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The regulation of glial-specific splicing of *Neurexin IV* requires HOW and Cdk12 activity

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SUMMARY

The differentiation of the blood-brain barrier (BBB) is an essential process in the development of a complex nervous system and depends on alternative splicing. In the fly BBB, glial cells establish intensive septate junctions that require the cell-adhesion molecule Neurexin IV. Alternative splicing generates two different Neurexin IV isoforms: Neurexin IV^{exon3}, which is found in cells that form septate junctions, and Neurexin IV^{exon4}, which is found in neurons that form no septate junctions. Here, we show that the formation of the BBB depends on the RNA-binding protein HOW (Held out wings), which triggers glial specific splicing of *Neurexin IV*^{exon3}. Using a set of splice reporters, we show that one HOW-binding site is needed to include one of the two mutually exclusive exons 3 and 4, whereas binding at the three further motifs is needed to exclude exon 4. The differential splicing is controlled by nuclear access of HOW and can be induced in neurons following expression of nuclear HOW. Using a novel in vivo two-color splicing detector, we then screened for genes required for full HOW activity. This approach identified Cyclin-dependent kinase 12 (Cdk12) and the splicesosomal component Prp40 as major determinants in regulating HOW-dependent splicing of *Neurexin IV*. Thus, in addition to the control of nuclear localization of HOW, the phosphorylation of the C-terminal domain of the RNA polymerase II by Cdk12 provides an elegant mechanism in regulating timed splicing of newly synthesized mRNA molecules.

KEY WORDS: HOW, Raf, Cdk12, Prp40, Neurexin IV, *Drosophila*, Glia, Splicing, Blood-brain barrier

INTRODUCTION

The regulation of gene expression is central to all aspects of development. This is particularly obvious when we consider that the number of genes present in the genome of *C. elegans*, *Drosophila* or human is surprisingly similar. The complexity of gene expression, however, dramatically increases due to differential splicing (Black, 2003; Keren et al., 2010; Li et al., 2007; Matlin et al., 2005). About 95% of all human pre-mRNAs are subject to alternative splicing, of which 4.3% carry mutually exclusive spliced exons (Koscielny et al., 2009; Pan et al., 2008; Wang et al., 2008). In *Drosophila*, roughly 88% of all genes are subject to splicing and 60.7% of these genes are subject to alternative splicing (Graveley et al., 2011).

Differential splicing is not only required for neuronal lineages but is also needed for the development of glial cells (Edenfeld et al., 2006; Wu et al., 2002). *Drosophila* glia arise during embryogenesis through the activity of the master regulatory gene *glial cells missing* (Jones, 2005; Soustelle and Giangrande, 2007). Once specified, two glial cell types can be recognized at the lateral boundary of the nervous system (Awasaki et al., 2008; Edwards and Meinertzhagen, 2010; Hartenstein, 2011; Pereanu et al., 2005; Stork et al., 2008). The outermost layer of the nervous system is formed by the perineurial glial cells, which participate in the formation of the blood-brain barrier (BBB) but interestingly lack any contact with neurons. Abutting the perineurial glia basally are the subperineurial glial cells. They establish the physical barrier that prevents paracellular leakage of small solutes into the nervous system. To perform this task, the subperineurial glia establish specialized cell-cell junctions called pleated septate junctions (pSJ) (Schwabe et al., 2005; Stork et al., 2008; Tepass and Hartenstein, 1994). In recent years a surprisingly high number of membrane proteins have been found to be expressed in pSJ (Laprise et al., 2009; Laprise et al., 2010; Wu and Beitel, 2004). In most cases, the deletion of one of these proteins results in the breakdown of the entire pSJ structure and, thus, in a defective BBB (Banerjee et al., 2006; Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004; Stork et al., 2008; Syed et al., 2011). The first protein identified as being required for the integrity of the BBB is Neurexin IV (Nrx-IV) (Baumgartner et al., 1996).

Nrx-IV is an evolutionary conserved protein that is also found in the vertebrate nervous system, where it is called Caspr (contactin-associated protein) (Peles et al., 1997a; Peles et al., 1997b). Caspr localizes to the paranodes that morphologically resemble the pSJ of *Drosophila* (Einheber et al., 1997; Rios et al., 2003). However, *Drosophila* pSJ are established between glial cells, whereas in the vertebrate nervous system septate-like junctions are formed between axons and the myelin-forming glial cells (Banerjee and Bhat, 2007).

We have previously identified mutations in the gene *crooked neck* (*crn*), which severely affects expression of Nrx-IV (Edenfeld et al., 2006). Crn is a well-conserved splicing factor that mediates spliceosome assembly by binding to the U1 snRNP protein Prp40 and the U2 snRNP auxiliary factor 65 (U2AF65) (Chung et al., 1999; Raisin-Tani and Leopold, 2002; Wang et al., 2003). *Drosophila* Crn interacts with one of the isoforms encoded by the *held out wings* (*how*) gene (Edenfeld et al., 2006). Three different HOW isoforms are generated that all share a KH RNA-binding domain and differ in their C-terminal tails. The short isoform, HOW(S), binds Crn in the cytoplasm and is able to shuttle to the nucleus. The long isoform, HOW(L), is only found in the nucleus and cannot bind Crn (Edenfeld et al., 2006; Volk et al., 2008). The

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middle form is predicted by FlyBase and is currently not further analyzed. Drosophila *how* mutants have altered glial differentiation and the HOW protein is able to bind *Nrx-IV* mRNA (Edenfeld et al., 2006). Mice that are mutant for the *how* homologue called quaking (*Qk* – Mouse Genome Informatics) develop severe demyelinization phenotypes resembling the *Drosophila* glial differentiation phenotypes shown by *crn* and *how* mutants (Ebersole et al., 1996; Edenfeld et al., 2006; Sidman et al., 1964; Wu et al., 2002; Zhao et al., 2010).

Quaking/HOW proteins are members of the STAR (signal transduction and activation of RNA) family of RNA-binding proteins implicated in the control of pre-mRNA splicing, mRNA stability and mRNA transport (Artzt and Wu, 2010; Edenfeld et al., 2006; Larocque et al., 2002; Vernet and Artzt, 1997; Volk, 2010; Zhao et al., 2010). STAR proteins, which are often regulated by post-translational mechanisms, bind to a short sequence motif in pre-mRNA molecules, which is similar to the recognition site for splice factor 1 (Artzt and Wu, 2010; Galarneau and Richard, 2005; Israeli et al., 2007; Matter et al., 2002; Ryder et al., 2004; Vernet and Artzt, 1997). In a microarray study, such a potential STAR protein-binding site was found enriched near the 5′ splice site of tissue specifically expressed exons (Sugnet et al., 2006).

In *Drosophila*, the *Nrx-IV* gene encodes two equally sized mutually exclusively spliced exons, of which one is flanked by HOW-binding sites. These exons encode two related Discoidin domains and their mutually exclusive splicing results in cell adhesion proteins with altered binding capabilities involved in different aspects of neuron-glia interaction (Edenfeld et al., 2006; Stork et al., 2009). The exon 4-containing *Nrx-IV* mRNA is expressed mainly in neurons. It generates a protein that interacts with the Ig-domain protein Wrapper, which is expressed by the midline glial cells. By contrast, we show here that the exon 3-containing *Nrx-IV* mRNA is expressed by cells that form pSJ.

Here, we have analyzed the role of HOW and its influence on differential splicing during glial maturation. We show that HOW is required for the establishment of the BBB and directly affects the splicing of *Nrx-IV* pre-mRNA. To further dissect the function of HOW, we generated several splicing reporters and assayed the relevance of individual HOW response elements. During mutually exclusive splicing, HOW is required for inclusion of exon3 and, thus, the presence of HOW is associated with the formation of the pleated septate junctions in glial cells. To link the activity of HOW to the general control of glial maturation, we performed a genetic screen and identified Raf kinase, Cyclin-dependent kinase 12 (Cdk12) and Prp40 as important regulators required for full activity of HOW.

MATERIALS AND METHODS

Fly work

All crosses were performed on standard food. The following genotypes were used: *Nrx-IV*^{4304} (Baumgartner et al., 1996), *how*^{stru} (Nabel-Rosen et al., 1999), *how(L)*^{f06788} (Exelixis), *repo*Gal4(II) (Lee and Jones, 2005), *repo*Gal4(III) (Sepp and Auld, 1999), *elav*^{c155}Gal4, *elav*Gal4(III) (Lin et al., 1995), *how*^{24B}Gal4 (Brand and Perrimon, 1993), UAS-*EGFR*^{DN} (B.-Z. Shilo, Weizmann Institute of Science, Rehovot, Israel), UAS-*how*^{RNAi}, UAS-*crn*^{RNAi}, UAS-*Cdk12*^{RNAi} (VDRC), UAS-hPABP^{Flag,} UAS-raf^{DN}, UAS::*lamEGFP*, GFP-balancers (Bloomington) and UAS-prp40$^{TriP\text{-}RNAi}$ (Ni et al., 2011). Germ line transformation was carried out as described previously (Bischof et al., 2007).

Dextran injections

Stage 17 embryos were injected as described previously (Stork et al., 2008).

Tissue fixation and histological analysis

Immunohistochemical methods were performed as described before (Stork et al., 2009). All fluorescent samples were recorded on a LSM 710 confocal microscope (Zeiss). Fluorescent measurements were carried out using ImageJ (http://imagej.nih.gov/ij/index.html).

Antibodies

The following antibodies were used: anti-EGFP (Invitrogen); anti-mCherry (Clontech); anti-repo (DSHB, B. Altenhein, University of Mainz, Germany); anti-dCdk12 (A. Greenleaf, Duke University, Durham, NC, USA); and anti-HRP-Cy5 (Molecular Probes). Alexa Fluor dyes 488, 568 and 633 were used (Molecular Probes).

Isolation of RNA

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Tissue-specific mRNA was isolated from embryos expressing human poly(A)binding protein in either glial cells or neurons (Yang et al., 2005).

RT-PCR

Total RNA (1 μg) or 100 ng of tissue-specific mRNA extracts were used for reverse-transcribed PCR using SUPERscript II polymerase with Oligo(dT)$_{12\text{-}18}$ primers (Invitrogen) following the manufacturer's protocol.

PCR and restrictions

The used primer combinations were partially designed with Primer3 (Untergasser et al., 2007) and standard PCR was performed using Taq DNA polymerase (NEB). Restrictions were carried out according to the manufacturer's instructions (Roche).

Neurexin IV detection
NrxIVSplice2for::CGCCTTCACGGACTATTTCT
NrxIVSplice7rev::CCTTAAGCTGCAGGGCATAG

Reporter cloning
nrxIV_2-5_for::caccCGCCTTCAtgGACTATTTCT
nrxIV_2-5_rev::GTAATCGCAGCCGTAGAGCTCCAC
mCherry_3.0_Nco_for::catgccatgggaGGAGGAGGAATGGTGAGCAAGG-GCGAGGAGGATAAC
mCherry_3.0_Nco_rev::catgccatggcCTACTTGTACAGCTCGTCCATGC
EGFP_3.0_xba_for::ctagTctagacGGAGGAGGAATGGTGAGCAAGGGC-GAGGAGCTGT
EGFP_3.0_spe_rev::ggactagtCTACTTGTACAGCTCGTCCATGC

Reporter detection
GFPrev2::AACTTGTGGCCGTTTACGT

Generation of constructs

UAS-HOW(S)-3HA and UAS-HOW(L)-3HA were generated using Gateway cloning in a custom-made pUASTattB_rfA_3HA vector. Wild-type DNA spanning *Nrx-IV* exons 2-5 was amplified with proofreading Phusion polymerase (Finnzymes). An artificial engineered ATG was included to ensure translation and the fragment was inserted into the expression vector pUASTattB_rfA_EGFP (R. Stephan, unpublished), containing a C-terminal EGFP-tag resulting in the reporter *nrxIV_2.0*.

Site-directed mutagenesis was carried out to eliminate a *Nco*I restriction site located in the intron 5′ of exon 3. For *nrxIV_3.0*, EGFP was inserted into the *Xba*I restriction site of exon3 and mCherry was inserted into the *Nco*I restriction site of exon 4. All constructs were integrated into the landing sites zh-68E and zh-86Fb$^{RFP\text{-}}$ (Bischof et al., 2007).

Site-directed mutagenesis (SDM)

To generate base pair changes, QuikChange II XL Site-Directed Mutagenesis Kit was used according to the manufacturer's instructions (Agilent Technologies/Stratagene). The primers for SDM were partially designed using QuikChange Primer Design Program (Agilent Technologies/Stratagene).

Δ3 HRE
Δ3HRE_Alw44I_for::GCGAGCCGCAGGTACTGTGCaCGGCTTAAAG-GATGGCGATG

Δ3HRE_AlwI44I_rev::CATCGCCATCCTTTAAGCCGtGCACAGTACCTGCGGCTCGC

Mutation HRE1
sh_HRE1_for::ccacactctgacactgcgTAAACctagattggtaaatgcaccttgtgc
sh_HRE1_rev::gcacaaggtgcatttaccaatctagGTTTAcgcagtgtcagagtgtgg

Mutation HRE2
sh_HRE2_for::GCCGCAGGTACTGTGtaaacTCCTAGAACTAAGACTAC
sh_HRE2_rev::GTAGTCTTAGTTCTAGGAgtttaCACAGTACCTGCGGC

Mutation HRE3
sh_HRE3_for::CGCAGGTACTGTGACTAATCCTAGAtaaacGACTACACTTAAAAC
sh_HRE3_rev::GTTTTAAGTGTAGTCgtttaTCTAGGATTAGTCACAGTACCTGCG

Mutation HRE2+3
sh_HRE2+3_for::GGTACTGTGtaaacTCCTAGAtaaacGACTACACTTAAAACAC
sh_HRE2+3_rev::GTGTTTTAAGTGTAGTCgtttaTCTAGGAgtttaCACAGTACC

Mutation HRE4
sh_HRE4_for::CTACACTTAAAACACAtaaacTCCCGGCTTAAAGGATGGC
sh_HRE4_rev::GCCATCCTTTAAGCCGGGAgtttaTGTGTTTTAAGTGTAG

Elimination *Nco*I in intron2 of *nrxIV_2.0*
nrxIV_2.0c1930a2_for::cgttgccacgaccaacAcatggacaattggcat
nrxIV_2.0c1930a2_rev::atgccaattgtccatgTgttggtcgtggcaacg

S2 cell culture

Drosophila S2 cells were grown and transfected as published previously (Bogdan et al., 2005). Generally, transfection efficiencies of ~30% were obtained. Cells were co-transfected with UAS-based constructs and act::Gal4 DNA, and kept for 2 days at 25°C.

RESULTS

HOW and Nrx-IV are essential for BBB function

Loss of *Nrx-IV*, which encodes a central component of pleated septate junctions, results in a leaky BBB (Fig. 1A,B). *Nrx-IV* encodes two mRNAs with mutually exclusive use of either exon 3 or exon 4 ($Nrx\text{-}IV^{exon3}$ and $Nrx\text{-}IV^{exon4}$) (Fig. 2A). The differentially spliced exons encode distinct N-terminal Discodin domains that provide differential adhesive properties (Stork et al., 2009). In glia, expression of $Nrx\text{-}IV^{exon3}$ predominates, whereas in neurons expression of $Nrx\text{-}IV^{exon4}$ is enriched. Restriction of $Nrx\text{-}IV^{exon3}$-containing cDNA products with *Xba*I generates 359 and 271 bp fragments ,whereas restriction of $Nrx\text{-}IV^{exon4}$-containing cDNA products with *Nco*I generates 423 and 207 bp fragments (Fig. 2A,C).

To test whether the mRNA-binding protein Held out wings (HOW) is required for the integrity of the BBB, we performed dye injection experiments. Loss of zygotic *how* expression (how^{stru}) compromises BBB function and 10 kDa fluorescent dextran penetrates into the CNS (Fig. 1A,C). However, the leakage is not as pronounced as that observed in *Nrx-IV* mutants, which lack all septate junctions (Baumgartner et al., 1996; Stork et al., 2008) (Fig. 1B). The *how* locus encodes at least three different isoforms that differ in a short C-terminal domain and share an identical RNA-binding domain. In the allele how^{f06788}, a transposon insertion occurred in the exon specific to the HOW(L) isoform (supplementary material Fig. S1). Homozygous how^{f06788} animals are lethal but have no open BBB phenotype (Fig. 1A,D). Thus, HOW(L) might not be required for BBB function or HOW(S) may in part compensate for HOW(L). To further test the role of the different HOW proteins in BBB formation, we performed rescue experiments. Upon pan-glial expression of HA-tagged HOW(S) or HOW(L), the BBB was partially restored (Fig. 1E). Generally, expression of HOW(S) resulted in a stronger rescue. The transgenes were integrated in the same landing site using the phiC31 technique (Bischof et al., 2007), and thus the differential effects of HOW(S) and HOW(L) are not expected to be due to different expression levels. In conclusion, both HOW isoforms appear to be required for BBB formation.

how mutants show abnormal splicing of *Nrx-IV*

In the next step, we characterized the *Nrx-IV* splicing pattern of *how* mutant embryos. Total mRNA from stage 16 embryos was reverse transcribed. Subsequently, PCR reactions with primers

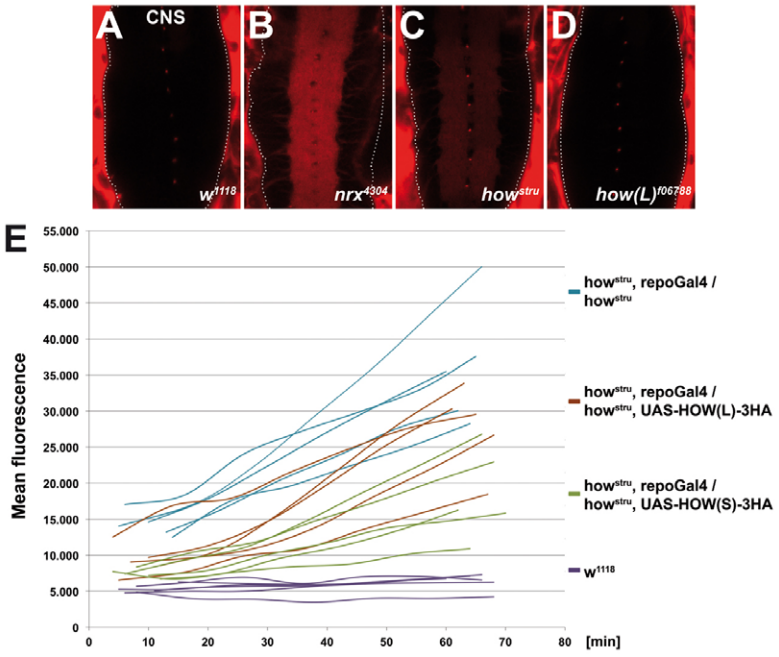

Fig. 1. *how* mutants exhibit a blood-brain barrier (BBB) defect. Analysis of BBB defects by injection of fluorescently labeled 10 kDa dextran into the hemolymph of stage 17 embryos. (A-D) Single confocal sections through the CNS of injected embryos (CNS indicated by white dotted line). Pictures were taken 15 minutes after injection with identical laser settings. (**A**) Wild-type (w^{1118}) embryos show no penetration of the dye into the CNS. (**B**) nrx^{4304} mutants lack a functional BBB. The dye penetrates into the CNS. (**C**,**D**) how^{stru} mutants show penetration of dye into the CNS (C), whereas, in $how(L)^{f06788}$ mutants, no dye penetrates into the CNS (D). (**E**) Isoform-specific rescue of the how^{stru} BBB phenotype. The uptake of labeled 10 kDa dextran into the CNS of individual embryos was monitored over 60 minutes. The mean fluorescence was determined in confocal *z*-stacks through the entire CNS. All experiments were performed on the same day, using identical conditions. The color coding indicates the different genotypes. In w^{1118} embryos (purple), no uptake of the dye can be seen. In mutant how^{stru} *repoGal4* embryos, (blue) the BBB is disrupted. Panglial expression of HOW(S) (green) results in slightly better rescue of the BBB integrity when compared with HOW(L) expression (brown).

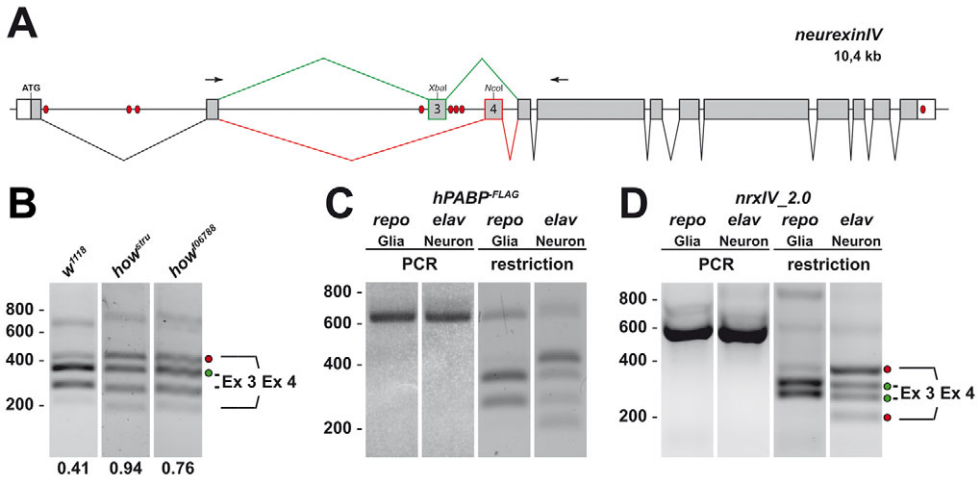

Fig. 2. Detection of tissue-specific splicing with *Nrx-IV* splicing reporters. (**A**) The *Nrx-IV* locus encodes 13 exons (gray boxes), of which exon 3 (green framed box and lines) and exon 4 (red framed box and lines) show mutually exclusively splicing. The exons contain unique endogenous restriction sites (*Xba*I and *Nco*I). HOW response elements (HREs) are indicated by red dots. The orientation and position of primers used for splicing analysis are indicated (arrows). (**B**) Analysis of *Nrx-IV* splicing in how^{stru} and how^{f06788} mutant embryos. w^{1118} flies are used as a wild-type control. Restriction analysis (*Nco*I/*Xba*I) of the *Nrx-IV*-specific PCR product shows a stereotypic splicing pattern. DNA fragments of 359 and 271 bp correspond to the $Nrx\text{-}IV^{exon3}$ mRNA, fragments of 423 and 207 bp correspond to the $Nrx\text{-}IV^{exon4}$ transcripts. The faint 630 bp band corresponds to traces of undigested DNA. In wild type, high levels of $Nrx\text{-}IV^{exon3}$ and low levels of $Nrx\text{-}IV^{exon4}$ are detectable. In how^{stru} and how^{f06788} mutants, higher amounts of $Nrx\text{-}IV^{exon4}$ corresponding bands can be observed. The red and green dots indicate the bands used to calculate the transcript ratio, which is given below the lanes. (**C**) Analysis of PCR products of glial (*repoGal4*) or neuronal (*elavGal4*) enriched cDNA pools and restriction with *Nco*I/*Xba*I. Primer positions are indicated by arrows in A. Glial-specific cDNA contain almost exclusively the $Nrx\text{-}IV^{exon3}$ product. Neuronal-specific cDNA exhibits higher amounts of $Nrx\text{-}IV^{exon4}$. (**D**) Analysis of tissue specifically expressing the *nrxIV_2.0* reporter in glial cells and neurons. PCR products detecting the reporter are shown in the left two panels and the corresponding *Nco*I/*Xba*I restrictions are shown in the right two panels. The restriction of the glial sample shows almost exclusively $Nrx\text{-}IV^{exon3}$, whereas neurons express higher amounts of $Nrx\text{-}IV^{exon4}$. The red and green dots indicate the DNA bands of the different splicing isoforms.

spanning the alternatively spliced region were performed (Fig. 2A, arrows). To discriminate between exon 3- and exon 4-containing isoforms, we used endogenous restriction sites (*Xba*I in exon3 and *Nco*I in exon4) (Fig. 2A). $Nrx\text{-}IV^{exon3}$ containing cDNAs generate 359 and 271 bp fragments, whereas restriction of $Nrx\text{-}IV^{exon4}$-containing cDNAs generates 423 and 207 bp fragments. how^{stru} mutants show a different distribution of the *Nrx-IV* isoforms compared with the wild-type splicing pattern, having more $Nrx\text{-}IV^{exon4}$ (Fig. 2B). To quantify the levels of the different splicing forms, we measured the intensity of the exon-specific DNA fragments (highlighted by red and green dots in Fig. 2B). In wild type, the exon4/exon3 ratio is 0.66 (±0.15 s.d.; quantification of DNA bands of five independent experiments), in how^{stru} mutants the ratio is 0.94 (±0.02) (Fig. 2B, n=6 independent experiments). Interestingly, how^{f06788} mutants also show a preferential splicing of $Nrx\text{-}IV^{exon4}$, although not as prominent as in the loss-of-function mutant how^{stru} (ratio is 0.86±0.05, n=6 independent experiments; Fig. 2B). This indicates that glial cells express both HOW(S) and HOW(L). It also demonstrates that HOW(L) is needed for *Nrx-IV* splicing, but Nrx-IV levels are still high enough to provide a functional BBB (Fig. 1D). Moreover, as the HOW null allele how^{stru} has a more severe splicing phenotype, we assume that HOW(S) is also involved in *Nrx-IV* splicing.

Differential expression of *Nrx-IV*

To demonstrate a cell type-specific expression of different *Nrx-IV* splicing isoforms, we isolated cell type-specific mRNA (Yang et al., 2005) (Fig. 2A,C). In embryos, glia almost exclusively express the $Nrx\text{-}IV^{exon3}$ form, whereas in neurons the expression of the $Nrx\text{-}IV^{exon4}$ isoform is enriched.

To further dissect glial-specific splicing, we generated a set of reporter constructs encompassing the differentially spliced region of *Nrx-IV* (Fig. 2D, Fig. 3A). In the construct *nrxIV_2.0*, exon 5 is partially replaced by a EGFP cassette to monitor expression of the transgene, and exon 2 is engineered to contain an ATG-start codon (Fig. 3A). The expression control is mediated via UAS elements, allowing expression of the reporter specifically in neurons, using *elav*Gal4, or in glial cells, using *repo*Gal4. In embryos, splicing of the *nrxIV_2.0* reporter closely mimics that found in the endogenous *Nrx-IV* gene (compare Fig. 2C,D).

HOW positive glia expresses $NrxIV^{exon3}$

To circumvent mRNA isolation, we generated the *nrxIV_3.0* reporter. We inserted a $EGFP^{Stop}$ sequence in exon 3, resulting in a translational stop whenever exon3 is used. In addition, we added a $mCherry^{Stop}$ sequence in exon4, resulting in a translational stop when exon4 is used (Fig. 3B). Upon transfection of such a construct into S2 cells, 45% of the cases we noted concomitant generation of exon 3- and exon 4-containing mRNAs. In the remaining cells, either exon 3 splicing or exon 4 splicing is observed (Fig. 3C-F; in 30% of the cells only exon 3 and in 25% of the cells only exon 4 splicing is detected). This indicates that even in S2 cells a stable commitment towards a specific splicing reaction can be made.

The *nrxIV_3.0* reporter can also be used to monitor splicing specificity with a single cell resolution in *Drosophila* embryos (Fig. 3G-N). When expressed in glia using *repo*Gal4, we noted only green fluorescence indicating splicing of the exon 3 (Fig. 3G,H; the CNS is indicated by broken lines). By contrast, following expression in neurons using *elav*Gal4, both *nrxIV_3.0* transcripts

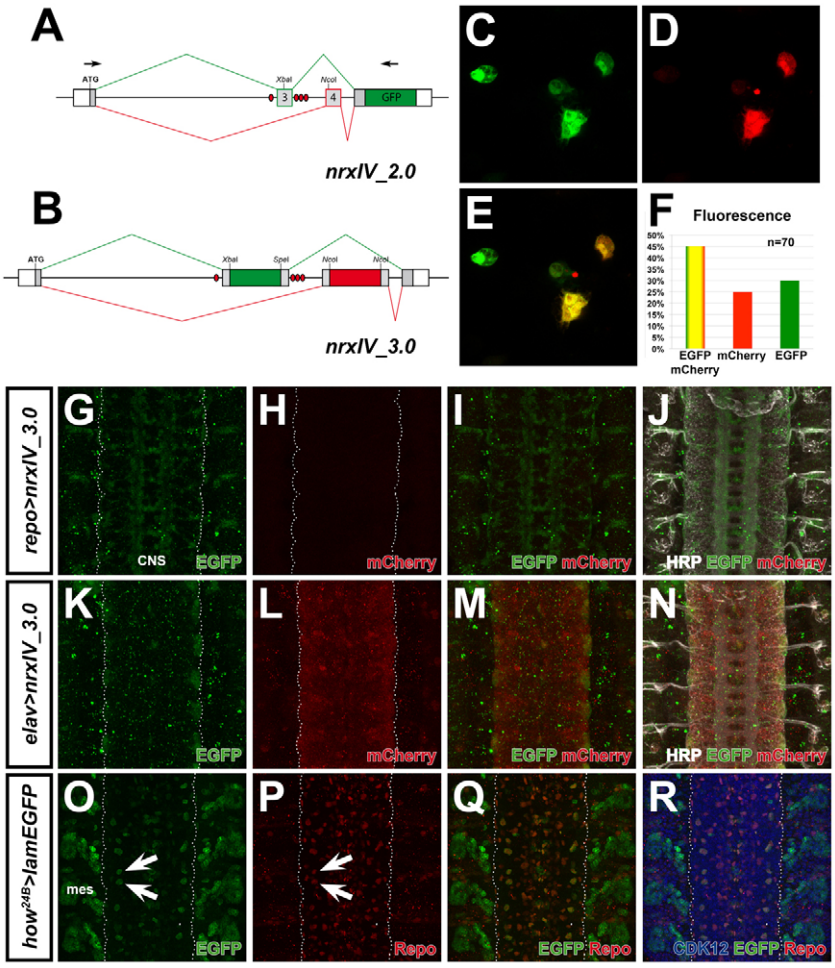

Fig. 3. Generation of splice reporter constructs. (**A**) The *nrxIV_2.0* reporter contains the genomic region covering exons 2-5 of *Nrx-IV*, fused to EGFP under the control of the UAS element. This allows cell type-specific expression of the reporters in glia using *repoGal4* or in neurons using *elavGal4*. The exclusive reporter detection is achieved by an EGFP-reverse primer (indicated by arrows). (**B**) The *nrxIV_3.0* reporter is a variant of *nrxIV_2.0*, which contains an in-frame EGFP sequence in exon 3 (green) and an in-frame mCherry sequence in exon 4 (red). Both fluorescent markers contain a stop codon, allowing the detection of alternative splicing of exon 3 or exon 4 by fluorescence detection. (**C-E**) Detection of endogenous fluorescence of S2 cells transfected with the *nrxIV_3.0* reporter and actinGal4. (C) EGFP fluorescence. (D) mCherry fluorescence. (E) Merge of EGFP and mCherry fluorescence. (**F**) Statistical analysis of the fluorescence distribution in S2 cells expressing the *nrxIV_3.0* reporter (*n*=70). Forty-five percent express both EGFP (exon 3) and mCherry (exon 4), 25% express mCherry and 30% express EGFP. (**G-R**) Projections of confocal sections through the CNS of stage 16 embryos. The outline of the CNS is indicated by a white dotted line. (G-J) Expression of *nrxIV_3.0* reporter in glia using *repo*Gal4 and (K-N) in neurons using *elav*Gal4. (G,H) Glial expression of *nrxIV_3.0* shows the detection of EGFP (exon 3) (G), but not of mCherry (exon 4) (H). (K,L) Neuronal expression of *nrxIV_3.0* shows some EGFP (exon 3) (K) and mCherry (exon 4) (L). (I,M) Merge. (J,N) Additionally, neuronal membranes are stained with anti-HRP (gray). (O-Q) The HOW-specific Gal4-driver *how*^{24BGal4} expresses nuclear localized lamin-EGFP to visualize the cells expressing HOW. In the CNS, HOW expression is found exclusively in Repo-positive glial cells (arrows). In addition, HOW expression is noted in the mesoderm (mes). (R) Cdk12 is expressed in the nuclei of all cells.

can be detected in the CNS (Fig. 3K-N, red and green). Similar results were obtained when we analyzed splicing in the larval nervous system (supplementary material Fig. S2A-H). In summary, this analysis supports the notion that the BBB-forming glia express only the *Nrx-IV*^{exon3} transcript.

To determine the HOW expression domain, we used the *24B*Gal4 insertion into the *how* locus (*how*^{24BGal4}), which faithfully recapitulates *how* expression (Brand and Perrimon, 1993; Fyrberg et al., 1997) (Fig. 3, supplementary material Fig. S3). Prominent *how* expression is noted in some CNS glia, which, based on their position, correspond to the subperineurial glia that constitute the BBB (Fig. 3O-Q). No *how* expression is noted in neurons. In addition, when we expressed the *nrxIV_3.0* reporter using the *how*^{24BGal4} driver in embryos, all cells express the green exon 3 cassette, indicating that, in embryos, the presence of HOW is associated with the selection of exon 3 (supplementary material Fig. S2M-P). When we expressed the *nrxIV_3.0* reporter in larvae using the *how*^{24BGal4} driver, most cells appear green, but we noted a few cells in the nervous system that express only the red exon 4 cassette (supplementary material Fig. S2I-L, arrow).

Nrx-IV splicing requires HOW response elements

We had previously shown that HOW is able to bind the *Nrx-IV* pre-mRNA (Edenfeld et al., 2006). Four potential HOW-binding sites (HOW response elements, HREs) are evolutionary conserved (Fig. 4). HRE1 is situated 5′ of exon 3, and three binding sites, HRE2 to

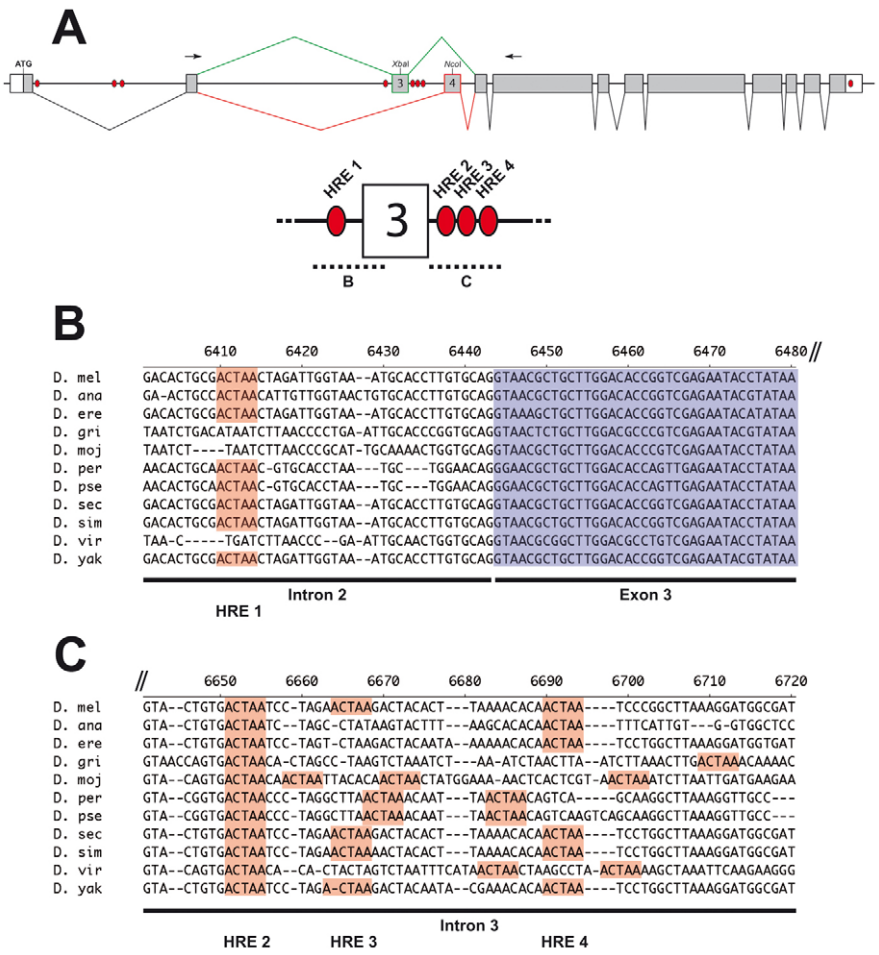

Fig. 4. Evolutionary conserved HRE in *Nrx-IV* pre-mRNA. (**A**) In the intronic sequence of the pre-mRNA of *Nrx-IV*, several HOW response elements (HREs) are found in close proximity to the alternatively spliced exon 3 (indicated by red dots). A magnification of the genomic region around exon 3 is shown. HRE1 is located 5′ of exon 3 and HRE2, HRE3 and HRE4 are found 3′ of it. (**B**,**C**) Alignment of the genomic regions of the *Nrx-IV* drosophilid orthologs containing the HREs (pink). HRE1 is conserved in all drosophilidae with exception of *D. grimshawi, D. mojavensis* and *D. virilis.* HRE2 is highly conserved in all drosophilidae. *D. ananassae* and *D. erecta* have two HREs in this region. All other drosophilidae have three HREs; *D. mojavensis* has four.

HRE4, are clustered within 40 bp just 3′ of exon 3. Within the splicing reporter *nrxIV_2.0*, we mutated all individual HREs, as well as several combinations, and tested the consequences for glial cell-specific splicing (Fig. 5, supplementary material Fig. S4).

In wild-type glia, the *nrxIV_2.0*^{wt} construct is almost exclusively spliced towards the exon 3-containing mRNA (Fig. 5C,D, green dots). Upon mutation of the three 3′ HREs, an aberrant splicing pattern is noted with mRNA molecules containing both, exon 3 and exon 4 (Fig. 5C, white dotted boxes, supplementary material Fig. S5). The different HREs appear to act in a redundant fashion and stronger effects are noted in the triple mutant (Fig. 5C, sh2+3+4). The deletion of all 3′ HREs (Δ3) has an even stronger impact on splicing.

The removal of the 5′ HRE increases the number of transcripts lacking both exon 3 and exon 4 (Fig. 5C, red dotted boxes). This phenotype is in particularly prominent in quadruple mutants, where all HREs are mutated (Fig. 5C, sh1+2+3+4 or sh1+Δ3). The restriction analysis of the PCR products shown in Fig. 5C indicated no shift of the glial splicing pattern (Fig. 5D). In conclusion, these data suggest that binding of HOW at the three 3′ HREs is needed to suppress inclusion of exon 4 and the 5′-binding site is required for general inclusion of one of the two exons in the final mRNA.

Reconstitution of glial splicing in neurons

To further demonstrate the instructive role of HOW in regulating *Nrx-IV* splicing, we switched to neuronal cells that endogenously express the exon 4 variant of Nrx-IV but no HOW protein (Stork et al., 2009) (Figs 2, 3, 6). To simplify RNA isolation, we used brains from third instar larvae, in which the *nrxIV_2.0* reporter shows a preferential expression of *Nrx-IV*^{exon3} in glia and an exclusive expression of *Nrx-IV*^{exon4} in neurons (Fig. 6A). Upon co-expression of *nrxIV_2.0* and HOW(S) in neurons using the *elav*Gal4 driver, no change in splicing pattern was detected. This was expected, as HOW(S) is predominantly found in the cytoplasm (Fig. 7B,D,F). By contrast, HOW(L) almost exclusively localizes to nuclei where it is differentially stabilized – similar to cytosplasmic HOW(S) (Fig. 7A,C,E,F, compare asterisk and arrow). Moreover, neuronal expression of HOW(L) was able to induce a glial-specific splicing pattern of the *nrxIV_2.0* splice reporter (Fig. 6, bands indicated by green dots). Thus, the presence of nuclear HOW seems to determine the alternative use of exons 3 and 4.

Identification of additional factors regulating HOW function

The above data are compatible with the following model underlying HOW function. HOW(L) acts as a constitutive factor promoting the selection of *nrxIV*^{exon3} in glia and also functions, when ectopically expressed, in neurons. However, in wild-type glia the formation of septate junctions occurs significantly later, after HOW expression is initiated. Thus, HOW is likely to be regulated post-translationally to instruct *Nrx-IV*^{exon3} splicing in glia. As HOW can be phosphorylated (Nir et al., 2012), we tested the influence of several kinases on the splicing activity following an RNAi-based (or expression of dominant-negative isoforms) screening approach (supplementary material Table S1).

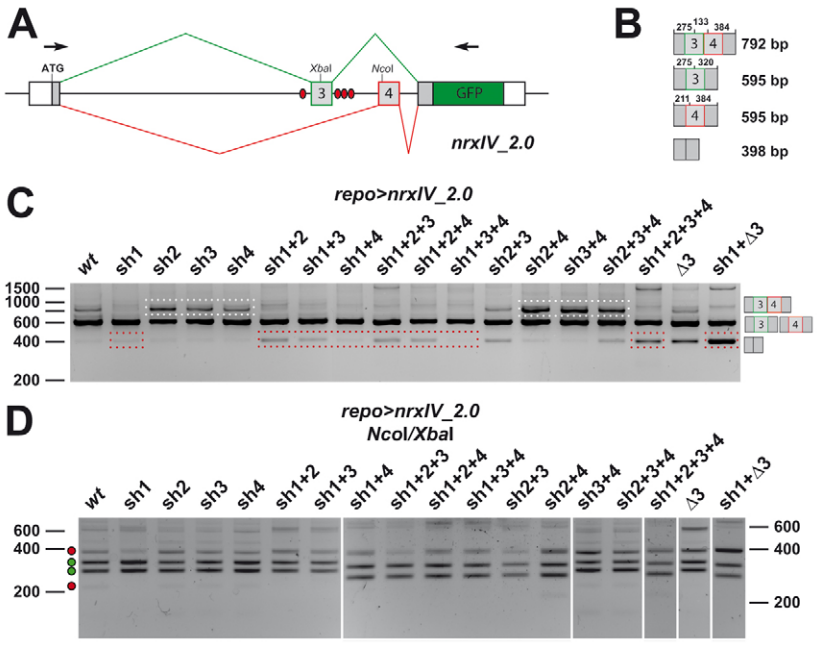

Fig. 5. Functional analysis of the HRE. (**A**) All HREs (red filled circles) in the splicing reporter *nrxIV_2.0* were mutated individually and in different combinations [ACTAA was mutated to TAAAC and named as 'shuffle' (sh); see supplementary material Fig. S4]. (**B**) The different splicing products of the *nrxIV_2.0* reporter are depicted and the fragment length is shown. (**C**) Splice patterns of wild-type (wt) and the different mutant *nrxIV_2.0* reporter constructs upon glial expression. Undigested PCR fragments are shown. All mutant *nrxIV_2.0* reporter constructs, which carry a HRE1 mutation (sh1), lose the basal inclusion of exon 3 and exon 4, compared with wild type. Instead, HRE1 mutants show increased exon skipping (red dotted lines), except the mutants for HRE 1+4 and HRE 1+3+4. The mutation of HRE 2+3 and the complete deletion of HRE2, 3 and 4 (Δ3) also show increased exon skipping. Mutants for HRE 2 or 3 or 4 alone and combinations of HRE 2+4, 3+4 and 2+3+4 show an increase of the splice variant containing exon 3 and exon 4 (white dotted lines). (**D**) Restriction analysis of the PCR products shown in C. All PCR products were subject to a *NcoI*/*Xba*I double restriction. No shift of the glial splicing pattern is observed in the HRE mutants. All changes in the pattern of the restriction bands correspond to the splicing patterns found in C. See supplementary material Fig. S5 for a better view of 133 bp band corresponding to a fragment generated by concomitant inclusion of exons 3 and 4. The gray lines indicate the corresponding molecular marker. The red and green dots indicate the DNA bands of the different splicing isoforms.

We determined the effects of gene silencing on the splicing of the *nrxIV_2.0* reporter specifically in larval glial cells. Fifteen kinases were tested and silencing of two of them, Raf and Cdk12, resulted in a clear shift of *Nrx-IV*^{exon3} to *Nrx-IV*^{exon4} splicing (Fig. 6B, supplementary material Table S1).

Cdk12 protein is a nuclear localized kinase that phosphorylates the C-terminal domain (CTD) of the RNA polymerase II during transcriptional elongation (Bartkowiak et al., 2010) (Fig. 9). The phosphorylated CTD is bound by Prp40, a subunit of the U1 snRNP (Morris and Greenleaf, 2000; Neubauer et al., 1997). Prp40, in turn, has been shown to interact with the HOW-binding protein Crn/Clf1 (Chung et al., 1999; Edenfeld et al., 2006). Thus, Cdk12 is in a position to facilitate splicing of pre-mRNAs that have bound the HOW protein (Fig. 9).

Provided that phosphorylation of the CTD by Cdk12 favors the recruitment of the U1 snRNP subunit Prp40 and thus the recruitment of Crn/HOW, one would postulate that knockdown of *prp40* gene function affects alternative splicing of *Nrx-IV*. To assay a cell-type specific reaction, we monitored the splicing of the *nrxIV_2.0* splice reporter following pan-glial co-expression of *prp40*^{RNAi}. Wild-type neurons predominantly express exon 4-containing *Nrx-IV* transcripts, whereas glia express both exon 3- and exon 4-containing *Nrx-IV* transcripts. Upon silencing of *prp40* function, we noted a significant increase in the level of the neuronal splicing form (Fig. 6L), indicating that Prp40 acts in the same pathway as Cdk12 and HOW.

Cdk12 is required for HOW-dependent splicing of *Nrx-IV*

To test the model that Cdk12 regulates HOW function, we first determined the relationship of the two genes in epistasis experiments. In a gain-of-function approach, we asked whether the ability of HOW(L) to induce glial-specific splicing in larval neurons requires Cdk12 activity. Cdk12 is expressed ubiquitously and localizes to the nucleus (Fig. 3R, supplementary material Fig. S3D) (Bartkowiak et al., 2010). We used the fluorescent-based *nrxIV_3.0* reporter, which, in contrast to the *nrxIV_2.0* reporter, shows some expression of the *Nrx-IV*^{exon3} isoform in neurons. Expression of ectopic HOW(L) in neurons results in late larval lethality. The surviving larvae show reduced brain size and reduced amounts of the neuronal splicing isoform (Fig. 8B-C′). To determine the ratio of transcripts, we determined the quotient of the mean fluorescence intensity of the EGFP and mCherry signals in the brain lobes using ImageJ (Fig. 8B,F). Expression of HOW(L) resulted in a significant shift of the isoform quotient from 0.63 to 1.67, reflecting the decreased expression of exon4 mRNAs ($P<10^{-7}$; Fig. 8C,F). Upon expression of HOW(L) and concomitant silencing of Cdk12, the larval lethality is rescued, demonstrating that HOW(L) acts at least in part through Cdk12. The splicing pattern of the *nrxIV_3.0* reporter is now shifted to a ratio of 1.19, suggesting that the presence of Cdk12 is needed for full HOW(L) activation ($P<10^{-7}$; Fig. 8D,F). Knock down of only Cdk12 does not alter the splicing pattern of the *nrxIV_3.0* reporter (ratio is 0.67;

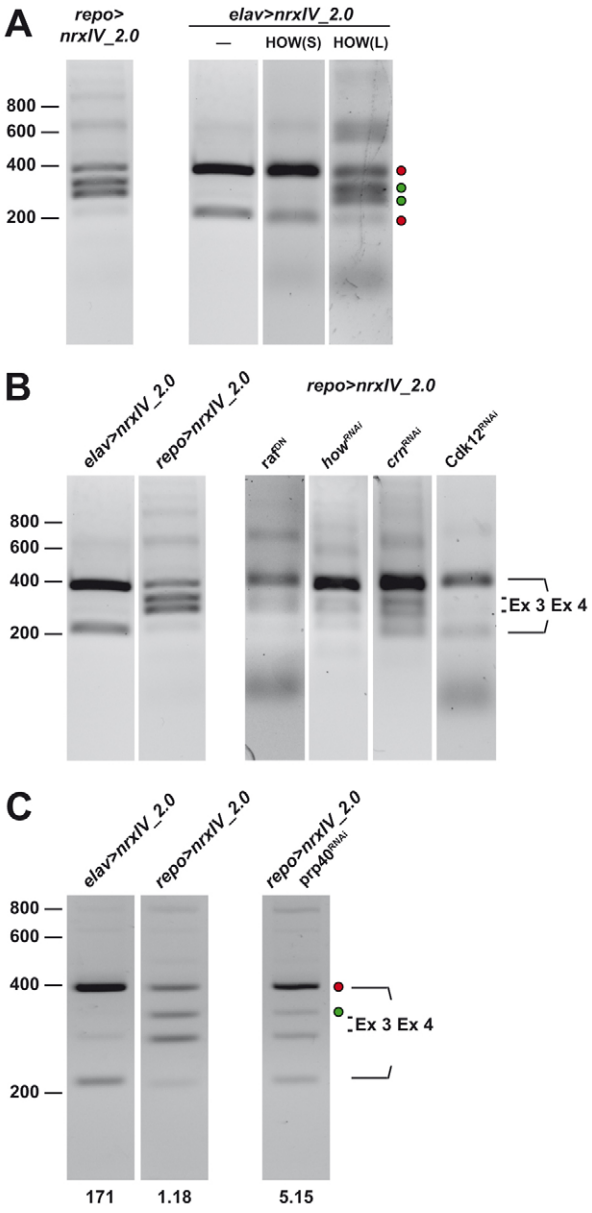

Fig. 6. HOW(L) and Cdk12 control glial splicing of *Nrx-IV*. (**A**) The expression of the *nrxIV_2.0* reporter in the central nervous system of third instar larvae has a stereotypic splice pattern. Expression in glial cells (left most panel) predominantly generates exon 3-containing transcripts. Expression in neurons leads to exon 4 inclusion (second panel). No change is visible upon the additional expression of HOW(S) (third panel). The co-expression of HOW(L) and the *nrxIV_2.0* reporter in neurons leads to an induction of the exon 3-containing isoform (green dots, fourth panel). (**B**) Analysis of the *nrxIV_2.0* reporter splicing in third instar larval brains. Splicing is shifted to *nrxIV*^{exon4} isoform upon glial expression of a dominant-negative *raf*, *how*^{RNAi}, *crn*^{RNAi} and *Cdk12*^{RNAi}, compared with control (elav>*nrxIV_2.0* and repo>*nrxIV_2.0*). (**C**) Analysis of the *nrxIV_2.0* reporter splicing in third instar larval brains. Splicing is shifted to *nrxIV*^{exon4} isoform upon glial expression of a prp40^{RNAi} construct compared with control. The red and green dots indicate the bands that were used to calculate the transcript ratio, which is given below.

Fig. 8E,F). These data indicate that HOW(L) requires Cdk12 activity for its function in promoting the presence of a glial-specific spliced *Nrx-IV*^{exon3} mRNA.

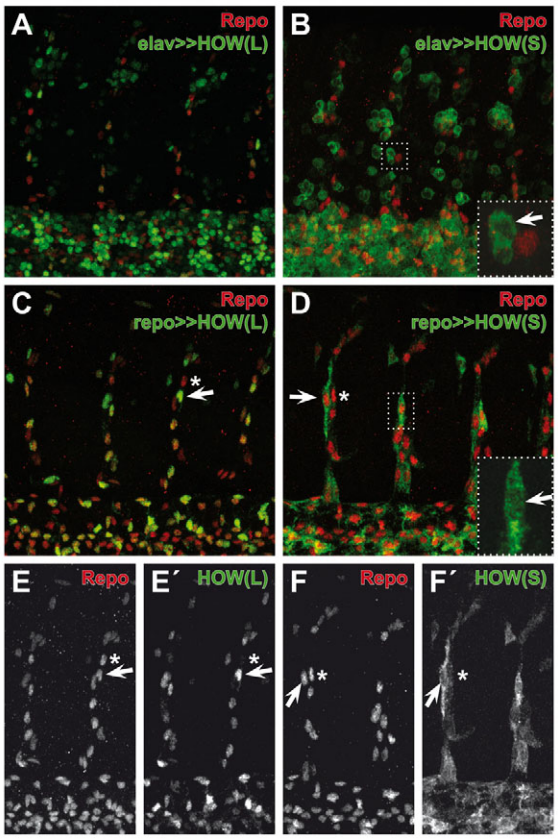

Fig. 7. Subcellular localization of HOW(S) and HOW(L). We expressed C-terminal HA-tagged HOW variants (green) during embryonic stages in neurons using *elavGal4* or in glia using *repoGal4*. Repo staining labels all glial nuclei for orientation (red). (**A**) In neurons, HOW(L) is found in the nucleus. (**B**) In neurons HOW(S) is predominantly found in the cytoplasm. The boxed area is shown at higher magnification in the bottom right. Some nuclear expression can be detected (arrow). (**C**) In glia, HOW(L) is stabilized in a subset of glial nuclei (compare asterisk and arrow). (**E**,**E′**) Single channels. (**D**) Expression of HOW(S) in glial cells. Expression mostly localizes to the cytoplasm. HOW(S) is differentially stabilized in glial cells (compare arrow and asterisks). The boxed area is shown at higher magnification in the bottom right. Some nuclear expression of the HOW(S) protein can be detected (arrow). (**F**,**F′**) Single channels.

DISCUSSION

Differential splicing is a key element in generating the amazing complexity of higher nervous systems. Through relatively few regulatory elements, a single gene can generate several different isoforms with potential distinct cellular functions. In *Drosophila*, differential splicing is required for the correct glial development. Here, we have dissected the role of the STAR-family member HOW in controlling such a differential splicing event at the *Nrx-IV* locus, which is pivotal for the generation of the BBB.

Nrx-IV exons 3 and 4 are spliced in a mutually exclusive manner. They share DNA sequence identity of 60% and encode related Discoidin domains, which provide distinct adhesive properties (Stork et al., 2009; Vogel et al., 2006). Within glial cells, expression of Nrx-IV^{exon3} predominates and participates in the formation of septate junctions. Interestingly, the binding partner of Nrx-IV at the *Drosophila* septate junctions, Neuroglian, or the Caspr-binding partner at the septate-like junctions in vertebrates,

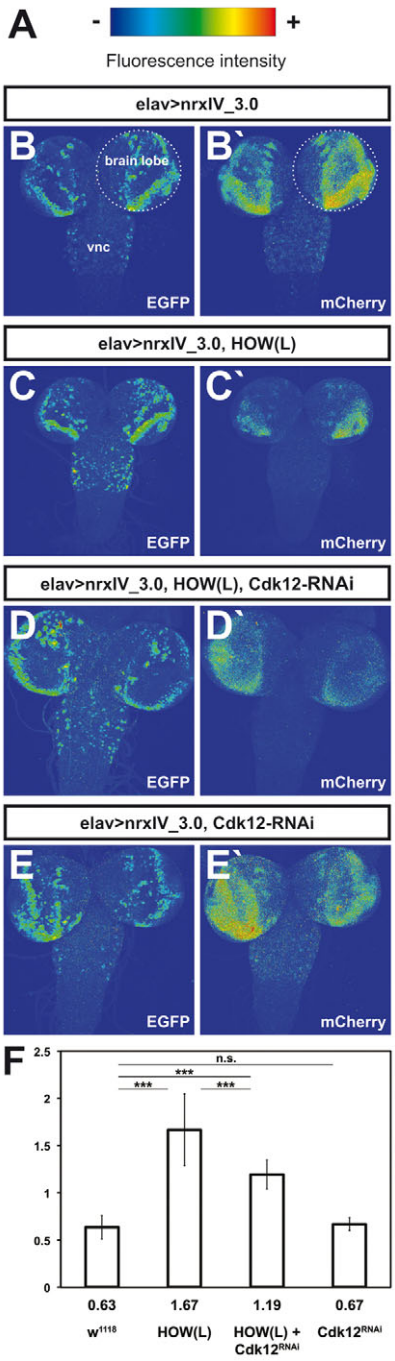

Fig. 8. Cdk12^{RNAi} changes the effect of HOW(L) on *nrxIV_3.0* splicing. Fluorescence intensity analysis upon neuronal expression of the *nrxIV_3.0* reporter in third instar larval brains. Staining with anti-EGFP and anti-mCherry were performed under the same conditions. *Z*-stacks of confocal images were taken using the same settings. False color-coded pictures are shown. (**A**) The fluorescence intensity code: dark blue (no fluorescence) to red (maximum fluorescence). Fluorescence intensity of the brain lobes (indicated by dotted lines) was measured using ImageJ. (**B**,**B′**) Neuronal expression of *nrxIV_3.0* reporter (control). The dimension of the brain lobes is indicated. The ratio of fluorescence EGFP/mCherry was measured and is given in F. The ventral nerve cord (vnc) was not analyzed. (**C**,**C′**) Neuronal expression of the *nrxIV_3.0* reporter with HOW(L). The fluorescence intensity of mCherry is reduced compared with the control. (**D**,**D′**) Neuronal expression of the *nrxIV_3.0* reporter upon co-expression of HOW(L) and Cdk12^{RNAi}. The fluorescence intensity of mCherry is not as reduced as upon expression of HOW(L) (C′). (**E**,**E′**) Neuronal expression of the *nrxIV_3.0* reporter and a Cdk12^{RNAi} construct. The ratio of EGFP and mCherry fluorescence intensity is unchanged. (**F**) Statistical analysis of the fluorescence ratio EGFP/mCherry. The different genotypes are indicated. The number of brain lobes used for the analysis are: w^{1118}, n=8; HOW(L), n=14; HOW(L) Cdk12^{RNAi}, n=14; HOW(L) Cdk12^{RNAi}, n=12. The changes were significant (***) [$P<10^{-7}$ comparing w^{1118}- and HOW(L)-expressing animals or HOW(L)- and HOW(L) Cdk12^{RNAi}-expressing animals; $P<10^{-4}$ comparing w^{1118}- and HOW(L) Cdk12^{RNAi}-expressing animals. n.s., non significant.

Neurofascin, are also subject to cell-type specific, differential splicing (Basak et al., 2007; Genova and Fehon, 2003; Hassel et al., 1997; Hortsch et al., 1990; Volkmer et al., 1996).

Differential splicing appears to be of more general relevance during the formation of septate junctions. The fly homologue of the membrane-skeleton protein 4.1, Coracle, binds to Nrx-IV and mediates the linkage of the septate junctions to the cytoskeleton (Fehon et al., 1994; Lamb et al., 1998; Ward et al., 1998). Differential splicing of *coracle* generates at least four different splice variants that encode four distinct proteins (Drysdale and Crosby, 2005). RT-PCR experiments indicate that the Coracle-PB isoform is generated in a HOW-dependent manner (F.R. and C.K., unpublished).

STAR proteins, like HOW, bind sequence motifs in the pre-mRNA of their targets (Galarneau and Richard, 2005; Israeli et al., 2007; Ryder et al., 2004; Ryder and Williamson, 2004). Following site-specific mutation of all HREs, we could show that HRE1 may be needed for general exon definition. The mutation of this sequence motif leads to increased exon skipping of both exon 3 and exon 4, suggesting a crucial role for HRE1 in general splicing, possibly affecting the branch point of this intron (Arning et al., 1996; Berglund et al., 2008; Kramer and Utans, 1991). The HRE2, HRE3 and HRE4 elements influence mutually exclusive splicing. Upon mutation of these motifs, both exons are left in the mRNA more frequently, which suggests their function in exon selection. Such an effect was not observed in neurons (data not shown). Thus, these HREs seem to play a role in exon selection.

The HOW isoforms share an identical KH RNA-binding domain (Volk, 2010; Volk et al., 2008). HOW(S) predominantly localizes to the cytoplasm and HOW(L) is found mostly in the nucleus of glial cells. Here, we showed that nuclear HOW is sufficient for the induction of glial-specific splicing in neurons. Interestingly, both HOW isoforms can partially rescue the *how* mutant phenotype. HOW(S) appears to have higher rescuing abilities. As both transgenes are inserted in the same chromosomal landing site (Bischof et al., 2007), resulting in identical expression levels, we assume that HOW(S) must be efficiently transported into the nucleus to promote *Nrx-IV* splicing. Because, following overexpression of the HOW(S), most of the protein stays in the cytoplasm, the shuttle mechanism(s) directing HOW(S) into the nucleus must be very tightly regulated. Possibly, HOW(S) has better rescuing abilities as HOW(S), but not HOW(L), can facilitate the nuclear import of the splice factor Crn (Edenfeld et al., 2006).

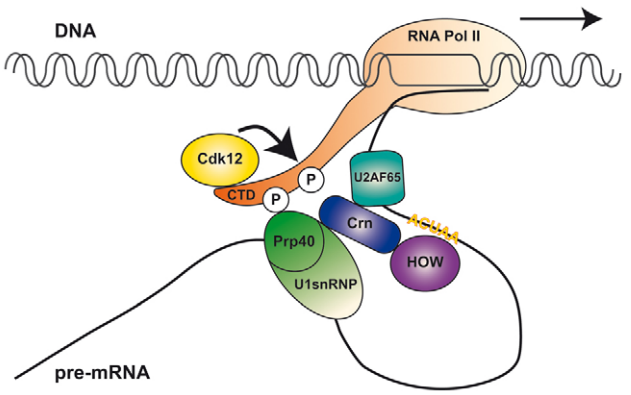

Fig. 9. Cdk12 primes HOW/Crn-dependent splicing in glial cells. The Cyclin-dependent kinase 12 (Cdk12) binds the CTD of RNAPII and leads to hyperphosphorylation (Bartkowiak et al., 2010). Prp40 binds the CTD (Morris and Greenleaf, 2000) and to Crn/Clf1 (Chung et al., 1999), which also binds to U2AF65 and HOW (Chung et al., 1999; Edenfeld et al., 2006). HOW is binding to specific target sites in the pre-mRNA (Israeli et al., 2007). For further details, see text.

STAR family proteins are phosphorylated on several residues (Matter et al., 2002; Stoss et al., 2004; Nir et al., 2012). In the past, it has been established that the HOW homolog Sam68 is phosphorylated by MAPK the regulation of which is controlled by Raf (Matter et al., 2002). Indeed, expression of a dominant-negative Raf protein in glia shifted the splicing pattern towards the neuronal form, suggesting a role for receptor tyrosine kinase signaling for glial differentiation as it has been demonstrated at several other instances (Franzdottir et al., 2009; Michailov et al., 2004).

In addition, we noted that silencing of Cdk12 resulted in a shift of the splicing pattern towards the neuronal form. Cdk12 is a broadly expressed serine/threonine kinase that also contains stretches of arginine- and serine-rich sequences (SR domains) known to be present in RNA-processing proteins, which regulate splicing, nuclear export and stability of the mRNA (Chen et al., 2006; Huang and Steitz, 2005). *Drosophila* Cdk12 is associated with the C-terminal domain (CTD) of the RNA polymerase II (RNAPII) and phosphorylates Ser2 (Bartkowiak et al., 2010) (Fig. 9). The CTD of RNAPII acts as an assembly platform that controls transcription and pre-mRNA processing (Egloff and Murphy, 2008). Phosphorylated CTD in turn is recognized by Prp40 (Morris and Greenleaf, 2000), which belongs to the U1 snRNP. Moreover, a direct interaction between PrP40 and Crocked neck like factor 1 (Clf1), which binds HOW, has been demonstrated (Chung et al., 1999; Edenfeld et al., 2006). Thus, phosphorylation of CTD by Cdk12 (Bartkowiak et al., 2010) recruits the assembly of the spliceosome at specific pre-mRNA targets defined by binding of HOW (Fig. 9). In line with this model, we noted that silencing of Prp40 also alters *Nrx-IV* splicing.

In *Drosophila*, Cdk12 associates with Cyclin K (Bartkowiak et al., 2010), which is required for its catalytic activity. The activity of cyclins can be regulated by RTK signaling and thus might present a link that connects the Raf/MAPK pathway with a direct control of splicing activity (Jorissen et al., 2003; Loyer et al., 2005; Yarden and Sliwkowski, 2001). Additionally, CTD phosphorylation could be linked to MAPK activity in former studies (Bellier et al., 1997; Bensaude et al., 1999; Dubois et al., 1994; Trigon et al., 1998; Venetianer et al., 1995). Cdk12 is expressed in the nucleus of almost all cells (Fig. 3R; supplementary material Fig. S3D). To further decipher the role of Cdk12 during splicing, we have used a loss-of-function allele. Homozygous mutant animals are lethal at the beginning of larval development. However, these mutants show no splicing defects, most probably owing to strong maternal contributions (data not shown).

The formation of the BBB implies the maturation of septate junctions only in fully differentiated subperineurial glial cells. Thus, the timing of splicing of pre-mRNAs encoding septate junction proteins is crucial and most likely regulated by two independent signaling cascades. We propose that the mRNA-binding protein HOW integrates these signaling events and is key in determining cellular differentiation.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table S1. List of the analyzed genes

Name	Symbol	Tool	Splicing change
crooked neck	*crn*	dsRNA	**Ex3 -> Ex4**
held out wings	*how*	dsRNA	**Ex3 -> Ex4**
CG3542 (prp40)	CG3542	dsRNA	**Ex3 -> Ex4**
Abl tyrosine kinase	*abl*	dsRNA	Ex3
Btk family kinase at 29A	*btkat29A*	dsRNA	Ex3
Cyclin-dependent kinase 4	*cdk4*	dsRNA	Ex3
Cyclin-dependent kinase 12	*cdk12*	dsRNA	**Ex3 -> Ex4**
fused	*fu*	dsRNA	Ex3
G protein-coupled receptor kinase 2	*gprk2*	dsRNA	Ex3
rolled/mapk	*rl/ mapk*	dsRNA / dominat negative	Ex3
MAP kinase kinase 4	*mkk4*	dsRNA	Ex3
cAMP-dependent protein kinase 1	*pka-c1*	dsRNA	Ex3
Phosphotidylinositol 3 kinase 59F	*pi3k59f*	dsRNA	Ex3
Phosphotidylinositol 3 kinase 68D	*pi3k68d*	dsRNA	Ex3
pole hole	*phl*	dsRNA / dominat negative	**Ex3 -> Ex4**
shaggy	*sgg*	dsRNA	Ex3
Src oncogene at 42A	*src42*	dsRNA	Ex3
Src oncogene at 64B	*src64B*	dsRNA	Ex3
RTK signaling components			
Epidermal growth factor receptor	*egfr*	dsRNA / dominat negative	**Ex3 -> Ex4**
downstream of receptor kinase	*drk*	dsRNA	Ex3
Son of sevenless	*sos*	dsRNA	Ex3
Ras oncogene at 85D	*ras85d*	dsRNA / dominat negative	Ex3
Downstream of raf1	*dsor1*	dsRNA	Ex3