dotted line; E,F). (A,B) Reducing the expression of Dephrin causes the interneuronal axons to exit the CNS (A, star) and severely disrupts the axonal scaffold (B, BP102). (C,D) DEph RNAi causes similar phenotypes as Dephrin RNAi. The interneuronal axons exit the CNS (C, star) or fasciculate loosely with each other. The axonal scaffold (D) is also severely disrupted. (E,F) Injection of dsCFP RNA rarely interferes with the projections of the MP2 neurones (E) or with the general layout of the axonal scaffold (F). Horizontal views of stage 17 embryos, anterior to the left.

is also present at the C terminus of DEph, the Eph-like receptor (Scully et al., 1999). In contrast, the cytoplasmic tail of Dephrin does not contain a consensus PDZ binding domain.

A novel assay to study protein localisation and function in live *Drosophila* embryos

To study the localisation and structure of Dephrin, we developed a new technique to transiently express proteins in living *Drosophila* embryos. Compared to the generation of stable transformants, which takes up to 6 weeks, transient expression enables protein localisation and potential phenotypes to be studied after only a few hours.

We injected syncytial blastoderm embryos with plasmids in which expression is driven by a constitutive promoter (Polyubiquitin; Fig. 1F-I, Fig. 2C) or by the GAL4 UAS system (Fig. 2B, Fig. 6G,H). The injections result in expression in small cell clusters located near the site of injection. The time and cell type of expression can be selected by choosing the site of injections according to the embryonic fate map of *Drosophila* (Polyubiquitin plasmids) (Hartenstein et al., 1985) or by injecting into a GAL4 transformant strain (Brand and Perrimon, 1993) with the desired expression pattern (UAS plasmids). Expression can be examined either 2 hours after the injection of Polyubiquitin plasmids or 3 hours after the onset of GAL4 expression (at 25°C). 80% of all embryos injected with the Polyubiquitin vector show expression. Expression of UAS plasmids depends on the GAL4 strain and varies between 40-80%.

Dephrin is a transmembrane protein with an extracellular ephrin domain and a cytoplasmic tail

The overall structure of Dephrin differs significantly from all other ephrins. The ephrin domain is not located at the N terminus but in the middle of the protein (Fig. 1A). Dephrin has no obvious signal peptide and an additional predicted transmembrane domain precedes the ephrin domain. To confirm Dephrin as a genuine member of the ephrin family we examined the structure of the protein in more detail.

We generated a polyclonal antibody against the ephrin domain of Dephrin. This antibody binds to the cell surface of

Table 1. The loss of Dephrin or DEph results in fused or lost commissures and breaks in the connectives

Phenotype	Dephrin RNAi	DEph RNAi	CFP RNAi	Buffer
Wild-type commissures	70% (140)	43% (61)	94.6% (140)	93.4% (156)
Commissures fused	20% (40)	45% (64)	4% (6)	6% (10)
Commissures lost	10% (20)	12% (17)	1.3% (2)	0.6% (1)
Wild-type connectives	90.5% (163)	86.7% (111)	99.2% (133)	98.7% (149)
Breaks in connectives	9.5% (17)	13.3% (17)	0.7% (1)	1.3% (2)

Axons were stained with BP102 antiserum. dsCFP RNA (CFP RNAi) or buffer were injected as controls. Numbers represent segments.

Table 2. The loss of Dephrin or DEph causes MP2 interneurones to exit the CNS

No. of hemisegments with outgrowing MP2 neurons	Dephrin RNAi	DEph RNAi	CFP RNAi	Buffer
0 (wild type)	5	3	16	17
1	3	4	4	
2	2	3		
3	2	3		
4	1	1		
5	2	1		
6	1			
7	1	1		
8		1		
9	1			
:				
12	1			
:				
20	1			

CY27; UAS-tauGFP6 embryos were injected with dsDephrin RNA (Dephrin RNAi), dsDEph RNA (DEph RNAi), dsCFP RNA (CFP RNAi) or buffer. Numbers in the left column are hemisegments (total number=20). All other numbers represent embryos.

non-permeabilised *Drosophila* S2 cells in vivo, which express Dephrin endogenously (Fig. 1C). Incubation of S2 cells with doublestranded (ds) Dephrin RNA (Dephrin RNAi) reduces Dephrin expression (Fig. 1E) and also diminishes the binding of anti-Dephrin to the cell surface (Fig. 1D). Thus, the binding of the antibody is specific for Dephrin and the ephrin domain is extracellular.

We also examined the localisation of the C terminus of Dephrin. We labelled the C terminus with a GFP tag (Dephrin-GFP). If the C terminus is extracellular, proteinase K treatment of non-permeabilised Dephrin-GFP-expressing cells should digest the GFP tag and the extracellular ephrin domain. If the C terminus is cytoplasmic, the intact membrane should protect the GFP tag, while the extracellular ephrin domain should be destroyed. Dephrin-GFP-expressing cells were generated by plasmid injection into the syncytial blastoderm of wild-type embryos. Only cells strongly expressing Dephrin-GFP can be recognised by GFP fluorescence. In flat preparations of living and non-permeabilised embryos the C-terminal GFP tag is always protected from proteinase K digestion (Fig. 1F, n=5), but the ephrin domain is always destroyed (Fig. 1G, n=6). Without proteinase K digestion, anti-GFP (Fig. 1H) and anti-Dephrin (Fig. 1I) bind to the membrane of Dephrin-GFP-expressing cells. The anti-GFP signal overlaps the GFP fluorescence, whereas the anti-Dephrin signal is confined to the outside of the cell. This differential staining and the proteinase treatment strongly suggest the existence of a C-terminal cytoplasmic tail in Dephrin.

The N terminus of Dephrin is essential for membrane localisation but not for membrane anchoring

Although Dephrin has no obvious signal peptide, the localisation of Dephrin to the membrane depends on its N terminus. Full length Dephrin expressed in S2 cells (Fig. 1J) and in embryos (Fig. 2B,C) accumulates at the membrane and in cytoplasmic vesicles. Deletion of the N terminus (aa 1-202) results in a diffuse distribution of the truncated protein in the cytoplasm of S2 cells (Fig. 1K) and embryos (data not shown).

Dephrin has three predicted transmembrane domains, one in the N terminus and two at the C terminus. If all domains are genuine membrane anchors, deletion of the C terminus of Dephrin should not interfere with membrane localisation. A C-terminal truncation still carries the N-terminal sequences necessary for membrane localisation and the predicted transmembrane domain preceding the ephrin domain. Expression of such a truncation (UAS-DephrinΔC-term, deletion of aa419-aa652) in S2 cells leads to an accumulation of the protein in the medium (Fig. 1L). In contrast, Dephrin can never be detected in the medium of S2 cells. We conclude that DephrinΔCterm is secreted, suggesting that the protein is still sorted correctly to the membrane but the hydrophobic domain at the N terminus is not able to anchor the protein at the membrane. Anchoring at the membrane most likely requires the predicted transmembrane domains at the C terminus.

Dephrin is cleaved at the N terminus

In western blots of S2 cell lysates, anti-Dephrin reveals two prominent bands at ~50 kDa (Fig. 1E) and frequently a weaker band at ~75 kDa, the predicted size of Dephrin. In embryonic lysates, anti-Dephrin detects a band at ~51 kDa and ~75 kDa (Fig. 2A). Since the Dephrin antisera was generated against the ephrin domain, these bands represent different forms of Dephrin which all contain the ephrin domain. Our cDNA analysis revealed only one *Dephrin* transcript. Therefore the two different isoforms of Dephrin might either result from protein cleavage or from alternative initiation of translation.

To examine the possibility of protein cleavage we generated GFP fusions to the N terminus (GFP-Dephrin) and the C terminus (Dephrin-GFP). Cleavage of the protein at either terminus should separate the GFP from Dephrin.

Expression of GFP-Dephrin in S2 cells or embryos results in a different subcellular distribution of GFP and Dephrin. GFP is mainly found in the cytoplasm, whereas Dephrin concentrates at the membrane (Fig. 2B). Interestingly, the GFP tag at the N terminus does not interfere with the membrane localisation of Dephrin. We failed to detect a GFP band in Western blots of lysates taken from GFP-Dephrin-expressing S2 cells or embryos. The absence of GFP might indicate a degradation of the N-terminal cleavage product. In contrast, GFP and Dephrin always co-localise in S2 cells or embryos expressing Dephrin-GFP (Fig. 2C). Western blots of lysates taken from Dephrin-GFP-expressing S2 cells confirm the absence of cleavage at the C terminus (Fig. 2D).

We noted that the *Dephrin* mRNA has a translation initiation consensus (Cavener, 1987) in front of the methionine doublet at position +544. A start of translation at this site would result in a 50 kDa protein with a signal peptide. To test this possibility we fused the first 630 bp of the *Dephrin* mRNA to GFP (NtermDephrin-GFP). If translation can start at the beginning and in the middle of the *Dephrin* mRNA, we would expect that transfection of Schneider cells would result in two proteins of different sizes. However, S2 cells only produce one protein migrating at around 51 kDa (Fig. 2E), a size expected from a translational initiation at the first methionine (24 kDa of Dephrin + 27 kDa of GFP).

We concluded that the two different isoforms of Dephrin result from N-terminal cleavage of the protein. This cleavage depends on the full length molecule; we cannot detect a

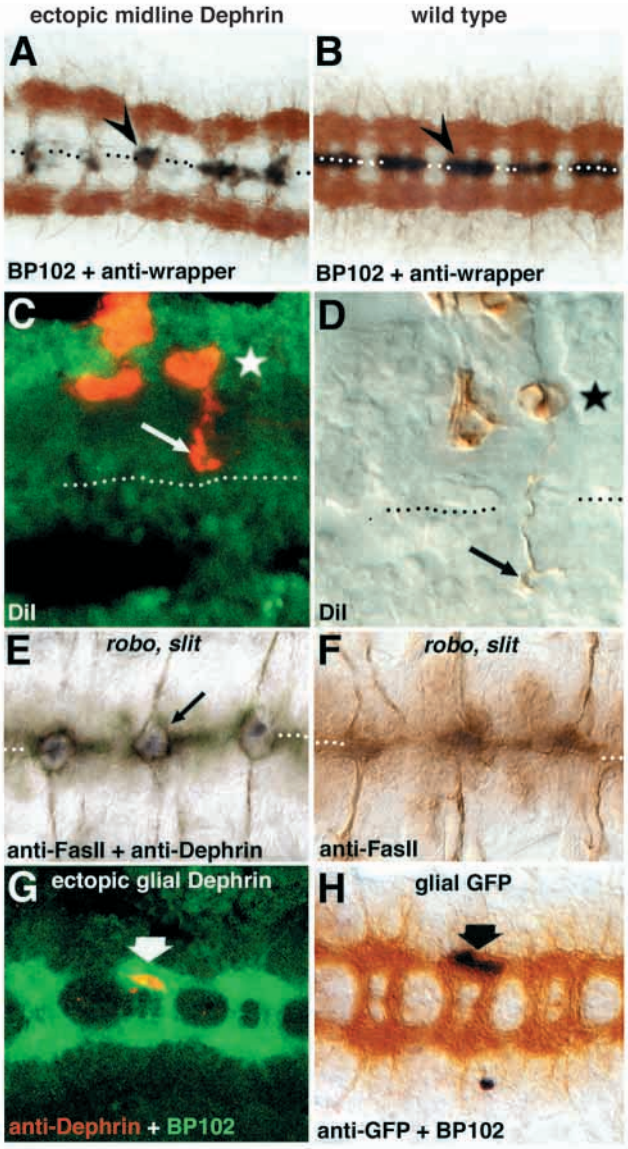

Fig. 6. Ectopic expression of Dephrin causes axonal repulsion. (A,B) Ectopic expression of Dephrin in midline cells (A) causes a severe thinning of the commissures (arrowhead) but does not interfere with the determination of midline glia (black, anti-Wrapper). As in wild type (B), midline glia still tightly enwrap the commissural fibres. (C,D) Ectopic expression of Dephrin in midline cells prevents axons from crossing the ventral midline. The lineage of one neural precursor includes glial cells and three contralateral projecting neurones (star). In embryos with ectopic midline expression of Dephrin (C), the projections of these neurones (thin arrow) are stalled at the midline. In a 1-hour younger wild-type embryo (D), the axons (thin arrow) have already crossed the midline. (E,F) Axonal repulsion by ectopic Dephrin does not depend on Slit or Robo1. Ectopic expression of Dephrin in midline cells of *slit, robo1* double mutants (E, purple, arrow) is able to push axons (FasII, brown) out of the midline. In *slit, robo1* double mutants (F), the longitudinal tracks collapse at the midline. (G,H) Ectopic expression of Dephrin in longitudinal glia cells (G, thick arrow) causes breaks in the axonal scaffold (green, BP102). Longitudinal glia cells expressing GFP (H, thick arrow) do not perturb the formation of the axonal scaffold (brown, BP102). Horizontal views of stage 17 embryos (except D, stage 16); anterior to the left. Dotted line marks the midline.

cleavage of the DephrinΔCterm truncation (Fig. 1L) or the NtermDephrin-GFP fusion (Fig. 2E). The cleavage of Dephrin yields a band of about 51 kDa in embryonic lysates. The doublet around 50 kDa in S2 cells might indicate different phosphorylation or glycosylation states of Dephrin.

Dephrin is widely expressed on neuronal cell bodies and binds to DEph

Dephrin mRNA and protein expression starts at the syncytial blastoderm (about 1.5 hours after fertilisation) and is ubiquitous. In situ hybridisation and antibody stainings of unfertilized eggs suggest that *Dephrin* is not expressed maternally. At gastrulation Dephrin expression is restricted to the invaginating mesoderm and to cells lining the cephalic furrow (Fig. 3A). No mRNA or protein can be detected during germband elongation. At the start of germband retraction, expression resumes in the ventral ectoderm, ventral muscles and the CNS. In the CNS the mRNA and protein can be found in a subset of 4-6 cells at the ventral midline, in medial and lateral cell clusters in the dorsal cortex and nearly all cells of the ventral cortex (Fig. 8D). After germband retraction (stage 13) the mRNA and protein are restricted to the CNS, with the highest level of expression along the outer border of the longitudinal axon tracts (Fig. 3B). The expression pattern of Dephrin complements that of DEph, a potential receptor for Dephrin (Scully et al., 1999). *Dephrin*, is transcribed in neurones and the protein is confined to the cell body and very low or absent on axons. *Deph* is also transcribed in neurones but the protein is confined to axons (Fig. 3C).

To test if Dephrin is able to bind to axons, we expressed the secreted DephrinΔC-term truncation, which has an intact receptor binding domain. Expression of DephrinΔC-term in muscles overlying the CNS (GAL4 line 24B) results in a specific accumulation of the truncated protein on axons (Fig. 3D). In vertebrates, injection of secreted forms of ephrins give a dominant negative phenotype (reviewed by Holder and Klein, 1999). However, expression of DephrinΔC-term in CNS or muscles (*elav-GAL4*, *sim-GAL4* and *GAL4*^{24B}) failed to cause any obvious defects. This lack of phenotypes is most likely due to insufficient levels of expression.

The accumulation of DephrinΔC-term around axons suggests that Dephrin may bind to DEph. We confirmed the binding between Dephrin and DEph in cell culture. *Drosophila* S2 cells were transfected with a UAS construct encoding the extracellular part of DEph fused to GFP (DEphex-GFP). After incubation with DEphex-GFP-containing medium, non-permeabilised Schneider cells can be labelled with anti-GFP (Fig. 4A). Hence, DEphex-GFP can bind onto the surface of S2 cells that express endogenous Dephrin. To confirm that DEphex-GFP binds to Dephrin we lowered the level of Dephrin expression by incubation of S2 cells with dsDephrin, Indeed, Dephrin RNAi treatment of S2 cells diminishes the binding of DEphex-GFP (Fig. 4B). Control incubation of Schneider cells with dsGFP RNA (GFP RNAi, data not shown) or dsDeph RNA (DEph RNAi, Fig. 4C) does not interfere with the binding.

Loss of Dephrin and DEph disrupts commissures and connectives

Both *Dephrin* and *DEph* map to the fourth chromosome, for which it is very difficult to obtain and maintain mutants by

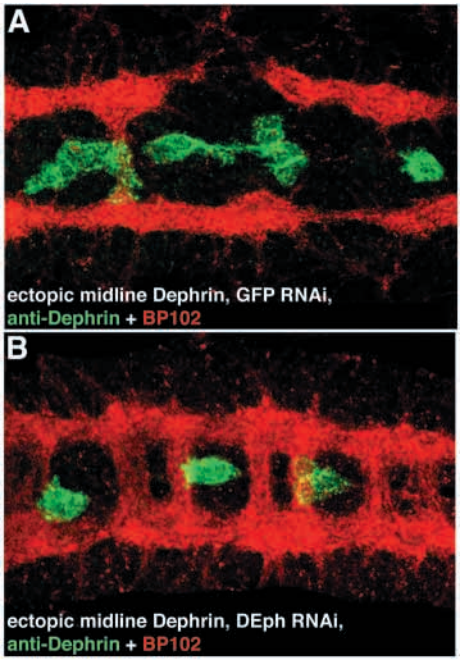

Fig. 7. Repulsion by Dephrin depends on DEph. (A,B) Injection of dsGFP RNA (A) increases the severity of the axonal phenotype caused by ectopic Dephrin in midline cells (compare with 6A). Commissures are often lost completely. In contrast, lowering the expression of DEph by DEph RNAi (B) allows axons (red) to cross over ectopic midline sources of Dephrin (green). Injection of dsDEph RNA into embryos with ectopic midline expression of Dephrin frequently restores the axonal scaffold to normal. Horizontal views of stage 17 embryos; anterior to the left.

classical genetic techniques. For this reason we have used RNAi (reviewed by Bosher and Labouesse, 2000; Nishikura, 2001) to inhibit expression. RNAi has rapidly become an accepted technique for generating mutant phenotypes (Fraser et al., 2000; Gonczy et al., 2000; Kalidas and Smith, 2002; Paddison et al., 2002). In test injections of dsDephrin RNA only two out of nine injected embryos show a nearly complete loss of Dephrin (http:/www.elc.cam.ac.uk/~brandlab/index.html), while the remainder retains about 20-50% of wild-type expression. Therefore, we did not expect that Dephrin RNAi would lead to a mutant phenotype in all injected embryos nor that all segments per embryo would be affected. Indeed, only 65% (13/20) of embryos injected with dsDephrin showed an aberrant phenotype and in total 39% (77/200) of all segments are affected. In four injected embryos all segments were affected. The phenotypes are fused commissures, loss of commissures and breaks in the connectives (Fig. 5B, Table 1). Although Dephrin RNAi impedes commissure formation, it does not interfere with the differentiation of midline glia (http:/www.elc.cam.ac.uk/~brandlab/index.html). Injection with dsCFP or buffer does not reduce Dephrin expression but occasionally results in phenotypes similar to dsDephrin injections (Table 1). However, only 30% (5/15) of dsCFP injected embryos and 23% (4/17) of buffer injected embryos show a phenotype. The number of affected segments is reduced to 6% (9/148, dsCFP) or 8% (13/167, buffer).

DEph RNAi also results in fused commissures, loss of commissures and breaks in the connectives (Fig. 5D, Table 1). The phenotype of DEph RNAi is more severe than for Dephrin RNAi. 80% (12/15) of all embryos have a phenotype and in total 69% (98/142) of all segments were affected. In five embryos all segments were abnormal. The difference in the strength of phenotype could either indicate that additional ligands besides Dephrin signal through DEph or the difference might be caused by the efficiency of RNAi, which varies between different genes (Kennerdell and Carthew, 1999).

Loss of DEph and Dephrin causes interneurones to exit the CNS

RNAi against *Dephrin* and *Deph* results in the fusion or loss of commissures and breaks in the connectives. Using a general axon marker, the origin of these phenotypes is not clear. We therefore decided to follow the behaviour of single axons in RNAi-treated embryos.

The Gal4 line CY27 primarily drives expression of UAS-taumGFP6 in 2 interneurones per hemisegment, the vMP2 and dMP2 neurone. The MP2 neurones are among the first neurones to extend their axons along the connectives (Jacobs and Goodman, 1989). In differentiated embryos the projections of these neurones form a tight fascicle which extends close and in parallel to the midline (Fig. 5E,F). Loss of Dephrin or DEph causes the axons of the MP2 neurones to project aberrantly out of the CNS (Fig. 5A,C). In 75% of embryos (15/20, Dephrin RNAi) and 82% of embryos (14/17, DEph RNAi), we find MP2 axons exiting the CNS (Table 2). In the GAL4 line CY27, additional interneurones (i.e. UMI neurones) start to express GFP in late embryogenesis (Fig. 5E). We did not attempt to examine these weak projections in detail but we noticed that many of these interneuronal axons also project out of the CNS (Fig. 5A,C). Buffer injections and dsCFP injections never, or rarely, interfere with the course of the MP2 axons (Table 2).

Therefore, signalling between Dephrin and DEph plays a role in confining interneuronal axons to the connectives.

Ectopic expression of Dephrin halts axonal growth

In vertebrates activation of Eph receptors in axonal growth cones is able to repel axons (reviewed by O'Leary and Wilkinson, 1999; Wilkinson, 2001). We speculated that despite the structural differences between vertebrate ephrins and Dephrin, the repulsive ability of Dephrin/DEph signalling might be conserved. Dephrin expression along the outer edge of the connectives and between the commissures could create barriers preventing axon extension. Absence of these barriers would be expected to result in fusion of commissures and the exit of interneuronal axons from the CNS, as we have observed in our RNAi experiments. To test whether Dephrin can act as an axonal repellent, we ectopically expressed Dephrin.

Only 4-6 out of about 20 midline neurones express Dephrin. Ectopic expression of Dephrin in all midline cells (*sim-GAL4*) causes fusion, severe thinning or loss of commissures without affecting midline glial cell differentiation (Fig. 6A,B). Single cell labelling of neural precursors (Bossing et al., 1996) reveals that ectopic Dephrin in midline cells is able to prevent the midline crossing of axons (Fig. 6C,D). In all clones with contralateral axons (n=18), the axons are stalled at the midline. Ectopic midline Dephrin does not affect the extension of ipsilateral axons immediately adjacent to the midline (n=5 clones; data not shown) or the determination of midline

neurones (judged by the expression of Engrailed, Futsch and Odd-skipped; data not shown).

Axonal repulsion by Slit, secreted from midline cells, is one of the major mechanisms controlling axons crossing the midline (Kidd et al., 1999). It is possible that Dephrin expression at the midline exerts its repulsive effect by upregulating the expression or secretion of Slit. To test if repulsion by Dephrin depends on Slit, we expressed Dephrin ectopically in the midline of embryos mutant for Slit and Robo1, one of the receptors for Slit (Kidd et al., 1998). Expression of Dephrin in *slit/robo* double mutants forces axons out of the midline (Fig. 6E,F). Therefore, Dephrin/DEph signalling at the ventral midline can act independently of Slit and Robo1.

Dephrin is expressed in nearly all neurones but not in the longitudinal glia that enwrap the connectives. We generated Dephrin-expressing longitudinal glia by injecting UAS-Dephrin plasmids into the syncytial blastoderm of *GAL4*^{MZ1580} embryos (Hidalgo et al., 1995). When longitudinal glial cells express Dephrin (n=7 embryos), we observe breaks in the connectives (Fig. 6G). The breaks are always located near the glial cell. No breaks are observed when neurones express Dephrin (n=5 embryos, data not shown). GFP-expressing longitudinal glial cells also do not disrupt axon extension (UAS-tau-mGFP6 plasmid; n=10 embryos; Fig. 6H). In summary, ectopic expression of Dephrin blocks axon extension.

Axonal repulsion by Dephrin can be supressed by lowering the expression of DEph

Activation of DEph on axons may be the reason that axons stall at Dephrin-expressing midline cells. In that case lowering the level of DEph activation by reducing DEph expression might allow these axons to overcome this repulsion and restore the commissures. To test this hypothesis we lowered DEph expression by DEph RNAi. Embryos with ectopic midline expression of Dephrin show a strong phenotype (Fig. 6A). Only 2% (2/100) of all segments have wild-type commissures and we never find embryos in which all segments have normal commissures. Injection of dsDEph RNA rescues the commissures. In 30% (8/27) of all injected embryos all segments were restored to wild type (Fig. 7B). In contrast, in all embryos injected with buffer (n=16) or dsGFP (n=19, Fig. 7A) we find segments with fused or absent commissures, indicating that ectopic Dephrin is still able to repel axons. In dsDEph-injected embryos 33% (75/230) of all segments have wild-type commissures, whereas in control-injected embryos only 9% (15/158, dsGFP) or 0% (0/157, buffer) of segments show normal commissures.

Presumably, we are able to rescue the commissures with DEph RNAi because dsDEph-injected embryos do not always show a loss or fusion of commissures. 20% of injected embryos and 31% of all segments have no phenotype (see above). In the rescued embryos, DEph expression might be lowered enough to overcome the repulsion by ectopic midline Dephrin but not low enough to result in fused or lost commissures.

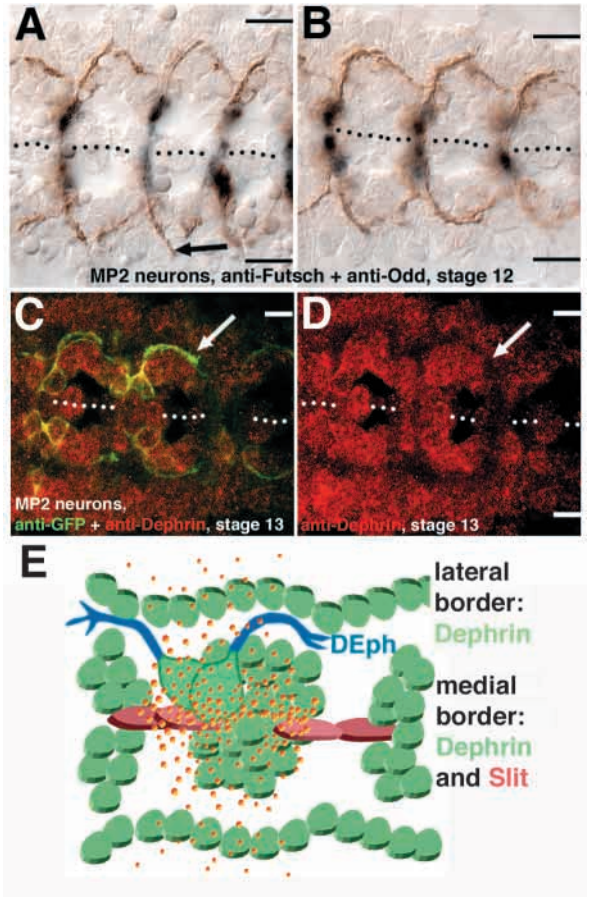

Fig. 8. The role of Dephrin/DEph signalling during CNS development in *Drosophila* embryos. (A,B) In stage 12 of embryogenesis, the first interneuronal axons project away from the midline (dotted line) and extend up to the lateral border (line) of the CNS (arrow labels growth cone in A). After reaching the border, the axons turn and continue to extend in parallel to the midline (B). At this stage anti-Futsch (brown) labels the axons of both MP2 neurones and anti-Odd (black) labels the nucleus of dMP2 and the MP1 neurones. (C,D) Anti-Dephrin staining at stage 13 shows that the MP2 axons (arrow labels growth cone) extend along a thin Dephrin-free channel (C, MP2 neurones are labelled by the expression of tau-GFP driven by the GAL4 driver CY27). D shows Dephrin expression only. (E) We propose that primary neurones project away from the ventral midline (pink) owing to the secretion of the long range repellent Slit (orange). When the growth cones reach the lateral border they are repelled by Dephrin (green). This repulsion induces growth cones, which carry the receptor DEph (blue), to turn and confines axon extension to within the CNS. Horizontal views, anterior to the left

DISCUSSION

Dephrin is the first transmembrane ephrin described in invertebrates. Our structural analysis indicates that Dephrin, which is cleaved at the N terminus, is composed of an extracellular ephrin domain, a transmembrane domain and a cytoplasmic tail with homology to B class ephrins. Dephrin is found on neuronal cell bodies outlining the presumptive axonal tracts. In contrast, DEph, the *Drosophila* Eph-like receptor, is found only on interneuronal axons. Dephrin binds to DEph and signalling between DEph and Dephrin is able to block axon extension. Axonal repulsion by Dephrin/DEph signalling plays a role in the separation of commissures. In addition, Dephrin/DEph signalling prevents the abnormal exit of

interneuronal axons from the CNS. We propose that Dephrin/DEph signalling is essential for the formation of the longitudinal tracts by delimiting the extension of interneuronal axons to the inside of the CNS.

Dephrin is a transmembrane ephrin

Ephrin signalling in vertebrates is mediated by two classes of receptors and ligands, A and B. In contrast, invertebrates appear to use a single ancestral ephrin-Eph signalling pathway. Four ephrins have been identified in *C. elegans*. Although these have a B-like receptor binding domain, they are all attached to the membrane by a GPI anchor, a feature characteristic of the A class ephrins (Chin-Sang et al., 1999; Wang et al., 1999). Similarly, the structure of Dephrin does not directly allow it to be classified as an A class or a B class ephrin. The receptor binding domain/ephrin domain shows the same degree of homology to both classes of vertebrate ephrins. As in vertebrates the ephrin domain in Dephrin is extracellular. In contrast to vertebrate ephrins, the domain is preceded by a stretch of 200 aa with no homology to any other ephrin.

This unusual N terminus has no obvious signal sequence but is essential for the membrane localisation of Dephrin. The N terminus also contains sequences needed for posttranslational control of Dephrin (T. B. and A. H. B., unpublished). Interestingly, we show that the N terminus is cleaved. The cleavage depends on the full length molecule, neither a truncation containing the N terminus and the ephrin domain (DephrinΔCterm) nor a fusion of the N terminus to GFP is cleaved. The function of this cleavage is unclear and we do not know if this cleavage occurs in all Dephrin-expressing cells. The cleavage could be necessary to create an additional membrane anchor at the N terminus by opening up the predicted myristoylation site (aa85-90) in the N terminus. Another possibility is that like Hedgehog ligands (Porter et al., 1996), cleavage of Dephrin is needed to attach a cholesterol anchor.

The C terminus of Dephrin encodes two closely spaced predicted transmembrane domains. Consistent with this prediction, we find that the C terminus of Dephrin is essential to anchor the molecule to the membrane. Currently we do not know which of the predicted transmembrane domains is used. The last predicted transmembrane domain is followed by 30aa, of which the last 19aa show homology to the cytoplasmic tail of B class ephrins. The tyrosine residue identified as a major in vivo phosphorylation target in vertebrate B class ephrins (Kalo et al., 2001) is conserved. We confirmed the existence of a cytoplasmic tail in Dephrin by proteinase K treatment and antibody staining against a C-terminal GFP tag.

Is Dephrin/DEph signalling bi-directional?

In vertebrates Eph receptors and ephrin B ligands are both able to transduce extracellular signals (reviewed by Mellitzer et al., 2000; Wilkinson, 2001). The phenotypes caused by ectopic expression of Dephrin or by the loss of Dephrin and DEph appear to be restricted to axonal pathfinding. The localisation of DEph on axons, appears to imply that DEph is the receptor and Dephrin only acts as a ligand. However, there are indications that Dephrin may also be able to transduce signals. The tyrosine involved in signal transduction by B class ephrins (Kalo et al., 2001) is conserved in Dephrin and we can immunoprecipitate Dephrin from embryonic lysates using anti-phosphotyrosine (V. Krishnan and A. H. B., unpublished). The function of Dephrin signalling might be obscured by the strong axonal phenotype. For example, Dephrin expression could play a role in the regulation of cell adhesion. In Dephrin and DEph RNAi-treated embryos, the embryonic CNS appears flat with a ragged outline. In contrast, the loss of other major components involved in axonal pathfinding, i.e. Commissureless (Tear et al., 1996), Roundabout (Kidd et al., 1998) or Slit (Kidd et al., 1999), does not affect the shape of the CNS.

Dephrin and its receptor DEph are expressed in different neuronal compartments

Dephrin is expressed by motor neurones and interneurones, whereas the expression of DEph is confined to interneurones (Scully et al., 1999). Our results show that DEph expression on interneurones restricts their axons to the CNS. It is tempting to speculate that the absence of DEph on motorneuronal axons, which have to project out of the CNS, might be essential to allow these axons to cross over the Dephrin barrier at the border of the connectives.

The expression of DEph and Dephrin is restricted to different subcellular compartments, although their RNA expression most likely overlaps. Dephrin is restricted to neuronal cell bodies, whereas DEph is confined to axons. DEph RNA appears not to be transported into axons (Scully et al., 1999), hence the differential sorting of the two components of ephrin signalling occurs at the protein level. We have shown that Dephrin and DEph bind each other and the separation of the two proteins may be essential to prevent a cell autonomous activation of signalling. Indeed, strong overexpression of Dephrin in interneuronal mosaic clones results in axonal accumulation of Dephrin and interferes with axonal pathfinding (data not shown). Recently, it has been shown that overlapping expression of ephrinA5 and its receptor EphA4 in retinal axons can desensitise the growth cone, allowing the axons to pass over Eph concentrations that repel axons that are not desensitised (Hornberger et al., 1999). Although desensitisation might play a role in pathfinding of a minority of axons in the embryonic CNS of *Drosophila*, our results suggest that the correct targeting of most axons depends on the exclusion of Dephrin from axons.

Dephrin/DEph signalling is essential for commissure formation

Ephrin/Eph signalling in *Drosophila* is involved in midline guidance, as are ephrins in vertebrates and *C. elegans* (Henkemeyer et al., 1996; Nakagawa et al., 2000; Yokoyama et al., 2001; Zallen et al., 1999). Ectopic expression of Dephrin in all midline cells prevents commissural axons from crossing the midline, supporting a role of Dephrin/DEph signalling in axon repulsion. The loss of Dephrin and DEph function results in the fusion of commissures. Dephrin is expressed in midline and non-midline cells located between the forming commissures in each segment. Dephrin may act as a repulsive force that is needed for the separation of commissures. Therefore, this loss of function phenotype can be explained by the loss of these repulsive barriers.

Ephrin/Eph signalling is bi-functional and can promote adhesion as well as repulsion (reviewed by Wilkinson, 2001).

This bi-functionality is especially striking in midline guidance. During the development of the spinal cord in mouse, the interaction of EphA4 on corticospinal axons with ephrinB3 at the midline of the neural tube prevents the midline crossing of collaterals (Yokoyama et al., 2001). In contrast, the expression of ephrinB2 on commissural axons and EphA4 on neurones at the anterior commissure of the brain is essential for the midline crossing of these axons (Kullander et al., 2001; Orioli et al., 1996). The vab-1/Eph receptor in *C. elegans* is needed on sensory axons for the ventral attraction towards the nerve ring, but it also functions as a repellent to prevent axonal crossover at the ventral midline (Zallen et al., 1999). A similar mechanism might apply for the formation of commissures in *Drosophila*, where loss of Dephrin or DEph can result in the loss of commissures. It may be that Dephrin at the midline is a permissive substrate to which growth cones of commissural axons can adhere, to be channelled towards the entry of the commissures. In vertebrates, the repulsive activity of ephrin/Eph signalling can be transformed into adhesion by the expression of different splice forms of an Eph receptor (Holmberg et al., 2000), by preventing the Kuzbanian-dependent cleavage of ephrins (Hattori et al., 2000) or by alternating the degree of receptor activation. It has been shown that adhesion of vertebrate cells in culture depends on the level of signalling by the EphB1 receptor. Low to medium level activation of the EphB1 receptor by ephrinB1 promotes adhesion, while high level activation blocks adhesion (Huyn Do et al., 1999). No different splice forms of the *DEph* mRNA have been reported (Scully et al., 1999), but the Kuzbanian cleavage site is conserved in the ephrin domain of Dephrin. Our observation that either loss of Dephrin or gain of Dephrin in midline cells can result in a loss of commissures, seems to indicate that the level of receptor activation at the midline is critical to distinguish between adhesion and repulsion.

The role of Dephrin expression at the midline differs from that of Slit, the second chemorepellent expressed in midline cells. In the loss of Slit axons linger at the midline (Kidd et al., 1999), whereas in the loss of Dephrin axons do not aberrantly enter the midline. We show that ectopic Dephrin/DEph expression at the midline does not repel axons through the upregulation of Slit/Robo1 signalling.

Dephrin/DEph signalling defines the outer borders of the connectives

The loss of Dephrin or DEph causes breaks in the connectives. Our examination of the projection pattern of primary axons in the loss of Dephrin and DEph explains the breaks in the connectives. Some of the first neurones to extend their axons in the CNS are the MP1 and MP2 neurones (Jacobs and Goodman, 1989). The axons of MP1 and MP2 neurones first project mediolaterally, away from the midline, until they nearly reach the outer border of the developing CNS (Fig. 8A). At the outer border they stop and turn to extend longitudinally (Fig. 8B). In the loss of Dephrin and DEph, the MP2 neurones do not turn but exit the CNS (Fig. 5A,C). It seems likely that an aberrant exit of interneurones can result in breaks in the connectives. The expression of Dephrin along the outside of the CNS seems to form a repulsive barrier confining the extension of interneuronal axons to the inside of the CNS. The repulsive capacity of Dephrin on longitudinal axons is evident because of the ectopic expression of Dephrin in longitudinal glia cells which is able to halt axonal growth along the connectives.

These results lead us to propose the following model for the formation of the connectives in the embryonic CNS of *Drosophila*. Repulsion by Dephrin at the outer border of the CNS and by Slit at the midline limits the extension of primary longitudinal axons to within the CNS (Fig. 8E). Restricting the first interneuronal axons to inside the CNS ensures that axon fascicles are in the correct place to enable selective fasciculation and axonal spacing in late embryogenesis (Rajagopolan et al., 2000; Simpson et al., 2000). In late embryogenesis, when the number of axons increases, repulsion by Dephrin/DEph signalling might well be restricted to the most lateral axons. This mechanism is similar to the establishment of the longitudinal tracts in vertebrates. During development of the vertebrate CNS, the medial repulsive border is defined by expression of Slits, Semaphorins and B class ephrins in the floorplate and the ventral spinal cord (Imondi et al., 2000; Zou et al., 2000). The outer repulsive border is formed by B class ephrins, Semaphorins and BMPs in the dorsal spinal cord (Augsburger et al., 1999; Imondi and Kaprielian, 2001).

Ephrin/Eph signalling plays a role in many important processes during vertebrate development. The number of receptors and ligands and their functional redundancy hinder the elucidation of the underlying signalling pathways. *C. elegans* and *Drosophila* use an ancestral signalling pathway, although many of the functions of ephrin/Eph signalling are conserved. This raises the possibility of identifying the downstream components of the pathway in genetically accessible model organisms. *C. elegans* has one Eph receptor and four ephrins, which show functional redundancy (Wang et al., 1999). *Drosophila* has only one ephrin and one Eph receptor. As with the Hedgehog, WNT and Ras signalling pathways, *Drosophila* might once again be a helpful tool to unravel an evolutionarily conserved signalling mechanism.

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