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Differential expression of neuroligin genes in the nervous system of zebrafish

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Abstract

The establishment and maturation of appropriate synaptic connections is crucial in the developmental of neuronal circuits. Cellular adhesion is believed to play a central role in this process. Neuroligins are neuronal cell adhesion molecules that are hypothesized to act in the initial formation and maturation of synaptic connections. In order to establish the zebrafish as a model to investigate the *in vivo* role of Neuroligin proteins in nervous system development, we identified the zebrafish orthologs of *neuroligin* family members and characterized their expression. Zebrafish possess seven *neuroligin* genes. Synteny analysis and sequence comparisons show that *NLGN2, NLGN3,* and *NLGN4X are* duplicated in zebrafish, but *NLGN1* has a single zebrafish ortholog. All seven zebrafish *neuroligins* are expressed in complex patterns in the developing nervous system and in the adult brain. The spatial and temporal expression patterns of these genes suggest that they occupy a role in nervous system development and maintenance.

Keywords

nlgn; NL; Nlg; cell adhesion; synapse; central nervous system

INTRODUCTION

Neuroligins (Nlgns) are transmembrane cell adhesion molecules that bind with high specificity and affinity to β-neurexins (Nrxns) (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Scheiffele et al., 2000). Cell localization studies suggest that, in general, Nlgns are localized in dendrites at synapses, i.e. at the postsynaptic density (PSD), whereas Nrxns are principally found at presynaptic terminals of axons (Dean et al., 2003; Rosales et al., 2005). These molecules have been hypothesized to mediate synaptogenesis and/or synaptic maturation (Sudhof, 2008). This interpretation is based on the ability of Nlgns expressed in non-neuronal cells to induce the formation of presynaptic terminals in contacting axons (Scheiffele et al., 2000) and the ability of Nrxns to induce the formation of postsynaptic specializations within dendrites of cultured neurons (Nam and Chen, 2005; Barrow et al., 2009).

In humans, 5 *NLGN* genes have been identified: *NLGN1*, *2*, *3*, *4X* and *4Y* (Bolliger et al., 2001). Both NLGN3 and NLGN4X are located on the X chromosome, while NLGN4Y is located on the male sex chromosome Y. Mice, on the other hand, possess only 4 *Nlgn* genes, with murine Nlgn4 being located on an autosome and demonstrating significant sequence divergence from the human proteins (Bolliger et al., 2008). In addition, *Nlgn* genes have

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been found in all vertebrates examined to date and are also present in invertebrates. 5 *NLGN* genes have been found in honey bees (Biswas et al., 2008) and 1 *NLGN* gene exists in the *Caenorhabditis elegans* genome (GenBank: NM_077882). It is possible that the increase in complexity of the *Nlgn* gene family has permitted the proteins to mediate the formation of different types of synapses and to potentially mediate synaptic specificity. While Nlgn1 is mostly localized to glutamatergic synapses (Song et al., 1999; Chih et al., 2005), Nlgn2 is concentrated at GABAergic synapses (Varoqueaux et al., 2004; Chih et al., 2005; Levinson et al., 2005). Corroborating a possible role of *Nlgn* genes during the development of the nervous system, *Nlgn* expression patterns are mostly confined to the nervous system and their expression levels increase during nervous system development in both honey bees and mice (Varoqueaux et al., 2006; Biswas et al., 2008).

The *Nlgn* family of genes has recently come under scrutiny because mutations in *NLGN3* and *NLGN4* genes have been found in patients with familial autism, Asperger syndrome and X-linked mental retardation (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005). The majority of these mutations are located in the extracellular acetylcholine-esterase (AChE)-like domain, abrogating the interaction with Nrxns and causing the protein to be retained in the endoplasmic reticulum (Chih et al., 2004). In addition to the AChE-like domain, Nlgns possess intracellular interaction domains that are also important for function. The C-terminal PDZ (postsynaptic density 95/Discs large/Zona occludens1) binding motif can interact with several PDZ domain containing proteins, most notably PSD-95 (Irie et al., 1997; Meyer et al., 2004). This small motif is necessary for PSD-95 recruitment to synapses and for the cotransport of Nlgn1 with NMDA-type glutamate receptors (NMDARs) (Barrow et al., 2009). In addition, a distinct intracellular motif can mediate an interaction with the synaptic scaffolding protein S-SCAM through its WW domain (Iida et al., 2004).

Ablation of individual *Nlgn* genes in the mouse have no overt effect on neural development or behavior, however combined deletion of *Nlgn* genes *1*, *2* and *3* results in neurotransmission deficits of inhibitory synapses in the hindbrain of mouse embryos, leading to respiratory difficulties (Varoqueaux et al., 2006). Knock-down studies in the amygdala of rats show a dependence of LTP and fear conditioning on *Nlgn1* expression (Kim et al., 2008). Furthermore, close examination of inhibitory synapses in the retinas of *Nlgn2* knockout mice reveal a decrease in the recruitment of postsynaptic GABA (A) receptors (Patrizi et al., 2008). These results suggest that Nlgn genes may not play a pivotal role in initial synapse formation, but may be critical for synaptic maturation. Alternatively, the synaptogenic function of Nlgn proteins may be redundant between individual Nlgn family members and other synaptic cell adhesion molecule families such as SynCAMs (Biederer et al., 2002) and netrin-G-ligands (Kim et al., 2006).

To shed more light on the role of Nlgns in the development of the nervous system, we have characterized the *nlgn* genes in zebrafish. Zebrafish have a relatively simple nervous system in which it is possible to detect expression patterns at the level of identifiable neurons. Furthermore, the teleost genome duplication has resulted in the duplication of roughly 50% of genes (Postlethwait et al., 2000). This duplication has permitted the partitioning of gene function or expression patterns (Cresko et al., 2003). Indeed, we have found that zebrafish possess 7 *nlgn* genes, 3 of which represent duplicates of mammalian *Nlgn* genes *2*, *3* and *4*. In contrast, *nlgn1* is present as a single copy. They are expressed throughout the nervous system during development and in the adult, with increasing expression levels during the course of nervous system development. Here we describe highly divergent expression patterns within the nervous system between the 7 *nlgn* genes during development and in the adult brain. Our data suggest that these expression patterns may provide the specificity necessary to dissect the roles of these genes during neural development *in vivo*.

RESULTS AND DISCUSSION

Isolation and Characterization of the Zebrafish *nlgn* Genes

There are five distinct *NLGN* gene family members in humans: *NLGN1*, *2*, *3*, *4X* and *4Y*. In contrast mice only possess four, with *Nlgn4* possessing great sequence divergence from other members of the *Nlgn* family (Bolliger et al., 2008). We have identified seven *Nlgn* orthologs in zebrafish by searching the zebrafish genome database and NCBI database using mammalian *Nlgns* as templates. Using reverse transcriptase PCR (RT-PCR) we have fully cloned and sequenced all seven zebrafish *nlgn* open reading frames, indicating all seven *nlgn* genes are expressed in zebrafish.

Alignment of the Nlgn amino acid sequences with those of human and mouse Nlgns showed that the primary structure of Nlgn proteins is largely conserved (see Supplementary Fig.1). This alignment revealed that for each human NLGN protein, except NLGN1, there are two zebrafish proteins that show a high degree of similarity. Because the ancestor of teleosts underwent genome duplication after branching from the tetrapod lineage (Postlethwait et al., 2000), we hypothesized these genes are duplicates. Consistent with this hypothesis, the amino acid identity as a percentage between the different sets of duplicates and their human orthologs is greater than that of the other Nlgns to each other (Table 1). Phylogenetic analysis confirms that the duplicates segregate into distinct clades with their mammalian orthologs (Biswas et al., 2008; Bolliger et al., 2008). We have named these co-orthologs *nlgn2a*, *nlgn2b*, *nlgn3a*, *nlgn3b*, *nlgn4a* and *nlgn4b*.

Interestingly, zebrafish Nlgn4a and 4b are more closely related, in terms of amino acid sequence identity, to the human orthologs NLGN4X and 4Y (between 78 and 82% identity, Table 1), than mouse Nlgn4 is to the human proteins (around 60 % identity; (Bolliger et al., 2008)). It will be interesting to further analyze the commonalities between the zebrafish Nlgn4 and the human NLGN4 duplicates, as only higher primates (*Homo sapiens* and *Pan troglodytes*) and teleost fish (*Danio rerio* and *Tetraodon nigroviridis*) possess duplicated *nlgn4* genes. BLAST searches of rhesus macaque (*Macacca mulatta*), dog (C*anis lupus familiaris*), rat (Rattus norvegicus), short-tailed opossum (*Monodelphis domestica*) and chicken (*Gallus gallus*) revealed only single copies of *Nlgn4* (see also Bolliger et al., 2008).

We detected only one *nlgn* gene similar to human *NLGN1* suggesting that this gene is a single ortholog and that its duplicate was likely lost during evolution. Furthermore, stickleback (*Gasterosteus aculeatus*) and puffer fish (*Tetraodon nigroviridis*) also possess a single *nlgn1* gene whereas the other *nlgns* are duplicated, as in zebrafish (Bolliger et al., 2008), lending weight to the observation that only a single ortholog exists in zebrafish.

We performed an analysis of conserved synteny between zebrafish and human genomes to further test the hypothesis of orthology and to determine if *nlgn2a*, *nlgn2b*, *nlgn3a*, *nlgn3b*, *nlgn4a* and *nlgn4b* genes are duplicates resulting from a genome duplication (Postlethwait et al., 2000; Jaillon et al., 2004; Postlethwait, 2007). This was performed using synteny analysis software (Catchen et al., 2009) based on the seventh zebrafish genome assembly (Zv7). Due to errors that still may be present in this version, our analysis may be imperfect. Our results suggest that zebrafish *nlgn1* is the single ortholog of human *NLGN1*, as several genes surrounding *NLGN1* on human chromosome 3 (Hsa3) have preserved co-localization on zebrafish chromosome 11(Dre11) in the regions around *nlgn1* (Fig.1A). Three of the six genes upstream of *nlgn1 (ect2, b3gnt5, zgc:110312)* possess duplicates (on Dre2), whereas downstream genes of nlgn1 (*rfc4, zgc92715, mfn1*) also returned to singleton status after the genome duplication.

The regions flanking *NLGN2* on Hsa17 display conserved synteny with regions flanking zebrafish *nlgn2a* and *nlgn2b* on Dre10 and 7, respectively (Fig.1B). For the most part, genes on Dre10 display the same order as those on Hsa17 and three genes are in the same order on Dre7, suggesting minimal chromosomal rearrangement in this region. Similarly, we also see conserved synteny between *NLGN3* on Hsa3 and *nlgn3a* on Dre14 and *nlgn3b* on Dre5 (Fig. 1C), with the flanking genes *ASB12* and *ZDHHC15* possessing orthologs on both zebrafish chromosomes.

We detected no conserved syntenies between HsaY and zebrafish *nlgn4* paralogs on Dre9 and Dre1. This is consistent with the loss of many genes from HsaY, that occurred after the generation of the sex chromosomes by duplication of a single autosomal ancestor (Graves, 1995). We did find conserved synteny between HsaX and the zebrafish *nlgn4* duplicates. In addition to *NLGN4X*, we found one other gene, *GPM6B*, which is a close neighbor to the *nlgn4* genes and has orthologs on both Dre9 and Dre1. We found four genes surrounding *nlgn4b* that appear to be single orthologs and three genes surrounding *nlgn4a* that appear to be single orthologs (Fig.1D). This represents a reduced amount of conserved synteny, as compared to the other *nlgn* genes. This may be due to the locations of NLGN4X and *nlgn4a* near the end of HsaX and Dre9, respectively, as chromosomal rearrangements, such as fissions, are more likely near chromosome ends (Bailey and Murnane, 2006). We conclude that all seven identified *nlgn* genes are indeed orthologs of four of the human NLGN genes.

Temporal Expression of *nlgn* Genes

We conducted RT-PCR to detect the presence of the *nlgn* genes during development and in the adult brain (Fig.2). All seven *nlgns* were expressed at or before 24 hours postfertilization (hpf), with expression remaining present or increasing as development progressed. In addition, all seven genes were strongly expressed in the adult brain. We found that *nlgn1* and *nlgn4a* were expressed at the 16 cell stage (1.5 hpf, Fig.2A,F), before midblastula transition, indicative of maternal mRNA expression. This expression was then lost until it was faintly detected at 24 hpf. Expression of *nlgn2a* was first detectable at 16 hpf and *nlgn2b* expression was first detectable at 3-somite stage (11 hpf, Fig.2B,C). We found that *nlgn3a* was also expressed maternally, however the low level made detection difficult (1.5 hpf, Fig.2D). This expression was then lost and was again detected at 16 hpf. Expression of *nlgn3b* was first detected at 90% epiboly (9 hpf, Fig.2E). This gene remained expressed at an intermediate level, and was then more strongly expressed at 48 hpf. *nlgn4b* was first detected at 3-somites (11 hpf, Fig.2G) and its expression then appeared to increase through development. We conclude that all *nlgn* genes are expressed during the development of zebrafish and that they show distinct temporal expression profiles.

Expression Patterns in the Developing Brain

To gain insight into possible anatomical specificity of expression patterns for the seven *nlgn* genes, we assayed the temporal and spatial distribution of their mRNAs by *in situ* hybridization (ISH) in whole-mount and sectioned zebrafish in the embryonic and adult zebrafish nervous system. We are confident that the probes used were specific and did not cross-react, as their expression patterns are highly specific and sense probes did not reveal any staining.

Our RT-PCR analysis suggested that, during early development, zebrafish embryos showed either no or very weak expression of most *nlgn* genes until after 16 hpf (Fig.2). Since we expected *nlgns* to be expressed in the developing nervous system, we decided to compare *nlgn* gene expression patterns at pharyngula stage (24–42 hpf) and hatching period (48–72 hpf) (Kimmel et al., 1995). At 24 hpf, the sensory-motor reflexive circuits are becoming functional (Drapeau et al., 2002), suggesting ongoing synapse formation in the spinal cord

(Pietri et al., 2009). At 48 hpf, sensory organs are becoming more mature. Olfactory placodes reveal beating cilia and hair cells have differentiated in the sensory maculae of the otic vesicle and in neuromasts (Kimmel et al., 1995). In the eye at 30 hpf, the first post-mitotic neurons have differentiated (Hu and Easter, 1999); and by 48 hpf, retinal axons reach the optic tectum (Burrill and Easter, 1995). We conclude our descriptive study by discussing the adult *nlgn* expression in the adult zebrafish brain and comparing the expression pattern to adult mouse brain *Nlgn* gene expression.

At both 24 and 48 hpf, the vast majority of expression for all *nlgns* was localized to the nervous system of zebrafish. Interestingly, close examination of the mRNA expression revealed very specific expression patterns, down to individual cells, particularly in the developing hindbrain and spinal cord (see below).

At 24 hpf, all seven *nlgn* genes were expressed in restricted regions in the developing forebrain [telencephalon (t) and diencephalon (d), Fig.3A]. We found weak and rather broad expression of *nlgn1* in the telencephalon, diencephalon and the eyes (Fig.3A,A′). In contrast, all other *nlgn* genes presented more robust and specific expression patterns. We found *nlgn2a* (Fig.3C,C′) and *nlgn2b* (Fig.3E,E′) mRNA transcripts expressed in the dorsal-rostral and the ventral-rostral regions of the telencephalon, whereas *nlgn3a* (Fig.3G,G′) and *nlgn3b* mRNA transcripts (Fig.3I,I′) were located more diffusely within the telencephalon. The expression of *nlgn4a* (Fig.3K,K′) was restricted to a dorsal and caudal aspect of the telencephalon, likely the olfactory bulbs (ob in Fig.3K′). Interestingly, we found *nlgn4b* expression (Fig.3M,M′) in a ventro-caudal region of the telencephalon, presumably the dorso-rostral cluster (drc in Fig. 3M). In addition, *nlgn4b* was expressed in the dorsal-most cell layers of the telencephalon (arrowheads in Fig.3M,M′). In general, all *nlgn* genes were expressed in the diencephalon in slightly different expression patterns. All *nlgn* genes except *nlgn1* were expressed in the ventro-rostral cluster (vrc; Fig.3C).

In the region of basal mesencephalon, we found clusters of cells expressing *nlgn2a, nlgn2b, nlgn3a, nlgn3b* and *nlgn4a*, suggesting expression of these *nlgn* genes in the ventro-caudal cluster (vcc in Fig.3E). Interestingly, *nlgn2b* was highly expressed in a small number of cells in the vcc (arrow in Fig.3E′). In general, we found faint and diffuse signal of all seven *nlgn* genes in the dorsal midbrain, suggesting only weak or no expression in this region. While all seven *nlgn* genes were expressed in the cranial ganglia (cg in Fig.3C′), the expression of *nlgn2a* (Fig.3C′) was significantly stronger compared to all other *nlgn* genes. As the expression of *nlgn* genes in the hindbrain was very restricted and selective at 24 hpf, we describe it in more detail in Fig.5.

At 48 hpf, *nlgn1* expression in the forebrain (Fig.3B,B′) appeared weaker compared to the other *nlgn* genes. Within the telencephalon, including the olfactory bulbs, *nlgn2* and *3* genes had similar expression patterns, whereas the *nlgn4* genes displayed more unique expression patterns. Also, *nlgn4b* showed a unique staining pattern in the diencephalon, likely in the posterior tuberculum, hypothalamus and hypophysis (arrows in Fig.3N). Within the diencephalon, all other *nlgn* genes were expressed in a similar and at least partially overlapping pattern, namely in the preoptic area, hypophysis, thalamus and hypothalamus. Further, all *nlgn* genes shared expression in the preoptic tectum, tegmentum, cerebellar plate, *medulla oblongata* and patches of otic sensory neurons in the hindbrain.

While expression for all *nlgn* genes was detected in the developing eyes, the expression levels and distributions differed. *nlgn1* demonstrated stronger expression in the ganglion cell layer at 48 hpf (Fig.4A_3); *nlgn4b* was expressed the strongest in the peripheral region of the retina (Fig. 4G_2,G_3). In contrast, expression for *nlgn2a, 2b, 3a, 3b* and *4a* was distributed evenly across all layers of retina at this age, albeit with different expression levels (Fig.4B_3-

F_3). *nlgn2a*, *2b*, and *3b* were expressed in the olfactory placode (op in Fig.$4B_1$,C_1 and not shown), while expression was not detected for *nlgn1*, *3a*, *4a* and *4b*. At 48 hpf, the optic tectum (ot in Fig.$4F_3$) was clearly visible as the most dorsal and caudal part of the mesencephalon. Expression was weak in the ot for *nlgn1, 2b, 3a* and *4a*, but strong in the tegmentum. This lack of expression was most clearly visible for *nlgn4a* (Fig.$4F_3$). Although broadly expressed, *nlgn2a* showed stronger expression in the tegmentum. *nlgn3b* appeared uniformly expressed throughout the midbrain. *nlgn1, 2a, 2b* and *3b* were expressed throughout the hypothalamus (ht in Fig.$4A_3$; Fig.$4B_3$,C_3,E_3), while *nlgn4*a was not expressed in this brain region (Fig.$4F_3$). In contrast, *nlgn3a* was almost exclusively expressed at its ventral portion (Fig.$4D_3$). At this level of the 48 hpf brain, this region corresponds to the ventral hypothalamus and the anterior-most portion of the hypophysis. *nlgn2a* also showed expression in the hypophysis (Fig.$4B_3$), whereas *nlgn4b* appeared to be exclusively localized to the hypophysis (hy in Fig.$4G_3$) and not to the hypothalamus. More posteriorly, expression was detected for all *nlgn* genes in the myelencephalon (Fig.$4A_4$-G_4), with very distinct expression in ventral regions of this structure for *nlgn2b* and 3a (Fig. $4C_4$,D_4) at the level of the otic vesicle (ov in Fig.$4A_4$). In the region of the hindbrain adjacent to the pectoral fin buds (pfb in Fig.$4A_5$), *nlgns 1, 3b* and *4b* displayed a rather uniform distribution. In contrast, *nlgn3a* was localized ventrally, while *nlgn2a*, *2b* and *4a* were localized laterally. We conclude that all seven *nlgn* genes are expressed widely throughout the developing brain of zebrafish in highly specific but also partially overlapping patterns. Also, we observed that the duplicate pairs (e.g. *nlgn2a* and *2b*) show distinct expression patterns, suggesting subfunction partitioning between the duplicates.

Cell specific expression in the reticulospinal neurons

Upon close examination of expression of the *nlgn* genes in the hindbrain of embryos at 24 hpf, we noticed very distinct and restricted expression patterns. It was possible to identify individual cells in particular rhombomeres (Fig.5). For example, the Mauthner (M) cells are conspicuously large neurons located in rhombomere 4 (r4), just anterior to the otic vesicle (ov; Fig.5A). The expression patterns detected for *nlgn1* and *nlgn4b* suggest that they may be almost exclusively expressed in large cells in r4, perhaps Mauthner cells (arrows in Fig. 5A,G). In contrast, *nlgn2a*, *2b*, *3a* and *3b* were expressed symmetrically in multiple cells throughout r2–6 on either side of the midline (Fig. 5B–F). The large reticulospinal neurons located throughout this part of the hindbrain are involved in generating specific swim behaviors, such as the escape response (Gahtan et al., 2002). Thus, it is interesting to speculate that the *nlgn* genes are expressed in the reticulospinal neurons and may be aiding in the formation of specific connections necessary for sensory-motor control in zebrafish embryos and larvae.

Differential expression in the spinal cord

Next we focused our attention on expression patterns of the *nlgn* genes in the developing trunk. *nlgn1* was not detectable in the trunk (not shown). However, all other *nlgn* genes were expressed in the trunk, with the vast majority of ISH staining localizing exclusively to the spinal cord (sc in Fig.6A) at 24 and 48 hpf. Interestingly, they showed highly varied patterns of expression that were also dynamic with development. *nlgn2a* was expressed in cells in the dorsal spinal cord, presumably Rohon-Beard sensory neurons (arrows in Fig. 6A) and dorsal interneurons at 24 hpf. This expression pattern transitioned to more ventrally located cells at 48 hpf (Fig.6B). In contrast, *nlgn2b*, was expressed in ventral cells of the spinal cord, presumably motoneurons and interneurons, at both 24 and 48 hpf (Fig.6C,D). Both *nlgn2* genes were expressed with an anterior to posterior gradient at 24 hpf (Fig.6A,C), suggesting that they are required during maturation of spinal cord development. *nlgn3a* and *3b* were weakly expressed in spinal cord cells. *nlgn3a* was stronger expressed in clusters in the ventral spinal cord, likely motoneurons; no expression was detectable at 48 hpf (Fig.6F). In

addition, *nlgn3a* showed strong staining ventral to the somites at 24 hpf (arrow in Fig.6E), consistent with expression in the mesoderm. This is interesting as NLGN3 was reported to be expressed outside of the nervous system in humans, but not in rodents (Philibert et al., 2000). While *nlgn4a* showed diffuse and weak expression at both 24 and 48 hpf (Fig.6I,J), similarly to *nlgn3b* (Fig.6G,H), *nlgn4b* showed a remarkably specific expression pattern in a limited number of interneurons at both time points (Fig.6K,L). In conclusion, our analysis of *nlgn* gene expression in the trunks of developing zebrafish embryos demonstrated highly divergent expression patterns for the different *nlgn* genes, suggesting that the Nlgn proteins may participate in cell-type-specific functions during nervous system development.

Expression in the adult brain

In contrast to the specific expression patterns displayed by the *nlgn* genes in the developing nervous system, these genes were expressed in highly overlapping patterns in the adult brain of zebrafish (Fig. 7). In general, ISH staining was seen in most regions where neuronal cell bodies are to be expected, including the periventricular gray zone of the optic tectum (OT), periventricular pretectal nucleus, thalamus, parvocellular preoptic nucleus, hypothalamus and the facial and vagal lobes of the hindbrain (see Fig. 7A′ for diagram of brain regions). However, we noticed that each *nlgn* gene was expressed in a distinct subregion of the telencephalon. For example, *nlgn1* was expressed in the ventral nucleus of the ventral telencephalon (Vv in Fig.7B), whereas *nlgn2a* was expressed in the dorsal nucleus of the ventral telencephalon (Vd) and in the medial zone of dorsal telencephalon (Dm, see Fig.7C). *nlgn2b* showed a similar pattern of expression in the telencephalon as *nlgn1*, which contrasted sharply with *nlgn3a*, being expressed exclusively in the Dm (Fig.7E). *nlgn3b* expression was broad and uniform in the forebrain (Fig.7F). *nlgn4a* and *4b* were sparsely expressed in this forebrain structure, being mostly restricted to the Vd (Fig.7G,H). In addition, we noticed that all but *nlgn 4b* were expressed in sparse cells in the dorsal layers of the optic tectum (OT, Fig.7B′-H′).

In general, *nlgn4a* and *4b* showed the most divergent expression patterns to all other *nlgn* genes. For example, *nlgn4a* (Fig.7G) was not expressed in the *torus longitudinalis* (Tl, see Fig.7C), whereas all other nlgn genes were expressed in this structure (expression of *nlgn2a* was reduced but present). *nlgn4b* expression was absent from the *corpus mamillare* (Cm in Fig.7H), a brain nucleus that presented expression for all other *nlgn* genes. Expression in the cerebellum also demonstrated differential patterning for the *nlgn4* genes. While most of the *nlgn* genes were expressed in the granule cell layer (GCL in Fig.7E′) of the *corpus cerebelli* (CCe, see Fig.7H′) and *valvula cerebelli* (VCe, see Fig.7G″), *nlgn4a* was only expressed in the Purkinje cell layer (PCL in Fig.7G′) of the CCe, with almost no expression in the VCe. Furthermore, *nlgn4b* was expressed in the granule cell layer of the CCe, but not in the VCe (Fig.7H′).

Our results suggest that, in general, the expression patterns of the *nlgn* genes in the adult zebrafish brain largely resemble the broad and overlapping expression patterns seen for *Nlgn1*, *2* and *3* in mice (http://mouse.brain-map.org), but do show some regional differences, especially for *nlgn4a* and *4b*. To date there is no information regarding the expression pattern of mouse *Nlgn4*, as this was only recently identified in the mouse genome due to unusually high variation in the mouse Nlgn4 sequence compared to other species (Bolliger et al., 2008). We conclude that the *nlgn* genes are expressed predominantly in the nervous system during zebrafish development and in the adult. Their dynamic and highly specific expression patterns suggest an important role in nervous system development.

EXPERIMENTAL PROCEDURES

Cloning of *nlgn* Genes

To clone the zebrafish *nlgn* genes, we searched the zebrafish genome assembly Zv7 from the Sanger Institute (http://www.ensembl.org/Danio_rerio) using the human NLGN1, NLGN2, NLGN3 and NLGN4X protein sequences (GenBank: NM_014932, NM_020795, NM_018977 and NM_020742 respectively). This search revealed seven zebrafish putative *nlgn* genes, with duplications at the *nlgn*2, *nlgn*3 and *nlgn*4 loci. The putative translated *nlgn* coding sequences contained signal peptide sequences and stop codons, indicating full-length open reading frames. PCR primers used in the cloning of the *nlgn* genes were designed 5′ and 3′ to these predicted coding sequences. RT-PCR was used to clone full-length zebrafish *nlgn* coding sequences from 6 days postfertilization cDNA and adult brain cDNA. The coding sequences for the zebrafish *nlgn* mRNAs have been deposited with NCBI under the following accession numbers: *nlgn1*, GQ892833; *nlgn2a*, GQ892834; *nlgn2b*, GQ892835; *nlgn3a*, GQ892836; *nlgn3b*, GQ892837; *nlgn4a*, GQ892838; *nlgn4b*, GQ892839.

Protein Alignment

Protein sequences deduced from the cloned sequences of the zebrafish *nlgn* genes were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). This alignment was used to predict the shared amino acid percent identity from the pairwise alignments of the seven Nlgn proteins.

Conserved Synteny

The chromosomal location of the *nlgn* genes were determined by blasting the nucleotide sequences against the zebrafish genome (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=7955). The HomoloGene tool at NCBI was used to identify zebrafish orthologs or co-orthologs of Homo sapiens genes in regions approximately 10–30 Mb flanking *NLGN1*, *NLGN2*, *NLGN3* and *NLGN4X*. The genes found in this search were then used to search a synteny database (http://teleost.cs.uoregon.edu/acos/synteny_db/), which is based upon the zebrafish genome assembly Zv7 (Catchen et al., 2009). The gene names and approximate locations indicated as megabases were determined using the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/).

Reverse Transcriptase PCR

RNA was isolated from whole embryos and adult brains using Trizol Reagent (Invitrogen). mRNA was isolated from total RNA and potential genomic DNA contamination using the Oligotex mRNA Midi Kit (Qiagen). First Strand cDNA was synthesized using the SuperScriptIII First-Strand Synthesis System (Invitrogen). The cDNAs were screened for genomic contamination by performing a PCR reaction with a reverse primer specific to intron 1 of the *nlgn1* gene (forward primer 5′-AACAACCAAACCGCCTGAGC-3′ and reverse primer 5′-GGATGGTGGGAAGGTTAGGACA-3′). Genomic DNA was used as a control. Concentrations of the cDNAs were determined and adjusted using primers specific to the tubulin-alpha gene (forward primer 5′-CTGTTGACTACGGAAAG AAGT-3′ and reverse primer 5′-TATGTGGACGCTCTATGTCTA-3′). cDNAs were amplified using forward primers designed to amplify a 3′ region of the *nlgn* gene low in nucleotide similarity and reverse primers in the 3′-UTR to ensure specificity. Primer sequences are listed in Table 1 in supplementary data online.

In Situ Hybridization

AB/Tübingen zebrafish embryos were raised at 28.5°C under standard procedures (Westerfield, 2000) and staged in hours post fertilization (hpf, Kimmel et al., 1995). To prevent pigment formation, embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU) in embryo medium at 8 hpf. Following the manufacturer's protocol (Roche), sense and antisense RNA probes were transcribed *in vitro* from linearized plasmids and tagged with digoxigenin. Purchased ESTs were used to synthesize the *nlgn1* and *nlgn3b* probes (Open Biosystems catalog numbers EDR1052-5635463 and EDR442-98316200 respectively). Probes for *nlgn2a*, *nlgn2b*, *nlgn3a*, *nlgn4a* and *nlgn4b* were synthesized from full length cDNAs cloned in pCRII-TOPO (Invitrogen). Full length probes were fragmented into 600–800 bp segments by incubating the probes in 2× carbonate buffer at 60°C for time = $(L_{initial}\text{-}L_{final})/(0.11\ kb\ min^{-1}\times L_{initial}\text{-}L_{final})$ where L=length in kb. Fragmentation was stopped with 3M NaOAc and the probes were precipitated using standard protocol. In order to test the specificity of the full length probes we also synthesized probes in the 3′-UTR region; although this staining matched that of the fragmented probes, the signal was weaker. Thus, in this manuscript we show whole-mount ISH stainings carried out with the full-length fragmented probes according to the procedures outlined in (Thisse and Thisse, 2008) with some minor modifications. ISH on frozen sections was performed as described in (Jensen et al., 2001) with some modifications. Sections were cut at 18 μm for 48hpf embryos and 20 μm for adult brains. Embryos and sections were viewed with a Zeiss Axioplan2 microscope and photographed with a Zeiss AxioCam MRc5 camera. The descriptions of expression patterns are based on high resolution close-up images and many additional sections that have not been reproduced due to limited space.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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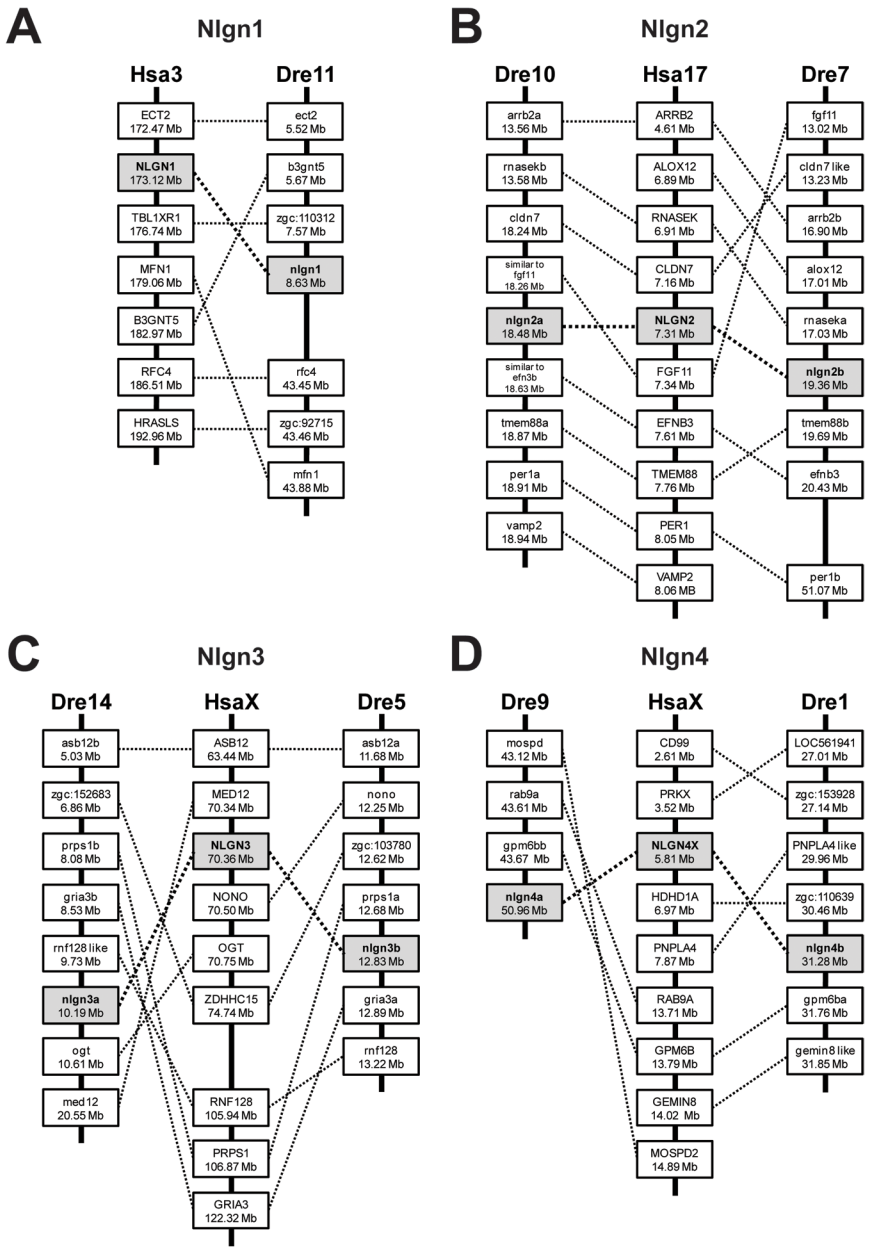

Figure 1. Genomic analysis of conserved syntenies for zebrafish *nlgn* genes

Chromosomal regions surrounding the human (Hsa) and zebrafish (Dre) Nlgn genes are represented with genes in boxes and their approximate locations in megabases (Mb). (**A**) *nlgn1*maps to Dre11 and is near several genes whose orthologs appear on Hsa3 near *NLGN1*. (**B**) *nlgn2a* maps to Dre10 whereas *nlgn2b* maps to Dre7 with several additional duplicates showing co-conserved synteny of these chromosome segments. (**C**) *nlgn3a* maps to Dre14 and *nlgn3b* maps to Dre5. For the purpose of this figure ENSDARG00000029890 was named *rnf128 like* because it was the reciprocal best BLAST hit. (**D**) *nlgn4a* maps near the end of Dre9 and *NLGN4X* maps near the end of HsaX. Gene names and locations indicated as megabases were determined using the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/). *Nlgn* orthologs are highlighted in grey boxes.

Larger gaps between genes on these chromosomal segments represent stretches of the chromosomes with no orthologous genes. Distances are not to scale.

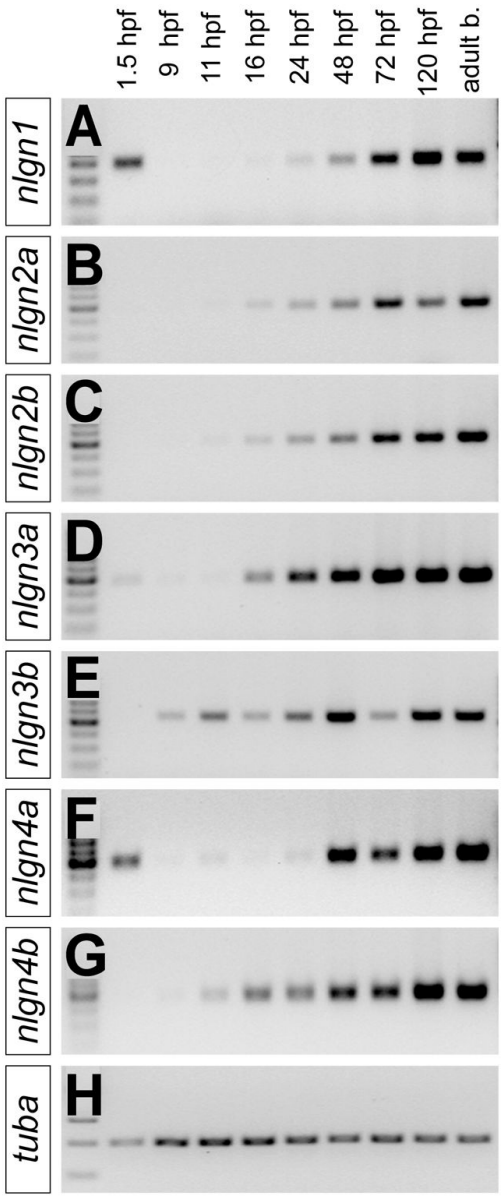

Figure 2. Analysis of *nlgn* expression levels during development

Expression levels were assayed by RT-PCR using primers specific to (**A–G**) the zebrafish *nlgns* across multiple developmental stages and in the adult brain and (**H**) tubulin alpha control. From left to right cDNA samples were derived from wild-type (AB/Tübingen) zebrafish at the 16 cell (1.5 hpf), 90% epiboly (9 hpf), 3-somite (11 hpf), 16 hpf, 24 hpf, 48 hpf, 72 hpf, 7 dpf stages and from adult brain.

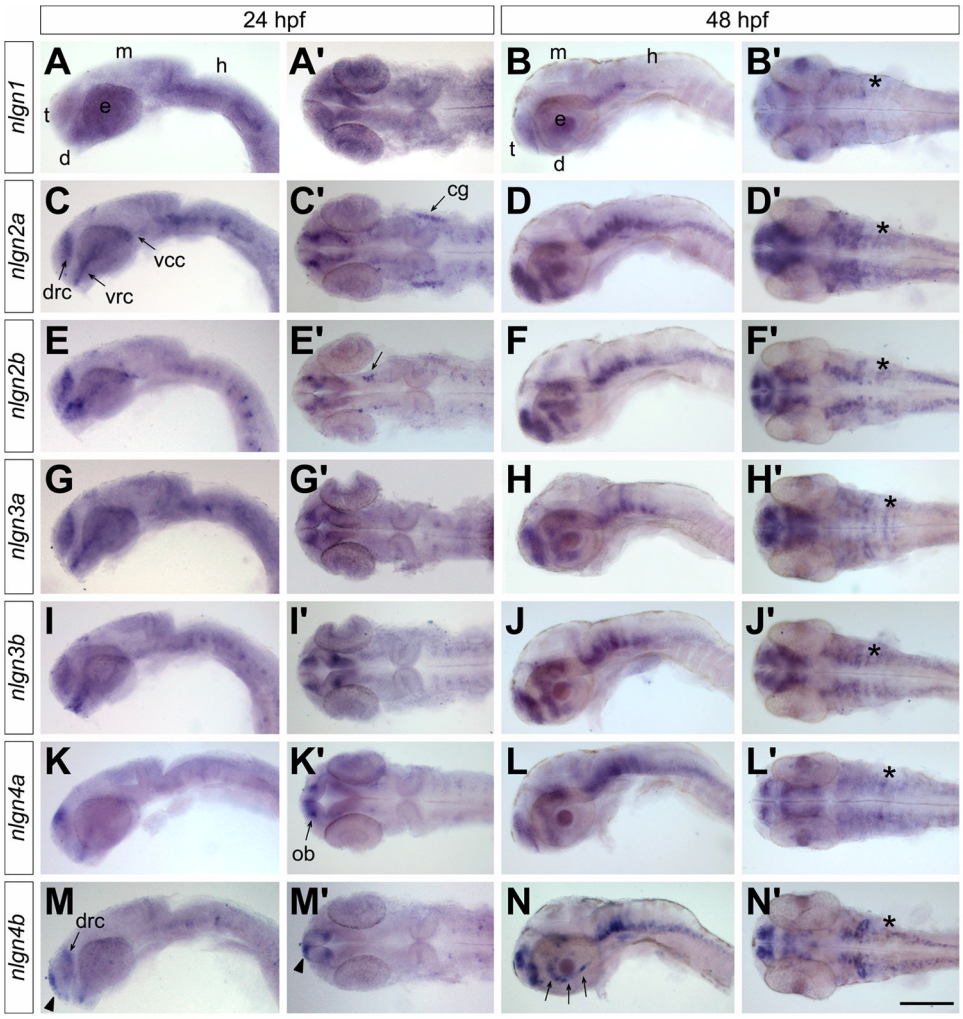

Figure 3. Expression of *nlgns* in the developing nervous system

The expression patterns for the *nlgn* genes were revealed by whole-mount ISH and are displayed as lateral views and dorsal views (A–N′) at 24 (A,C,E,G,I,K,M) and 48 hpf (B,D,F,H,J,L,N). (d, diencephalon; e, eye; h, hindbrain; m, midbrain; t, telencephalon; cg, cranial ganglia; ob, olfactory bulb; drc, dorsal-rostral cluster; vcc, ventral-caudal cluster; vrc, ventral-rostral cluster; asterisks denote the otic vesicles). Scale bar = 110 μm in 24 hpf embryos and 90 μm in 48 hpf embryos.

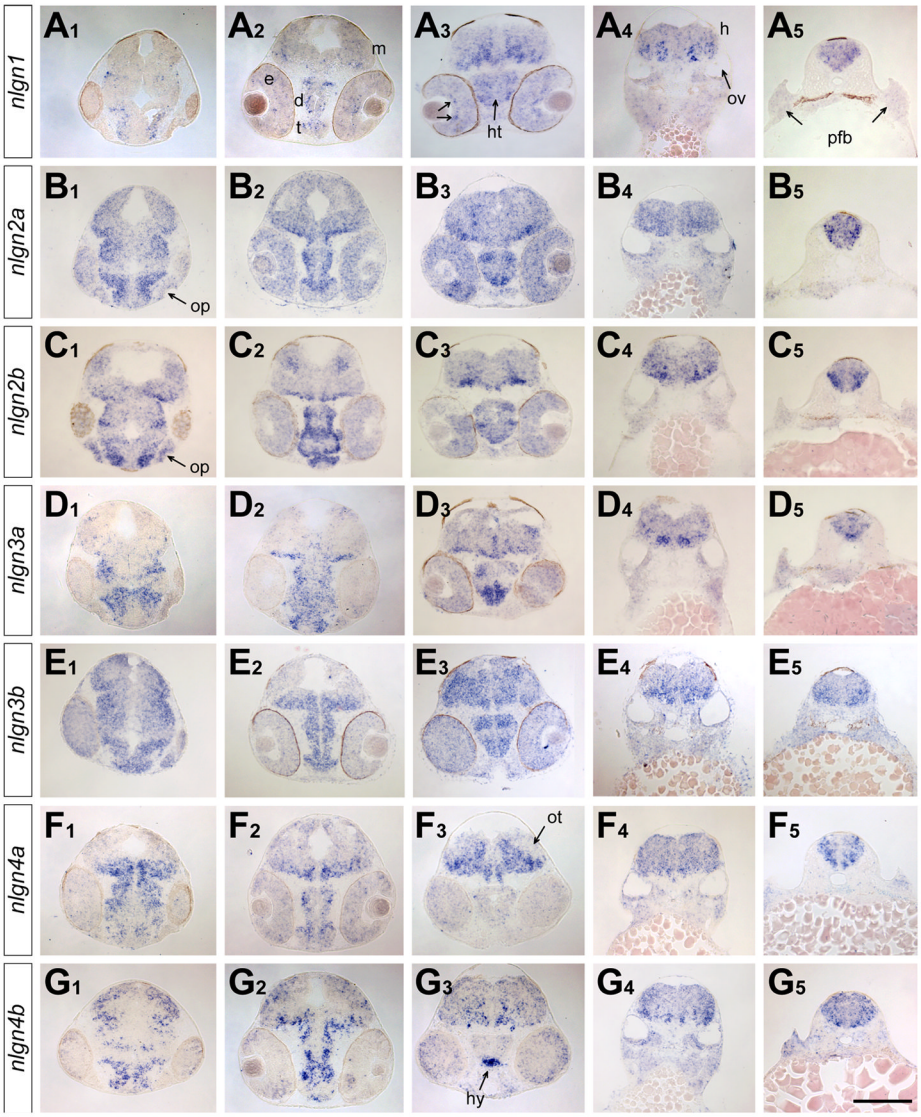

Figure 4. Expression of *nlgns* in the developing brain at 48 hpf

The expression patterns for the *nlgn* genes were examined by ISH in cross sections of embryos at 48 hpf. The levels of the sections (1–5) correspond to sections 2, 3, 5, 8 and 12, respectively, in the ZFIN atlas of zebrafish anatomy (zfin.org/zf_info/anatomy/48hrs/48hrs.html). (d, diencephalon; e, eye; m, midbrain; h, hindbrain; ht, hypothalamus; hy, hypophysis; op, olfactory placode; ot, optic tectum; ov, otic vesicle; pfb, pectoral fin bud). Scale bar = 105 μm in A_1 G_4 and 100 μm in A_5–G_5.

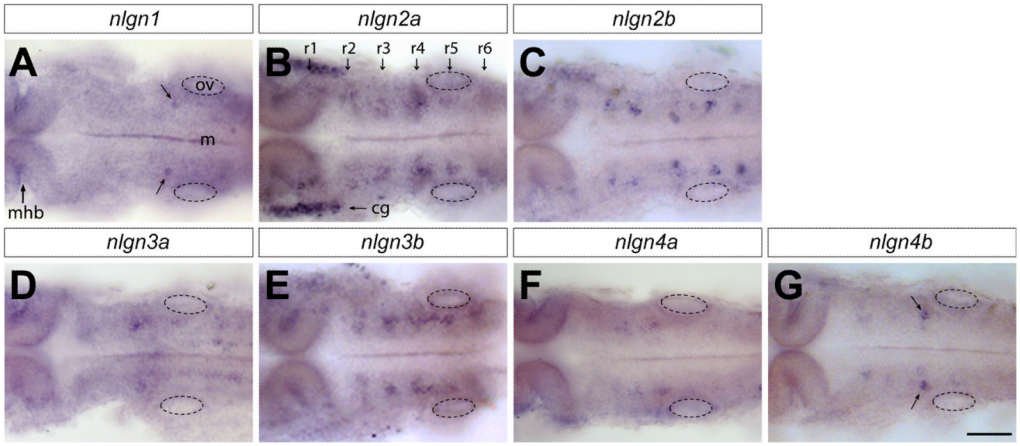

Figure 5. Expression of *nlgns* in the developing hindbrain

The expression patterns for the *nlgn* genes were examined by whole-mount ISH in the hindbrain of zebrafish embryos at 24 hpf. This revealed cell-specific expression patterns in large neurons of the reticulospinal tract. Individual cells located in r4, possibly Mauthner cells, are highlighted with arrows in A and G. Rhombomeres of the hindbrain are labeled with arrows in B. (m, midline; r, rhombomere; cg, cranial ganglia; mhb, midbrain-hindbrain boundary). Scale bar = 50 μm.

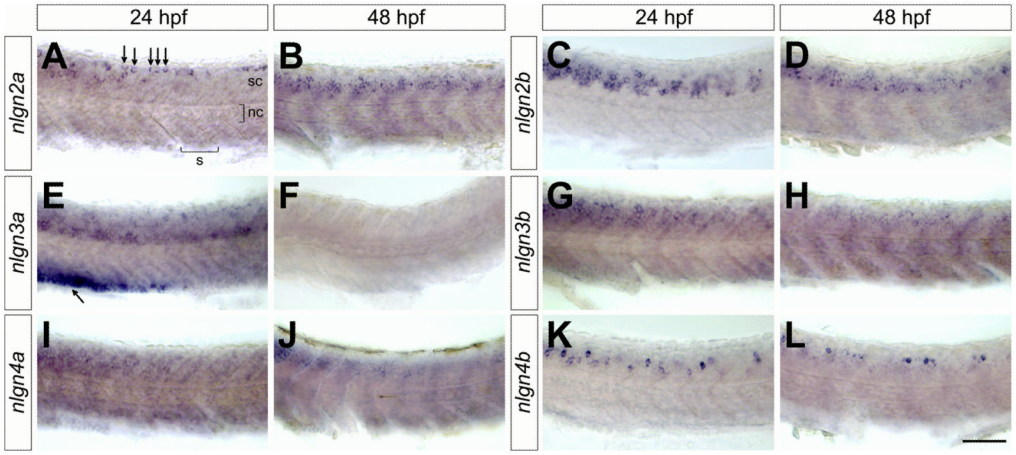

Figure 6. Expression of *nlgns* in the developing trunk

ISH performed on 24 and 48 hpf whole-mount zebrafish embryos reveals that the *nlgn* genes are expressed in the developing spinal cord in a dynamic and cell specific manner. Dorsal cells that are presumably Rohon-Beard neurons are highlighted with arrows in A. (s, somite; nc; notochord; sc, spinal cord). Scale bar = 35 μm.

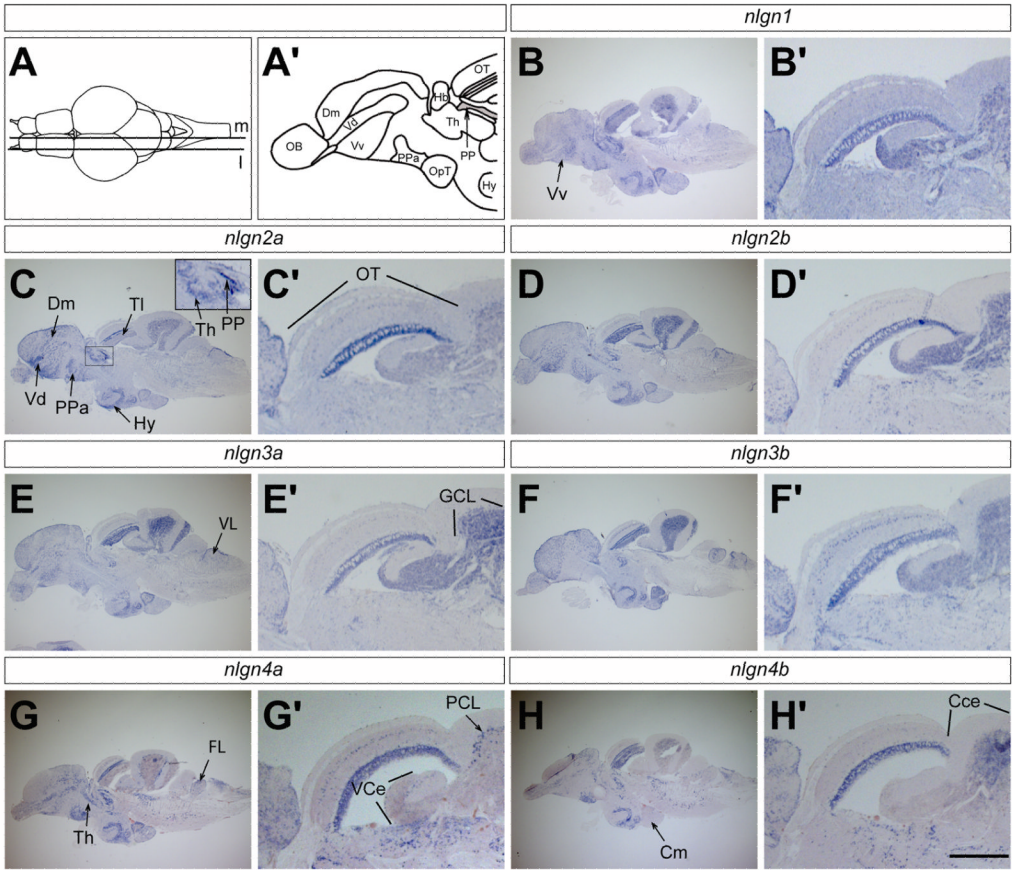

Figure 7. Expression of *nlgns* in adult brain

(A) ISH staining is displayed in saggital sections of adult brain at a medial (m) and a more lateral level (l) as indicated in the dorsal view of adult brain. Medial sections are displayed in B through H and more lateral sections are displayed in B′ through H′. (A′) Schematic of the forebrain displays subregions of the telencephalon. (B–H) show the entire brain and (B′-H′) show an enlargement of optic tectum, valvula and corpus cerebelli. The inset in C is an enlargement of the thalamus and periventricular pretectal nucleus. (CCe, corpus cerebelli, Cm, corpus mamillare; Dm, medial portion of the dorsal telencephalon; Hy, hypothalamus; OT, optic tectum; PP, periventricular pretectal nucleus; Th, thalamus; Tl, torus longitudinalis; Vd, dorsal portion of the ventral telencephalon; Vv, ventral portion of the ventral telencephalon; GCL, granule cell layer; PCL, Purkinje cell layer; VCe, valvula cerebelli). Scale bar = 750 μm in medial sections and 320 μm in lateral sections.

Table 1
Amino acid conservation of Nlgn proteins

Percentage amino acid identity for pairwise alignments of the seven zebrafish Nlgn proteins (*Danio rerio*) and the five human NLGN proteins (*Homo sapiens*). Shaded boxes highlight comparisons between presumed paralogs within the zebrafish genome (left) and comparisons between human and zebrafish orthologs (right).

Percent amino acid identify												
		Danio rerio							Homo sapiens			
		1	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**	**NLGN1**	**NGLN2**	**NLGN3**	**NLGN4X**	**NLGN4Y**
Danio rerio	nlgn1		62	66	65	59	67	67	81	62	68	70	69
	nlgn2a			81	63	57	62	62	64	71	63	63	62
	nlgn2b				63	59	65	66	66	72	64	67	65
	nlgn3a					72	69	69	67	62	83	71	71
	nlgn3b						62	62	60	58	70	64	63
	nlgn4a							81	68	63	69	76	75
	ngln4b								69	61	68	82	81