Dynamic expression of *neurexophilin1* during zebrafish embryonic development

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**A B S T R A C T**

Neurexophilin 1 (*Nxph1*) is a specific endoligand of α-neurexins that is essential for trans-synaptic activation. Here, we report its dynamic expression during development in zebrafish. Our study revealed an early onset of expression of *nxph1*. RT-PCR on a series of embryonic stages showed that it is maternally deposited, although only readily detectable by whole mount in situ hybridization by 22 hpf. During embryogenesis and larval stages, the zygotic transcript is expressed dynamically in various clusters of post-mitotic neurons and in glia in the central nervous system.

Neurexophilin is a highly conserved secreted cysteine-rich glycoprotein in vertebrates. It was discovered as a 29-kDa protein that is co-purified with neurexin 1α on immobilized α-latrotoxin (Petrenko et al., 1993). The binding of the protein to neurexin is very tight, hence the name neurexophilin (Petrenko et al., 1996).

The protein neurexophilin comprises of four domains: a signal peptide, a non-conserved N-terminal region, a highly conserved central N-glycosylated domain, and an equally conserved C-terminal cysteine-rich domain. Expression and protein purification experiments revealed that neurexophilins are secreted glycoproteins that are proteolytically processed from the primary translation product, first by signal sequence cleavage and then by cleavage at the boundary between the N-terminal non-conserved and the central conserved domain. The proteolytic processing observed in neurexophilin, reminiscent of maturation processes in neuropeptides, together with their tight binding to neurexins indicates that they are endogenous ligands for α-neurexins (Missler and Sudhof, 1998). Neurexins (NRXNs) are neuronal cell surface proteins that are required for trans-synaptic activation of synaptic transmission but not synapse formation (Missler et al., 2003). They are polymorphic synaptic receptors encoded by at least three genes (NRXN1, NRXN2 and NRXN3) in mammals, each of which has two independent promoters directing transcription of long α-neurexins and short β-neurexins.

Molecular cloning revealed that there are at least four neurexophilins genes (*nxph1*, *nxph2*, *nxph3* and *nxph4*) in mammals (Missler and Sudhof, 1998; Petrenko et al., 1996). Comparisons of neurexophilins show that they are closely related to each other in the C-terminus regions but diverge considerably in their N-terminal regions (Missler and Sudhof, 1998). Rodents have all four genes but only neurexophilins 1, 3, and 4 are expressed at detectable levels. *Nxph2* is however expressed in bovine, strongly suggesting evolutionary changes in *nxph* gene expression. *Nxph4* has a different linker region and doesn’t bind to α-neurexins, unlike other neurexophilins (Missler et al., 1998).

Although neurexophilin gene family has been studied in mammals, there is lack of data for these genes in lower vertebrates. Very little is known about the expression of neurexophilins in vivo. Zebrafish (*Danio rerio*) has a quite simple and well-characterized nervous system with broad developmental and physiological similarities to humans. Recent studies in zebrafish showed that *nxph1* is expressed in discrete clusters in the habenula, pallium, and ventral thalamus at 72 hpf and is involved in Notch signaling (Hortopan and Baraban, 2011). We provide here a comprehensive description of *nxph1* expression during zebrafish development.
Fig. 1. Phylogenetic tree and amino acid alignment of neurexophilins. (A) Phylogenetic tree of the neurexophilin homologues of human (NP_067712.2, NP_009155.1, NP_009156.2, NP_009157.1), mouse (NP_032777.3, NP_032778.1, NP_570928.1, NP_899120.2), rat (NP_037126.1, XP_001055001.1, XP_002726131.1, NP_067711.1, frog (NP_001120374.1, XP_002933980.1, NP_001002733.1, XP_691473.2) and zebrafish (NP_001096477.1, XP_001337769.1, XP_002667270.2) was generated based on the full-length amino acid sequence available at NCBI. (B) Multiple sequence alignment of nxph1 in human, rat, mouse, frog and zebrafish. The nxph1 aminoacid sequences of Danio rerio, Homo sapiens, Mus musculus, Rattus norvegicus and Xenopus tropicalis were aligned based on Clustal W(2.1) algorithm. Conserved amino acids are indicated by an asterisk (identical in all cases), colon (conserved substitutions observed) or dot (semi-conserved substitutions observed).
1. Results and discussion

1.1. Phylogenetic study of vertebrate neurexophilins

A phylogenetic tree was constructed (Fig. 1) based on the multiple sequence alignment of all neurexophilin orthologues of *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, *Xenopus tropicalis* and *Danio rerio* generated using COBALT alignment tool (Papadopoulos and Agarwala, 2007). The following orthologues were selected for analysis: human (NP_689958.1, NP_009157.1, NP_009156.2, NP_009155.1), mouse (NP_032777.3, NP_032778.1, NP_570928.1, NP_899120.2) rat (NP_037126.1, XP_001055001.1, XP_002726131.1, NP_067711.1, NP_067712.2), frog (NP_001120374.1, XP_002933980.1, NP_001096477.1) and zebrafish (NP_001002733.1, NP_698222.2, XP_001337691.1, XP_691473.2, XP_002667270.2). The phylogenetic analysis confirmed that these genes share a common ancestor.

The zebrafish genome contains one confirmed neurexophilin, *nxph1* (ENSDARG00000070694) and four predicted paralogues, *nxph2a* (ENSDARG00000063224), *nxph2b* (ENSDARG00000079188), *nxph3* (ENSDARG00000070694) and *nxph4* (ENSDARG00000086716). Zebrafish *nxph1* (NM_001002733.2; GI: 402692395) is located on chromosome 19 in the zebrafish genome ENSEMBL database. Comparison of z-nxph1 amino acid sequence with those of other vertebrate species of interest using Clustal W1.8 multiple sequence alignment (Fig. 1B) revealed a high conservation amongst vertebrates with a high amino acid homology of z-nxph1 with *H. sapiens* (87%), *M. musculus* (87%), *R. norvegicus* (86%) and *X. tropicalis* (80%) nxph1 proteins.

1.2. *nxph1* transcript is maternally inherited in zebrafish

The presence of zebrafish *nxph1* transcripts during embryonic development was shown by RT-PCR analysis of total RNA extracts at different embryonic stages: 32-cell (1.75 hpf), 64-cell (2 hpf), 128-cell (2.25 h), 256-cell (2.5 hpf), 1000-cell (3 hpf), shield (6 hpf), 5-somite (10.5 hpf), 15-somite (14 hpf), prim-5 (24 dpf), and prim-25 (36 dpf). Zebrafish *nxph1* transcripts could be detected from as early as the 32-cell stage, indicating the presence of maternally deposited transcripts in the egg (Fig. 2A). Tissue-specific zygotic expression was detectable from 22 somite stage onwards in the pineal gland and soon after (prim-5) in post-mitotic neurons (data not shown and Fig. 2B).

1.3. Neuronal expression of *nxph1* in zebrafish

Embryos at Prim5 express *nxph1* in discrete patch of cells in the ventral telencephalon (subpallium), pre-optic area, pineal gland/
epiphysis, tegmentum, hindbrain rhombomeres and all along the spinal cord (Fig. 2B). The expression gets stronger and dynamic during the later stages of development except in the spinal cord where it gets down regulated from 48 hpf onwards. The pallial expression of nxph1 initially observed at 32 hpf by fast red staining gets stronger by 36 hpf (Fig. 3M, O; Fig. 5G, I), but not detected at the same stage by NBT/BCIP coloration (Fig. 3). In the 5 dpf larvae, the nxph1 expression around the anterior and posterior commissure (arrows in Fig. 3C) and in the hindbrain radial glia (asterisks in Fig. 3C, F) suggests a late expression in glia. The location of nxph1 in basal clusters inside the embryonic central nervous system (CNS) strongly suggested a neuronal expression, confirmed by co-localization with Hu-C (Fig. 4A–G).

1.4. nxph1 is mostly expressed in brain and spinal cord interneurons

Co-staining with Anti-HuC revealed that nxph1 is expressed ventrally to the Rohon-Beard sensory neurons suggesting their expression in the interneurons in the spinal cord (Fig. 4C, G). At prim15 stage, all neurons labeled by the Hu-C express nxph1, but all regions of nxph1 expression overlap with neuronal area except in the pineal gland (Fig. 4A–G). Transgenic lines, Tg(isl1:GFP) and Tg(mnx1:GFP)ml2, expressing GFP in a subset or all motor neurons respectively, were used to test whether nxph1 is expressed in these neuronal populations. With the exception of a sub-domain of facial motor neuron (nVII), nxph1 populations do not overlap with GFP expressing motor neurons at prim5 (Fig. 5). The nxph1 labeling

**Fig. 3.** Dynamic expression of nxph1 during zebrafish development. (I) Lateral (A–C) and dorsal (D–F) view of 24 hpf (A, D), 36 hpf (B, E) and 5 dpf (C, F) embryos showing nxph1 expression (blue). Black arrowhead indicates anterior and post-optic commissure and asterisk indicates hindbrain radial glia in C, F. Abbreviations: t, telencephalon; pOA, post-optic area; e, epiphysis; tg, tegmentum. (II) Transverse section showing nxph1 expression in telencephalon (t) and pre-optic area (pOA) (G, H) at 24 hpf and 36 hpf. Transverse section through the diencephalon (I, white arrowhead indicates the pineal gland). Dorsal view of hindbrain (J), lateral view (K) and transverse section (L) of the spinal cord at 24 hpf. (III) Lateral (M) and dorsal (N) view of brain and transverse section of spinal cord (O) of nxph1 (red) at 36 hpf. Scale bar = 40 μm.
in the spinal cord did not co-localize, but appeared dorsal to the motor neurons marked by *mnx1* at prim5, confirming its expression in spinal interneurons.

1.5. Conclusions and discussion

Expression patterns of different neurexophilin mRNAs showed tissue-specific variations between species (Missler and Sudhof, 1998). In humans, *nxph1* expression is detected in spleen but not in brain unlike *nxph2*, *nxph3* and *nxph4* (Missler and Sudhof, 1998). In zebrafish, *nxph1* is widely distributed in the central nervous system from late somite stage onwards, getting highly localized in distinct regions of the brain by 72 hpf and in more domains by 5 dpf (data not shown). Though *nxph1* is maternally deposited in zebrafish, it can be detected by *in situ* hybridization by only 22 hpf (data not shown). In contrast to neurexins that are expressed in all neurons, we found that *nxph1* is expressed at high levels only in scattered subpopulations of neurons that probably represent inhibitory interneurons. This finding support the localization in interneurons in adult rat brain (Petrenko et al., 1996), making the mouse a possible exception and indicating some level of conservation in the specificity of *nxph1* expression in interneurons in vertebrates.

Previous studies demonstrated that in rats and mice, only NxpH1 and NxpH3 interact biochemically with α-neurexins (Missler et al., 1998). *nxph3* is highly localized in mouse cortical and cerebellar regions and is functionally important for sensorimotor gating and motor coordination, but, the function of *nxph1* in the CNS can’t be assessed in mouse where the gene is not expressed in the CNS (Beglopoulos et al., 2005). Due to the expression of most neurexophilins in the human brain, it is important to know the function of *nxph* gene family in a developing brain.

The function of NXPH is poorly understood; however, previous data suggest that they inhibit NRXN-mediated signalling (Beglopoulos et al., 2005; Zhang et al., 2005). Recent studies in animal models demonstrate the role of interneuron dysfunction in psychiatric

![Fig. 4.](image) nxph1 is expressed in neurons, mostly interneurons. (I) Anti-HuC staining for post-mitotic neurons in 30 hpf embryos stained for *nxph1* (A–G). Lateral view of zebrafish brain (A), frontal view of forebrain (D), transverse view of midbrain (E), dorsal (B) and transverse (F) view of hindbrain, lateral (C) and transverse (G) view of spinal cord at 36 hpf. Abbreviations: nMLF, nuclei of the media longitudinal fascicle; ov, otic vescicle; e, epiphysis. Scale bar: A–C (40 μm) and D–G (20 μm). (II) Lateral (A–F), dorsal view (G–I) and transverse section of spinal cord (J–L) of *Tg(mnx1:GFP)* zebrafish at 24 hpf, anterior to the left. Scale bar: A–C, J–L (40 μm) and D–I (20 μm). *nxph1* is expressed ventral (blue) to the Rohon-Beard sensory neurons (+) stained by HuC (I) and dorsal (red) to the motor neurons marked by *Tg(mnx1:GFP)* (II), thus indicating its expression in the interneurons.
disorders such as schizophrenia, autism spectrum disorder (ASD) and intellectual disabilities (ID) (Belforte et al., 2010; Chao et al., 2010; Fazzari et al., 2010). The haploinsufficiency of NRXN1 causes the ASD/ID and schizophrenia spectrum phenotypes in mice and involves synaptic dysfunction (Missler et al., 2003). It has become increasingly evident that the disruption of inhibitory circuits in these psychiatric disorders is linked to specific defects in the development and function of interneurons – the cells that are responsible for establishing inhibitory circuits in the brain. In zebrafish, nxph1 was found to be down regulated in the transcriptome analysis of mind bomb (mib) E3 ubiquitin ligase mutant that exhibits electrographic and behavioral seizure activity (Hortopan and Baraban, 2011). In humans, loss-of-function mutations in E3 ubiquitin ligase cause a neurogenetic disorder, Angelman syndrome.
(Kishino et al., 1997) which includes motor dysfunction leading to an ataxic gait, frequent severe seizures, profound learning disability, absent speech, and characteristic happy demeanor (Lossie et al., 2001). nxph1 being highly restricted to distinct neuronal clusters (mostly interneurons) in the zebrafish CNS, interfering with its function would give valuable insights about the role of interneurons and the function of NXPH genes in such neurogenic or psychiatric disorders. Thus, zebrafish is a good model to study the in vivo function of neuroxophins and the current study provides the basis for future functional loss of function studies using TALEN-induced knockout technology to assess the role of neuroxophins in synapse development and dysfunction in vertebrates and explore its possible role in glia.

2. Experimental procedures

2.1. Zebrafish embryos maintenance

Zebrafish (Danio rerio) was maintained at 28 °C on a 14 h light/10 h dark cycle as described in the book (Brand et al., 2002). Embryos collected were cultured in fish water containing 0.003% 1-phenyl-2-thiourea to prevent pigmentation and 0.01% methylene blue to prevent fungal growth. Embryos were staged beginning from 4 hpf according to Kimmel (Kimmel et al., 1995) and fixed for further studies.

2.2. Cloning of zebrafish nxph1 gene

The IMAGE clone (IMAGE: 7155997) of nxph1 complete cDNA sequence was obtained (genservice) in vector pDNR-LIB, which did not have any T7/sp6/T3 priming site that is necessary for in situ hybridisation. In order to reclone the insert into an appropriate vector, the insert was amplified by PCR using the oligonucleotides synthesized based on the information obtained on nxph1 gene sequence from the zebrafish genome database ENSEMBL available on the Sanger website (http://www.sanger.ac.uk/Projects/D_rerio/); nxph1 (GenBank Accession number: BC076424). The PCR product was recloned to PCR-4-TOPO vector using TOPO TA Cloning Kit for sequencing (Invitrogen) according to supplier's protocol.

2.3. Whole mount in situ hybridisation and immunohistochemistry

Whole mount in situ hybridisation was performed on wild type embryos as previously described by (Macdonald et al., 1994) using Digoxigenin labeled antisense RNA probes synthesized from linearized plasmid nxph1 (Not1) and NBT/BCIP coloration (Roche). For neuronal cell type experiment, transgenic GFP embryos were labeled with digoxigenin, stained with Fast Red (Roche) and further processed for immunostaining. Antibody staining was performed (Shanmugalingam et al., 2000) using anti-GFP antibody (Torrey Pines Biolabs, USA) and secondary antibody Alexa 488 (Molecular Probes, Invitrogen, UK).

2.4. RT-PCR

Total RNA was extracted from WT embryos at various stages using TRIzol® RNA isolation reagent (Invitrogen) and cDNA was synthesized (First strand CDNA synthesis kit, GE Healthcare) and amplified by PCR using the nxph1 specific primers, nxph1F (5′-TCTCATACGCGGCGTCTAC-3′) and nxph1R (5′-ACGCGATG TATGAGACTACG-3′) as the internal control for RNA quality and quantity, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript expression was amplified using the primer pair GAPDH F (5′-TCAATTGGATTGGCGCTATT-3′) and GAPDH R (5′-GAGCTGAGCTTCTTCAATG-3′) in the same reaction. PCR amplification of cDNA included 28 cycles with an initial denaturation at 94 °C (5 min) and final extension at 72 °C (10 min). Each reaction cycle consisted of incubations at 94 °C (60 s), 58 °C (60 s), and 72 °C (60 s) with Taq DNA Polymerase (GoTaq, Promega). The products were resolved on a 1.5% agarose gel with 1 μg/ml of ethidium bromide and analyzed in a gel-doc system.

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