The Synaptic Proteins β-Neurexin and Neuroligin Synergize With Extracellular Matrix-Binding Vascular Endothelial Growth Factor A During Zebrafish Vascular Development

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Objective—The goal of this study was to determine the in vivo functions of the synaptic proteins neurexins and neuroligins in embryonic vascular system development using zebrafish as animal model.

Methods and Results—In the present study, we show that the knockdown of the α-form of neurexin 1a induces balance defects and reduced locomotory activity, whereas β-neurexin 1a and neuroligin 1 morphants present defects in sprouting angiogenesis and vascular remodeling, in particular in the caudal plexus and subintestinal vessels. Coinjection of low doses of morpholinos for β-neurexin 1a and neuroligin 1 together or in combination with morpholinos targeting the heparin-binding isoforms of vascular endothelial growth factor A (encoded by the VEGFAb gene) recapitulates the observed abnormalities, suggesting synergistic activity of these molecules. Similar coinjection experiments with morpholinos, targeting the enzyme heparan sulfate 6-O-sulfotransferase 2, confirm the presence of a functional correlation between extracellular matrix maturation and β-neurexin 1a or neuroligin 1.

Conclusion—Our data represent the first in vivo evidence of the role of neurexin and neuroligin in embryonic blood vessel formation and provide insights into their mechanism of action. (Arterioscler Thromb Vasc Biol. 2012;32:1563–1572)

Key Words: neurexins ■ neuroligins ■ vascular endothelial growth factor A ■ extracellular matrix ■ vascular system
The molecular cues shared between the nervous and vascular systems are mainly involved in axon/vessel guidance, boundary formation, and cell fate specification.15 Nevertheless, we recently demonstrated that NRXNs and NLGNs are expressed in the vascular system and contribute to its function.16 In parallel, we identified the members of zebrafish (Danio rerio) NRXN and NLGN gene families.17,18 To deepen the functional characterization of these 2 gene families in vascular development, we took advantage of the zebrafish model system to perform an in vivo study. Given the lack of in vivo functional data for β-NRXNs and starting from our previous findings,16 we focused our analyses on the β-nrxn1a and nlgn1 genes. Expression analyses indicate that both genes are expressed in endothelial and mesenchymal cells of the trunk/tail region of zebrafish embryos. The knockdown with morpholinos (MOs) for β-nrxn1a or nlgn1 but not for α-nrxn1a specifically reduces sprouting angiogenesis of the caudal plexus (CP) and, at later stages of development, it impairs the formation/stability of blood vessels also in other vascular districts. Furthermore, we show a genetic interaction of β-nrxn1a and nlgn1 with both ECM-dependent VEGFA isoforms and the matrix modifying heparan sulfate 6-O-sulphotransferase 2 (HS6ST2). Our data represent the first in vivo report of the function of these genes in the vascular system, giving new insights into their possible mechanism of action.

Methods
Zebrafish Lines and Maintenance
Zebrafish lines and embryos were raised and maintained according to established conditions.18,19 The following lines were used: Tg(gata1:dsRed)25 and Tg(flkl:EGFP).22 The double transgenic line Tg(gata1:dsRed);Tg(kdr:EGFP)gs843 was a generous gift from Dr Massimo Santoro (Laboratory of Cardiovascular Biology, Molecular Biotechnology Center, Torino, Italy). Wild-type fish used in this study include AB and TL lines.

Morpholinos
Antisense morpholinos were purchased from Gene Tools (LLC, Philomath, OR). Different doses of MOs were diluted in Danieau buffer or water and then injected into 1- to 2-cell stage embryos. The phenotypic effects of each MO was tested by dose escalation. See Table I in the online-only Data Supplement for a complete list of MOs and doses.

Reverse Transcriptase–Polymerase Chain Reaction
Total RNA samples preparation, reverse transcription, and PCR reactions were performed, as previously described.17 Products were then separated on agarose gels and visualized by ethidium bromide staining.

Sorting of Endothelial EGFP+ Cells and Quantitative PCR
Endothelial cells were isolated from Tg(kdr:EGFP)gs843 embryos at 30 to 35 hours postfertilization (hpf) by fluorescence-activated cell sorting, as described previously.22 Total RNA from EGFP+ and EGFP− populations was isolated in Trizol (Invitrogen) according to manufacturer’s instructions. cDNA strands were synthesized using SuperScript III following manufacturer’s instructions (Invitrogen). The products were amplified in real-time PCR reactions with StepOne Plus Real-Time PCR System (Applied Biosystems) using PCR master mix PowerSYBR Green (Applied Biosystems). The results of a representative experiment are shown in Figure IF in the online-only Data Supplement. See Table II in the online-only Data Supplement for a list of primers used in quantitative PCR.

In Situ Hybridization
Whole mount in situ hybridizations were performed, as elsewhere described.23 Specific probes for nlgn1, edh5, vsigl, flkl, tie2, and flt4 were synthesized and used, as previously described.16,22 All the probes were purified using Spin Post-Reaction Clean-Up Columns (Sigma-Aldrich, St Louis, MO) before use. We used 2 different probes for the nrxn1a gene: a probe specific for both α- and β-forms and a second probe specific only for β-forms. For αβ nrxn1a probe, primers were designed to cover the 3′-end of the coding sequence and part of the 3′ untranslated region of the nrxn1a gene; for β NRXN1a probe, primers cover the 5′untranslated region and the β-specific exon.

Imaging
Images were taken with a Leica MZFLIII epifluorescence stereomicroscope equipped with a DFC 480 R2 digital camera or with a Leica DM6000 B microscope equipped with a DFC 360 FX digital camera and LAS Leica imaging software (Leica, Wetlazar, Germany). For confocal microscopy, embryos were mounted in 0.5% to 1% low melting agarose and the medium was supplemented with 0.1% Tricaine. Imaging was performed on a Leica TCS SP2 confocal microscope (Leica Microsystems) using a 10x or 20x objective. In timelapse confocal analysis, time points were recorded every 17 to 20 minutes. Images were processed using Adobe Photoshop software (Adobe, San Jose, CA) whereas movies were processed using Sorenson Squeeze 5 (Sorenson Media Inc) or edited using VEGAS 9.0 (Sony Creative Software Inc).

Alkaline Phosphatase Staining
Alkaline phosphatase staining was performed, as previously described.23

Semithin Plastic Sections
Embryos for histological analysis were fixed and embedded, as previously described.21

Statistical Analysis
Unless otherwise indicated, the percentage of affected embryos observed in wild-type or ST-CTRL MO-injected embryos have been subtracted from the percentages observed in morphants. Results are expressed as the mean±standard deviation or standard error of the mean. Differences were determined by repeated-measures ANOVA with Dunnett post hoc analysis using GraphPad PRISM version 5.0 (GraphPad, San Diego, CA). Embryos coinjected with the α-NRXN1a MO have been used as reference groups. A P<0.05 indicates a statistically significant effect.

Results
β-nrxn1a and nlgn1 Are Expressed in Endothelial Cells and Modulate Vascular Development
The spatiotemporal analysis of αβ-nrxn1a by whole mount in situ hybridizations showed that around 33 hpf they are expressed in different regions of the nervous system (Figure I in the online-only Data Supplement). Moreover, we also observed a faint signal in the ventral region of the embryos below the notochord (black arrows in Figure IA in the online-only Data Supplement). Further analyses, using ISH probes specific for β-nrxn1a and nlgn1 followed by histological sectioning of hybridized embryos, indicated that CP and the surrounding mesenchymal cells express both transcripts (Figure IB–IE in the online-only Data Supplement). To further sustain the expression of β-nrxn1a and nlgn1 genes in endothelial cells, we performed quantitative reverse transcriptase-polymerase chain reaction on EGFP+ cells sorted from Tg(kdr:EGFP)gs843 embryos at 30 to 35 hpf, confirming that only β-forms of
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To determine the role of **nrxn1a** and **nlgn1** in zebrafish embryo development, we targeted both the major forms (α and β) of **nrxn1a** and the **nlgn1** gene using specific MOs (see Table I in the online-only Data Supplement). The injection of a specific translation-blocking MO for α-**nrxn1a** (α-NRXN1a MO) produced no evident vascular defects (Figures II, IV, VB, VI, and IX in the online-only Data Supplement). However, at 3 days postfertilization (dpf) α-NRXN1a MO-injected embryos (morphants) displayed a severe impairment of their locomotory activity without visible defects in the formation or maintenance of trunk muscles (data not shown). In particular, they usually failed to maintain body balance and presented a very low performance in touch response tests (see Videos I and II in the online-only Data Supplement). Different reports indicate that MO injection can induce off-target effects through p53 activation,24,25 in particular neural toxicity. However, the coinjection of α-NRXN1a MO with p53 MO did not rescue the phenotype in touch-response assays, indicating that locomotory defects are not attributable to a generic p53 activation (Figure II in the online-only Data Supplement). The molecular bases of the locomotory defects caused by α-NRXN1a MO were not analyzed further. Moreover, given the lack of significant vascular phenotypes, α-NRXN1a MO was used as internal control in the following experiments of β-**nrxn1a** and **nlgn1** knockdown.

To knockdown β-**nrxn1a** gene, we used 2 different MOs: a translation-blocking (β-NRXN1a MO1) and a splice-blocking MO (β-NRXN1a MO2). The effectiveness of the splice-blocking MO was confirmed by reverse transcriptase-polymerase chain reaction (Figure 1 and Figure IK in the online-only Data Supplement). The injection of either 1 of these 2 different MOs targeting β-**nrxn1a** transcripts into Tg(**flk1**:EGFP) embryos showed that up to 24 hpf the vascular system develops normally (data not shown), but by 26 hpf a specific CP alteration is evident (Figure 2A). In zebrafish, caudal vein morphogenesis requires 2 steps: first, around 26 hpf, the caudal part of the axial vein undergoes sprouting angiogenesis and becomes a plexus of vessels (Figures 2A and 3) that continues to grow until 48 hpf; then its maturation through progressive remodeling will produce a well-structured and hierarchic vascular network (Figure 2B).26,27 The confocal analysis performed on Tg(**flk1**:EGFP)-injected embryos between 26 and 32 hpf showed that CP reduction in β-**nrxn1a** morphants is attributable to a limited ventral sprouting and branching of the caudal vein (Figure 2A; Figure II and Videos III and IV in the online-only Data Supplement). Notably, other vascular structures such as the intersegmental vessels (ISVs) form normally. At 32 hpf ISVs still appear largely unaffected, whereas the CP remains drastically reduced therefore excluding a possible developmental delay (see white brackets in Figure 2A and Figure II in the online-only Data Supplement). The injection of a translation-blocking MO targeting **nlgn1** transcripts produced similar vascular phenotypes (data not shown). Recently, Mouillesseaux and Chen28 showed that LA1908 mutants present CP defects attributable to the lack of utp15 activity. This deficiency induces a p53-mediated antiangiogenic effect on CP development. Moreover, they showed that blocking p53 activation in LA1908 mutants by p53 MO injection rescued the vascular defects in the CP region. To further prove the specificity of our observed vascular phenotypes, we coinjected p53 MO with high doses of β-NRXN1a or NLGN1 MOs. The coinjection experiments indicate that p53 activation is not involved in vascular phenotypes...

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**Figure 1.** β-NRXN1a MO2 dose dependently impairs the normal splicing of **nrxn1a** transcripts. The schematic depiction describes the positions of splicing morpholino (MO) (β-NRXN1a MO2) and primers on β-**nrxn1a** transcripts. Alternative spliced exons are marked with an asterisk. e1 Beta spec. indicates the first exon of β-**nrxn1a** gene. The injection of a splicing-blocking MO targeting the donor splice site of intron 1 (F1) of β-**nrxn1a** transcripts specifically blocks the correct splicing (black arrow in lane 2 of uninjected embryos) of the targeted pre-mRNAs, inducing the inclusion of I1 in the majority of the transcripts. Intron retention is expected to induce multiple premature STOP codons which activate nonsense-mediated mRNA decay (NMD) as suggested by the lack of transcripts with primer couple number 1 in all the samples with the exception of positive control on genomic DNA. Only in a small amount of transcripts, the injected MO induces the activation of different cryptic splice sites in exon 1 or intron 1 (faint bands indicated by red and green asterisks, respectively) producing aberrant mRNAs. Notably, with the increase of the dose the pattern of bands changes from lower to higher forms. MW indicates molecular weight size marker; NRXN, neurexins.
observed in both β-nrxn1a and nlgn1 morphants (Figure III in the online-only Data Supplement). To gain insight into the molecular events induced by the knockdown of β-nrxn1a or nlgn1, we analyzed the expression of several endothelial markers by whole mount in situ hybridizations (Figure IV in the online-only Data Supplement). At 32 hpf, in both β-nrxn1a and nlgn1 morphants the hybridization signal of all tested markers (cdh5, flk1, flt4, and tie2) appeared thinner exclusively in the CP region, further supporting the idea that β-nrxn1a and nlgn1 are involved in CP formation.

The confocal analyses of Tg(gata1:dsRed)sd2; (kdrl:EGFP)s843 embryos at around 54 hpf (Figure 2B) show that CP of control embryos consists of a major venous vessel (CV in Figure 2B) located in its ventral-most part. However, a plexiform portion is still present in the dorsal-most part, beneath the dorsal aorta (DA in Figure 2B). Notably, from this stage on circulation is mostly restricted to the single major venous vessel and only few blood cells flow into the plexiform portion (Figure 2B). At the same stage, β-nrxn1a and nlgn1 morphants presented an anomalous blood circulation in their tail region (Figure 2B and Figure IXA in the online-only Data Supplement). Indeed, blood circulation is mainly restricted to the dorsal-most part of the plexiform region and some tracts of the ventral-most vessels present a partial or complete lack of blood circulation (arrows in Figure 2B), suggesting an altered organization of the CP. To verify this hypothesis, we analyzed vessels morphology of β-NRXN1a MOs and NLGN1 MO-injected embryos by histological sectioning (Figure VA in the online-only Data Supplement). Semithin plastic sections showed that the observed blood flow variation in the CP region of β-nrxn1a and nlgn1 morphants is attributable to a specific dilation of the dorsal-most vessels at the expense of the ventral ones (black arrows and arrowheads, respectively in Figure VA in the online-only Data Supplement). Altogether, the confocal and histological analyses suggest that this altered organization of CP is attributable to vascular defects during its formation and subsequent remodeling.

Because of progressive remodeling of the CP, at later stages of development the vascular defects in CP appear less evident, but additional vascular defects arise. Around 3 dpf, some parts of the dorsal longitudinal anastomotic vessels and some ISVs of β-nrxn1a morphants lose blood circulation and start to regress, detaching from adjacent endothelial cells (see insets in Figure VI and Videos V and VI in the online-only Data

Figure 2. Knockdown of β-nrxn1a and nlgn1 induces specific vascular defects in caudal plexus (CP). A, Confocal analysis of vascular development in trunk/tail portion of Tg(flk1:EGFP) embryos between 26 and 32 hours postfertilization (hpf). β-nrxn1a morphants present a specific reduction of CP (white arrowheads). Dashed rectangles indicate the portion of images in corresponding insets. In all insets, red and white brackets indicate dorsal aorta (DA) and CP of St-Control (St-CTRL) injected embryos, respectively (see also Figure UJ in the online-only Data Supplement). Magnification ×20. B, Confocal side-views of the trunk and the tail of ≈54 hpf Tg(gata1:dsRed) embryos. β-nrxn1a morphants present abnormal vessels inducing a blood flow variation in CP region (white arrows in insets). Magnification ×10. CV indicates caudal vein; NRXN, neurexins; MO, morpholinos.

Figure 3. Caudal plexus originates through vascular sprouting angiogenesis. Confocal side-views of the tail region of transgenic zebrafish embryos. The ventral sprouting of endothelial cells begins around 26 hours postfertilization (hpf) (arrowheads). Afterward, some endothelial cell sprouts approach each other and form anastomosis. The formation of lumen within endothelial cells produces a primitive network of vessels that allows blood circulation (27–29 hpf). Magnification ×20.
Supplement). Finally, we observed that β-nrxn1a and nlgn1 morphants showed a range of defects in the subintestinal veins (SIVs) as revealed by endogenous alkaline phosphatase staining at 3 dpf (Figure VB in the online-only Data Supplement). The defective SIVs presented a severe reduction with sporadic elongated spikes extending toward the yolk. Notably, SIVs of α-nrxn1a morphants appear substantially unaffected (Figure VB in the online-only Data Supplement). Overall, these data indicate that nlgn1 and only the β-forms of nrxn1a are involved in embryonic vascular development and in particular in CP and SIVs formation.

Synergistic Interaction of β-nrxn1a and nlgn1 in CP Formation and Their Correlation With VEGFA
Although zebrafish represents 1 of the most recent animal models used in vascular biology, it provides a very useful tool to study the vascular system. Different studies have highlighted a strong similarity between zebrafish and other animal models in basic molecular mechanisms of vascular development and in particular in sprouting angiogenesis. Nevertheless, the majority of these studies focused on ISVs formation and few of them drew attention to the CP defects associated with a reduced expression of α-nrxn1a. We focused our attention on this structure. Using a previously published MO for VEGFAb, we observed vascular defects in CP and SIVs formation. Moreover, we found a more than additive effect by coinjecting β-nrxn1a MO1 or NLGN1 MO with both VEGFAa and VEGFab MOs, albeit with a different degree of penetrance (Figure 4). Notably, a low dose of α-NRXN1a MO alone or in coinjection with β-NRXN1a MO1, NLGN1 MO, or VEGFab MO produced milder vascular defects and minimal additive effects (Figure 4B and Figure IXC and IXD in the online-only Data Supplement).

Taken together, these data highlight a distinct role between VEGFAa and VEGFab isoforms in vascular development. In particular, our data suggest a pivotal function of VEGFab in CP formation, whereas VEGFAa isoforms are more involved in ISVs and only partially in CP formation, as previously indicated by other studies. Moreover, these results indicate a synergy of action of β-NRXN1a and NLGN1 MOs together or in coinjection with VEGFAb MOs, suggesting an interplay between all these molecules in the vascular system.

β-nrxn1a and nlgn1 Are Involved in CP Formation in Synergy With the Heparin-Binding Isoforms of VEGFA and the Heparan Sulfate 6-O-Sulphotransferase 2 Enzyme
It is interesting to note that the coinjection of VEGFAa MO with β-NRXN1a or NLGN1 MOs presents a lower degree of effectiveness (≈35%, n=87 and ≈33%, n=134, respectively) than the coinjection of VEGFab MO with β-NRXN1a MO1 or NLGN1 MO (≈59%, n=162 and ≈55%, n=178, respectively) (Figure 4B). We supposed that a possible explanation might reside in the differential affinity for HSPGs of the different isoforms produced by VEGFAa and VEGFab genes. A perturbation of HSPGs can result in a variation of morphogen gradients in ECM or alternatively it can interfere with stabilization of signaling complexes. To test this hypothesis, we perturbed the overall HSPGs maturation by injecting a previously published MO targeting HS6ST2. Sulphotransferases contribute to the heterogeneity of heparan sulphates and ECM maturation, which is essential for the modulation of ligand-receptor binding. In particular, the knockdown of zebrafish HS6ST2 induced a reduction of vascular branching in the CP compromising a correct VEGFA gradient formation. Moreover, at 5 dpf hyaloid vessels of HS6ST2 morphants fail to remodel, producing vessels very similar to the vascular phenotype observed in the retina of VEGFA120/120 knock-in mice (ie, with an increased lumen caliber and a very low number of branch points). In our experiments, the injection of HS6ST2 MO alone drastically impaired the correct CP formation (≈73%, n=99) (Figures 5A and IXB in the online-only Data Supplement). The induced vascular and consequently blood flow defects were analogous to the CP abnormalities observed in β-nrxn1a and nlgn1 morphants and in VEGFab MO-injected or β-NRXN1a MO1-VEGFab MO-coinjected embryos (compare the histological sections in Figures VA and VIII in the online-only Data Supplement). Whereas a low dose of HS6ST2 MO produced a low percentage of affected embryos (Figure 5A and Figure IXD in the online-only Data Supplement), it prevented a correct formation of CP in coinjection with low doses of β-NRXN1a MO1 (≈58%, n=102), NLGN1 MO (≈48%, n=231), VEGFab MO, or VEGFAa MO (≈55%, n=123 and ≈36%, n=107, respectively) (Figure 5).
Notably, the percentage of affected embryos in coinjection with VEGFAa MO was lower and they presented mild vascular defects (Figure 5). These experiments suggest that the establishment of a VEGFA gradient is necessary for a correct CP formation during embryo development. Moreover, they indicate that the effects of the injection of β-NRXN1a and NLGN1 MOs can be enhanced by defects in ECM maturation and the consequent alteration of morphogen gradients, in particular that of VEGFAb.

**SIVs Development Is Also Affected by the β-nrxn1a/nlgn1/VEGFAb/HS6ST2 Axis**

As previously described, the knockdown of β-nrxn1a, nlgn1, or VEGFAb induced defects in SIVs formation (Figures VB and VIIA in the online-only Data Supplement). To further sustain these data, we tested the effects of the different combinations of MOs on SIVs formation using endogenous alkaline phosphatase staining at 3 dpf (Figure 6). The single injection at low doses of each different MO (data not shown) or their coinjection with α-NRXN1a MO did not produce significant
vascular defects at SIVs (see Figure 6A and 6B). The injection of a high dose of HS6ST2 MO impaired a correct formation of SIVs (Figure 6A) and, as previously observed for the CP, the coinjection of VEGFAb MO with β-nrxn1a MO1 (≈48%, n=110) or NLGN1 MO (≈51%, n=132) proved to be more effective in affecting SIVs growth than the coinjection with VEGFAa MO (Figure 6B). Notably, the effect of the coinjections with VEGFAb are comparable to those observed cooinjecting β-nrxn1a MO1 and NLGN1 MO (≈50%, n=260). Moreover, HS6ST2 MO impaired SIVs morphogenesis when associated with β-nrxn1a MO1 (≈38%, n=95), NLGN1 MO (45%, n=144) or VEGFAb MO (33%, n=89) (Figure 6A and 6C). Finally, VEGFAa MO failed to impair SIV morphogenesis in coinjection with HS6ST2 MO (Figure 6A and 6C). Taken together, these data indicate that VEGFAb genetically interacts with β-nrxn1a and NLGN1 also during SIV morphogenesis and highlight the importance of the ECM maturation for a correct formation of the SIVs.

Discussion

In this study, we took advantage of the zebrafish animal model to deepen the knowledge on the role of the synaptic proteins NRXNs and NLGNs in vascular development. Through whole mount in situ hybridizations and real-time reverse transcriptase-polymerase chain reaction analyses, we observed that during embryo development β-nrxn1a and nlgn1 are expressed in endothelial cells of CP and surrounding menenchymal cells, whereas α-forms of nrxn1a are mainly expressed in different regions of brain and spinal cord. Consistent with our expression data, the knockdown of α-forms of nrxn1a resulted in a reduced locomotory activity of the injected embryos without evident vascular defects. Notably, previous independent studies have indicated that in Drosophila melanogaster the single α-NRXN protein is important for neurotransmission at neuromuscular junctions and consequently for larvae locomotion.45,46 In contrast, from 26 hpf the knockdown of β-nrxn1a and nlgn1 specifically induced vascular defects in CP region. The observed defects consist in a reduction of vessel sprouting and a concurrent decrease in branching which affect plexus morphology also at later stages. These results suggest a role of β-nrxn1a and nlgn1 in sprouting angiogenesis and vascular plexus remodeling and confirm our previous data obtained in the chicken chorioallantoic membrane model using an anti–β-NRXN blocking antibody.16 Furthermore, our recent experiments show that downregulation of neurexin 1 by siRNA in human endothelial cells causes a strong decrease of their tubulogenetic/morphogenetic capacity in the in vitro matrigel angiogenesis assay (A.V. Samarelli, manuscript in preparation, 2012). The alteration of a correct plexus formation in β-nrxn1a and nlgn1 morphants has consequences in the later phases of embryo development. At 54 hpf, the dorsal-most vessels of CP present a specific dilation at the expense of the ventral part, which in some tracts presents a reduced or absent blood flow attributable to the reduced lumen of the vessels as assessed by confocal and histological analyses. In β-nrxn1a morphants, although ISVs grow normally indicating no vascular patterning or guidance defects, around 72 hpf some of them present a reduced circulation and apparently begin to lose their cellular associations with neighboring endothelial cells and then regress. The blood vessels regression in CP, dorsal longitudinal anastomotic vessels, and ISVs can be explained by a reduction/lack of blood flow and of the resultant shear stress on endothelial cells. Blood flow-induced shear stress can indeed regulate different endothelial cell functions such as migration, proliferation, remodeling, and apoptosis.47 As previously observed by Isogai et al.,48 in zebrafish the emergence of secondary sprouts from the cardinal vein is genetically programmed. These sprouts require blood flow to sustain and finally fix the new venous identity of ISVs and in the absence of blood flow the new connection usually undergoes regression.48 More recently, Choi et al.49 showed that circulation is necessary for the maintenance of the CP structure only in the late phases of its development. As demonstrated by different studies in other animal models,49 blood flow plays also a pivotal role in vessels lumen regulation. Finally, given that in β-nrxn1a morphants only a subset of ISVs and dorsal longitudinal anastomotic vessels undergo
Figure 6. The genetic interaction of β-nrxn1a and nlgn1 with VEGFAs and heparan sulfate 6-O-sulphotransferase 2 (HS6ST2) is important for sub-intestinal veins (SIVs) formation. Qualitative (A) and quantitative (B and C) analysis of the effect on SIVs formation of the injection of different morpholinos (MOs) at low and high doses. A. Alkaline phosphatase staining was performed in order to visualize vessels in the region of the SIVs baskets at 3 days post-fertilization (dpf). The SIVs baskets appear substantially unaffected in control embryos (white on a black background). High doses of HS6ST2 MO (red) or the coinjection (black on white background) of low doses of VEGFAβ MO and β-NRXN1a MO1 or NLGN1 MO (data not shown) induces defects in SIVs formation. Moreover, SIVs appear disorganized in embryos coinjected with low doses of HS6ST2 MO and β-NRXN1a MO1, NLGN1 MO, and VEGFAβ MO, whereas they present no or very mild defects in coinjection with VEGFAα MO. (B and C) Quantitative analysis of the percentage of embryos with defects in SIVs formation in embryos injected with different combinations of MOs at low doses. α-NRXN1a MO has been used to test the extent of the mere additive effect in coinjection experiments. n indicates the total number of analyzed embryos. Values are expressed as means±SD. *P<0.05, **P<0.01, ***P<0.001; embryos coinjected with the α-NRXN1a MO have been used as reference groups. NLGN indicates neuroligins; NRXN, neurexins.
vascular regression, another possibility is that arterial versus venous fate decision might be involved. Future analyses will be finalized to characterize the nature of these defective vessels and to investigate the molecular mechanisms involved in this specific lack of blood circulation.

Using a loss-of-function approach, we show that β-nrxn1a and nlgn1 synergistically cooperate during CP and SIVs formation. This event logically follows 2 facts: the physical association of β-NRXN and NLGN in blood vessels40 and their innate partnership at the synapse.50 Moreover, we show that in both vascular structures the action of both β-nrxn1a and nlgn1 is genetically linked to VEGFAs. As shown by co-injection experiments, the role of β-nrxn1a and nlgn1 is associated to the isoforms more dependent on the ECM such as the VEGFaa165 and in particular to both isoforms produced by the VEGFAb gene.14 Previous studies indicated that VEGFaa, and in particular the VEGFaa121 isomorph, is mainly involved in ISVs formation,40,42 whereas the VEGFaa165 isoform has been more implicated in CP and SIVs formation.40 Our data and a previous independent study14 indicate that the knockdown of VEGFab induced vascular defects at the SIVs. We expanded these last observations by pointing out that VEGFAB is also strongly involved in CP formation. Both VEGFAB isoforms interact with HSPGs creating a gradient in the ECM, and our data indicate that a correct formation of the gradient is important for VEGFAB activity and consequently for CP and SIV morphogenesis. Recently, Wiley et al42 showed that also Bmp2 signaling is involved in CP formation; notably, BMPs-mediated processes involve their interaction with HSPGs in the ECM. As for VEGFAB isoforms, the interaction of BMPs with HSPGs restricts their diffusion creating a morphogen gradient in the extracellular environment.

In conclusion, how are NRXN and NLGN connected to VEGF? In preliminary experiments show that β-NRXN1a and VEGFAs are correlated at the transcriptional level (Figure X in the online-only Data Supplement) in zebrafish embryos between 24 and 30 hpf: when β-NRXN1a is downregulated, the VEGFAB (but not VEGFAa) transcripts are upregulated. Although this event needs further investigation into its meaning and mechanism, it strongly substantiates our above findings, ie, the existence of a specific link between β-NRXN1a and heparin-binding VEGFA, and the possibility that NRXNs and VEGFA act within the same cellular pathway, so that if 1 of them is downregulated, the other undergoes a compensatory rise in expression. In conclusion, our findings have 2 major implications for the field of neurovascular biology. First, they represent the first in vivo data about the role of NRXNs and NLGNs in vascular system development, further supporting our previous results in other species16 and expanding the number of molecules important in neuronal and vascular systems functions. Second, they indicate that members of a heterogeneous and complex family of neuronal proteins with cellular functions distant from the most classic angiogenic behaviors2 (proliferation, migration, and adhesion) are linked to a master regulator of angiogenesis such as VEGFA.

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Disclosures
None.

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The Synaptic Proteins β-Neurexin and Neuroligin Synergize With Extracellular Matrix-Binding Vascular Endothelial Growth Factor A During Zebrafish Vascular Development

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Supplement Figures Legends

Figure I: Expression of nrxn1a and nlgn1 genes during embryo development.

WISH with antisense and sense probes for α- and β-forms of nrxn1a (A, G), β-forms of nrxn1a (B-C, H) and nlgn1 (D-E, I) at different stages of development. Black arrows indicate signal in the CP region and ventral mesenchyme. Dashed rectangles indicate the portion of images in corresponding insets. (A) Dorsal view (upper left) and lateral views (upper right and lower panel) of wild-type embryos at 33 hpf. Red and white arrowheads indicate positive neurons in telencephalon and diencephalon, respectively. Black arrowheads indicate positive signal in rhombencephalic or spinal neurons. (B) Lateral views of wild-type embryos at ~ 30 (b¹) and ~ 33 hpf (b⁻). (C) Transverse sections images of the trunk of β-nrxn1a WISH at ~ 33 hpf. Sections were stained with eosin. (D) Lateral views of wild-type embryos at 26 hpf (d¹), 33 hpf (d⁻) and 36 hpf (d³). (E) Transverse sections images of the trunk of nlgn1 WISH at 33 hpf. (F) The expression of α-nrxn1a, β-nrxn1a and nlgn1 was examined by real time RT-PCR on mRNA extracted from endothelial cells isolated from Tg(kdrl:egfp)¹¹⁶ embryos at 30-35 hpf by cell sorting. The mRNA levels were normalized on β-actin and are presented as relative expression levels in EGFP⁺ compared to EGFP⁻ cells. Only β-forms of nrxn1a and nlgn1 were enriched in endothelial cells (EGFP⁺ cells). Error bars indicate SD. (G-I) WISH with sense probes for α- and β-forms of nrxn1a and β-forms of nrxn1a at different stages of development. (I) WISH with sense probe for nlgn1 on two embryos at 36 and 48 hpf, respectively. In all the panels head is on the left and dorsal is up. In all the sections neural tube and notochord are highlighted by black dotted and red lines respectively. (J) Confocal
analysis of vascular development in trunk/tail portion of Tg(flk1:EGFP) uninjected embryos and α-NRXN1a morphants between 26-32 hpf. Dashed rectangles indicate the portion of images in corresponding insets. In all insets red and white brackets indicate the Dorsal Aorta and CP of St-Control injected embryos showed in Figure 2A. Lateral views, anterior to the left. Magnification: 20X. (K) β-actin cDNA was amplified by PCR as an internal control for the quality of cDNA using a couple of primers specific for zebrafish β-actin\(^1\) which demonstrates the lack of genomic contamination in RNA preparations. Mw, Molecular weight size marker.

**Figure II: Reduced motility of α-nrxn1a morphants is not dependent on p53 activation.**

Quantitative analysis of the percentage of embryos with a partial or complete reduction of locomotion after stimulus in a touch response assay (see also Videos S7-S10 for the results of representative embryos for each class in touch response test). In order to test the off-target activity of MOs injection in our experiments, we coinjected high doses of α-NRXN1a MO, β-NRXN1a MO1 and NLGN1 MO with a 1.5 fold dose of p53 MO. The results are the sum of three different experiments. The total number of analyzed embryos are indicated above each bar. Error bars represent the SD.

**Figure III: Vascular phenotypes in caudal plexus of β-nrxn1a and nlgn1 morphants are not dependent on p53 activation.**

Quantitative analysis of the percentage of embryos with vascular defects in CP at 54 hpf. In order to test the off-target activity of MOs injection in our experiments, we coinjected high doses of α-NRXN1a MO, β-NRXN1a MO1 and NLGN1 MO with a 1.5 fold dose of p53
The results are the sum of three different experiments. The total number of analyzed embryos are indicated above each bar. Error bars represent the SD.

**Figure IV:** The knockdown of β-nrxn1a or nlgn1 affects the expression of several endothelial markers in the CP region.

The expression of a panel of vascular markers has been evaluated by whole-mount *in situ* hybridization on control embryos (St-CTRL and α-NRXN1a MO injected embryos) and β-nrxn1a and nlgn1 morphants. Around 32 hpf *flk1* is predominantly expressed in arterial endothelial cells (EC), whereas *cdh5* is a pan-endothelial marker and *flt4* and *tie2* are mainly expressed by venous endothelial cells. All the markers analyzed presented a reduction of signal limited to the CP region (arrowheads) of both β-nrxn1a and nlgn1 morphants. Moreover we obtained identical results with the following vascular markers: *vsgl* and *ephB4a* (data not shown). Images shown correspond to the trunk/tail region of 32 hpf embryos, lateral views, anterior to the left. Dashed rectangles indicate the portion of images in corresponding insets. “n” indicates the number of affected embryos over the total number of embryos.

**Figure V:** Analysis of vascular defects in CP and Subintestinal veins (SIVs) of β-nrxn1a and nlgn1 morphants.

Wild-type embryos were injected with high doses of St-CTRL MO, β-NRXN1a MOs or NLGN1 MO and at 54 hpf representative morphants were selected for histological analysis. (A) Bright field images (left) and histological semithin plastic sections (right) of the trunk/tail around 54 hpf embryos. Vertical black bars (a-c iii) indicate the approximate positions of the
corresponding sections. $\beta$-nrxn1a and nlgn1 morphants present a dilatation of the dorsal portion of the CP (black arrows) instead of the ventral one (black arrowheads). Notably, as shown by the respective bright field images (Figure S5A, left), the trunk/tail regions of the $\beta$-nrxn1a and nlgn1 morphants presented no evident morphological defects. *= dorsal aorta, nc= notochord, s= somite, CP= caudal plexus. (B) Alkaline phosphatase staining of embryos injected with different MOs. Both nlgn1 and $\beta$-nrxn1a morphants show a wide range of defects in the SIVs (black arrows). Frequencies of phenotypes are indicated in parentheses.

**Figure VI: Vascular defects induced by $\beta$-NRXN1a MOs injection visualized in 72 hpf double transgenic embryos.**

Confocal analysis of double transgenic Tg(gata1:dsRed)$^{6d2}$,(kdrl:EGFP)$^{843}$ embryos injected with high doses (0.6 pmol/embryo) of control MOs (St-CTRL MO and $\alpha$-NRXN1a MO) or $\beta$-NRXN1a MOs. From ~ 72 hpf some intersegmental vessels (ISVs) and DLAVs (see white arrowheads in insets) in the caudal portion of the trunk of morphants present a reduced or completely absent blood circulation. Moreover, they gradually lose their cellular contacts with adjacent cells beginning to regress (see also Supplemental videos S5-S6). Lateral views, head in on the left dorsal is up. Magnification: 20X.

**Figure VII: Knockdown of VEGFAb induces vascular defects in SIVs and CP.**

Embryos injected with high dose (0.6 pmol/e) of a previously published MO for VEGFAb (see Table S1). Frequencies of phenotypes are indicated in parenthesis. (A) Dorsal view of a representative VEGFAb morphant embryo at 72 hpf. The vascular system has been visualized by alkaline phosphatase staining. Black arrows indicate defects in SIVs. Similar defects have
been previously observed by Bahary and colleagues. (B) Lateral view (head is on the left, dorsal is up) of a Tg(gata1:dsRed) VEGFAb morphant at 54 hpf. Notably, the observed phenotype in the CP resembles that observed in β-nrxn1a and nlgn1 morphants, i.e. a dilation of the vessels in the dorsal portion of the plexus (red asterisk) and a lack of blood flow in some ventral regions of the plexus (white arrows) (see also Figure S8 for the histological analysis). Rhodamine has been used as tracer. Frequencies of phenotypes are indicated in parentheses. (C) Expression analysis of different vascular markers by WISH on St-CTRL MO injected embryos and VEGFAb morphants. VEGFAb morphants showed a reduction of signal limited to the CP region (arrowheads). Images shown correspond to the trunk/tail region of 32 hpf embryos, lateral views, anterior to the left. Dashed rectangles indicate the portion of images in corresponding insets. “n” indicates the number of affected embryos over the total number of analyzed embryos.

**Figure VIII: Histological analysis of vascular defects induced in CP by the injection of different MOs.**

The trunk morphology of Tg(gata1:dsRed) embryos injected with high doses MOs (red) or coinjected with low doses (black) of VEGFAb MO and β-NRXN1a MO1 was analyzed at ~54 hpf by semithin plastic sections (0.8 micron each). The injection of VEGFAb MO, NLGN1 MO or HS6ST2 MO produced vascular defects in CP in several points along the rostral-caudal axis, as previously observed in β-nrxn1a morphants (see also Figure S5A). The injection of similar doses of ST-CTRL MO or VEGFAa MO did not produced defects in CP. Also VEGFAb-β-nrxn1a double morphants showed a dilatation of dorsal-most vessels of the CP (arrows). Sections were stained with gentian violet and basic fuchsin. *= dorsal aorta, nc=
notochord, s= somite, CP= caudal plexus. Black arrows and arrowheads indicate the dorsal and ventral part of the CP, respectively.

**Figure IX: Qualitative and quantitative analyses of the injection of high or low doses of different MOs in Tg(*gata1*:dsRed) embryos.**

All the embryos are shown in a lateral position, anterior to the left and dorsal is up. In each injection, Rhodamine has been used as tracer. (A) Tg(*gata1*:dsRed) embryos injected with high doses of different MOs were analyzed at 54 hpf. To perform an extensive analysis of the phenotypes, we decided to use Tg(*gata1*:dsRed) embryos instead of a vascular transgenic line. The morphological features of the ventral part of the tail (where CP resides) and the fluorescence of the transgenic fish line allowed an easy and fast analysis of phenotypes in a large amount of embryos. The injection of single MOs at high doses has been used as a positive internal control in coinjection experiments. Notably, CP is substantially unaffected in high dose α-NRXN1a MO or St-CTRL MO injected embryos. (B) Quantitative analysis of the percentage of embryos with vascular defects in CP in embryos injected with high doses of different MOs. The total number of analyzed embryos in different experiments are indicated above each bar. Values are expressed as means ± SD. ** P<0.01, *** P<0.001; embryos injected with the α-NRXN1a MO have been used as reference groups. (C) Vascular Plexus formation is only minimally affected in embryos injected/coinjected with low doses of different MOs. (D) Quantitative analysis of the percentage of embryos with vascular defects in CP in embryos injected or coinjected with low doses of different MOs. The total number of analyzed embryos in different experiments are indicated above each bar. Error bars represent the SD.
Figure X: Knock-down of β-forms of nrxn1a specifically induces the up-regulation of VEGFAb transcript.

Here we compare VEGFAa and VEGFAb expression levels in embryos injected with a high dose of β-NRXN1a MO1. In order to test the effect of down-regulation of β-nrxn1a on VEGFAs transcripts during CP development, β-NRXN1a MO1 and St-CTRL MO injected embryos were collected at different stages of development from 24 to 30 hpf. Total RNA samples preparation and reverse transcriptions were performed as previously described. cDNAs were amplified with Icycler w/MyIQ PCR (Bio-rad) using iQ SYBR Green Supermix and the products of the reactions were then separated on agarose gels and visualized by ethidium bromide staining. Expression levels were normalized on St-CTRL injected embryos and rpl8 has been used as housekeeping gene. The results of a representative experiment are shown. Error bars represent the standard error of the mean (SEM). See Table S2 for a list of primers.

Video I: Effects of touch response test on St-CTRL MO injected embryos.

St-CTRL MO injected embryos (1 pmol/embryo) were analyzed by the touch response assay at 72 hpf. St-CTRL morphants show a rapid escape movement from the stimulus. The video was recorded using a Leica MZFLIII stereomicroscope equipped with a DFC 480 R2 digital camera.

Video II: Abnormal balance and touch response phenotypes in α-nrxn1a morphants.

A tactile stimulus is applied by a needle to α-NRXN1a MO injected embryos (1 pmol/embryo) at 72 hpf. The embryos present balance defects and a reduced or completely
absent motility before and after repeated touch stimulations. The video was recorded using a Leica MZFLIII stereomicroscope equipped with a DFC 480 R2 digital camera.

Video III: Vascular caudal plexus development from 36 to 48 hpf.

Time-lapse confocal movie of the trunk/tail of a wild-type Tg(\textit{flk1}:GFP) embryo from approximately 36 and 48 hpf (time points were recorded every 17 minutes) showing the formation of the CP. Anterior to the left, dorsal is up. Magnification: 20X.

Video IV: Caudal plexus development from 36 to 48 hpf in \textit{Beta nrxn1a} morphants.

Time-lapse confocal movie of the trunk/tail of a wild-type Tg(\textit{flk1}:GFP) embryo injected with 0.6 pmol/embryo of β-NRXN1a MO1. The video starts at around 36 hpf and ends at approximately 48 hpf (time points were recorded every 17 minutes). Anterior to the left, dorsal is up. Magnification: 20X.

Video V: Vascular caudal plexus development from 48 to 72 hpf.

Time-lapse confocal movie of the trunk/tail of a wild-type Tg(\textit{flk1}:GFP) embryo from approximately 48 and 72 hpf (time points were recorded every 20 minutes). The caudal plexus begins to be remodeled to a single vascular tube. Anterior to the left, dorsal is up. Magnification: 20X.

Video VI: Caudal plexus development from 48 to 72 hpf in \textit{Beta nrxn1a} morphants.
Time-lapse confocal movie of the trunk of a β-NRXN1a MO1 injected Tg(flk1:GFP) embryo from approximately 48 and 72 hpf (time points were recorded every 20 minutes). Arrows indicate regressing vessels in CP. White and open arrowheads indicate vascular abnormalities and regressing vessels in DLAV and ISV, respectively. Anterior to the left, dorsal is up. Magnification: 20X.

**Video VII: Effects of touch response test on St-CTRL MO injected embryos.**

St-CTRL MO injected embryos were analyzed by the touch response assay at 72 hpf. St-CTRL morphants show a rapid escape movement from the stimulus. The video was recorded using a Leica MZFLIII stereomicroscope equipped with a DFC 480 R2 digital camera.

**Video VIII: Effects of touch response test on α-NRXN1a MO and p53 MO coinjected embryos at 72 hpf.**

α-nrxnlα morphants show a completely absent motility before and after repeated touch stimulations. The video was recorded using a Leica MZFLIII stereomicroscope equipped with a DFC 480 R2 digital camera.

**Video IX: Effects of touch response test on β-NRXN1a MO1 and p53 MO coinjected embryos.**

β-NRXN1a MO1 and p53 coinjected embryos were analyzed by the touch response assay at 72 hpf. Coinjected embryos are able to escape from the stimulus. The video was recorded using a Leica MZFLIII stereomicroscope equipped with a DFC 480 R2 digital camera.
Video X: Effects of touch response test on NLGN1a MO and p53 MO coinjected embryos.

NLGN1 MO and p53 MO coinjected embryos were analyzed by the touch response assay at 72 hpf. Coinjected embryos are able to escape from the stimulus. The video was recorded using a Leica MZFLIII stereomicroscope equipped with a DFC 480 R2 digital camera.

REFERENCES

Figure I

A) \(\alpha/\beta\text{nrxn1a}\) WISH at \(~33\) hpf

B) \(\beta\text{-nrxn1a}\) WISH

C) \(\beta\text{-nrxn1a}\) WISH at \(~33\) hpf

D) \(nlgn1\) WISH

E) \(nlgn1\) WISH at \(33\) hpf

F) Transcript levels EGFP+/EGFP- cells

G) \(\alpha/\beta\text{nrxn1a}\) sense probe WISH

H) \(\beta\text{-nrxn1a}\) sense probe WISH

I) \(nlgn1\) sense probe WISH

J) 26 hpf, 28-29 hpf, 32 hpf

K) Uninjected, \(\beta\text{-NRXN1a MO2} 1\) pmole, \(\beta\text{-NRXN1a MO2} 0.8\) pmole, \(\beta\text{-NRXN1a MO2} 0.5\) pmole, \(\alpha\text{-NRXN1a MO} 1\) pmole

Rostral \(\rightarrow\) Caudal
Touch Response test at 3 dpf

- St-CTRL MO
- α-NRXN1a MO + p53 MO
- β-NRXN1a MO1 + p53 MO
- NLGN1 MO + p53 MO

% of embryos with very low performance
Figure III

% embryos with vascular defects

- α-NRXN1a MO + p53 MO
- β-NRXN1a MO1 + p53 MO
- NLGN1 MO + p53 MO
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Figure V

A

B

Alkaline phosphatase staining at 3 dpf

- Uninjected
- St-CTRL MO
- α-NRXN1a MO
- β-NRXN1a MO1 (75%)
- β-NRXN1a MO2 (56%)
- NLGN1 MO (52%)
Figure VI

\[ \text{Tg(gata1:dsRed)}^{sd2},(kdrl:EGFP)}^{s843} \text{ at 3 dpf} \]
Figure VII

A

VEGFAb MO High dose (87%)

B

VEGFAb MO High dose (82%)

C

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Figure VIII

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Tg(gata1:dsRed) at ~54 hpf

**Legend:**
- S: Somites
- nc: Neural Crest
- CP: Caudal Pole

**Direction:**
- R: Rostral
- C: Caudal
Figure IX

A

Tg(gata1:dsRed) at 2.5 dpf

Uninjected

ST-Control MO High dose

β-NRXN1a MO High dose

β-NRXN1a MO1 High dose

β-NRXN1a MO2 High dose

NLGN1 MO High dose

B

% embryos with vascular defects

α-NRXN1a MO

β-NRXN1a MO1

β-NRXN1a MO2

NLGN1 MO

VEGFAb MO

VEGFAa MO

HS6ST2

C

Tg(gata1:dsRed) at 2.5 dpf

Uninjected

VEGFAb MO

VEGFAa MO

β-NRXN1a MO1 + α-NRXN1a MO

NLGN1 MO + α-NRXN1a MO

VEGFAb MO + α-NRXN1a MO

D

% embryos with vascular defects

α-NRXN1a MO

β-NRXN1a MO1

NLGN1 MO

VEGFAb MO

VEGFAa MO

HS6ST2 MO

α-NRXN1a MO + β-NRXN1a MO1

α-NRXN1a MO + NLGN1 MO

α-NRXN1a MO + VEGFAb MO

α-NRXN1a MO + HS6ST2 MO
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<td>A. Nasevicius et al., Yeast 17, 2000</td>
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<td>N. Bahary et al., Blood 110, 2007</td>
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<td>E. Chen et al., Dev Biol 284, 2005</td>
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<td>5’-GCGCCATTGCTTGTGAAGATTG-3’</td>
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<td>M.E. Robu et al., PLOS Genet. 3(5):e78, 2007</td>
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