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VEGF Modulates Synaptic Activity in the Developing Spinal Cord

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ABSTRACT: **Although it has been documented that the nervous and the vascular systems share numerous analogies and are closely intermingled during development and pathological processes, interactions between the two systems are still poorly described. In this study, we investigated whether vascular endothelial growth factor (VEGF), which is a key regulator of vascular development, also modulates neuronal developmental processes. We report that VEGF enhances the gamma-aminobutyric acid (GABA)/glycinergic but not glutamatergic synaptic activity in embryonic spinal motoneurons (MNs), without affecting MNs excitability. In response to VEGF, the frequency of these synaptic events but not their amplitude was increased. Blocking endogenous VEGF led to an opposite effect by decreasing frequency of synaptic events. We found that this effect occurred specifically at early developmental stages (E13.5 and E15.5) and vanished at the prenatal stage E17.5. Furthermore, VEGF was able to increase vesicular inhibitory amino acid transporter density at the MN membrane. Inhibition of single VEGF receptors did not modify electrophysiological parameters indicating receptor combinations or an alternative pathway. Altogether, our findings identify VEGF as a modulator of the neuronal activity during synapse formation and highlight a new ontogenic role for this angiogenic factor in the nervous system.** © 2014 Wiley Periodicals, Inc. Develop Neurobiol 74: 1110–1122, 2014

Keywords: **vascular endothelial growth factor; angiogenic factors; neuronal activity; development**

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INTRODUCTION

Spinal cord motor activity relies on the maturation of accurate synaptic connectivity between interneurons and motoneurons (MNs) that control motor output. First synaptic activities appear in the spinal cord at embryonic (E) day 12.5 (E12.5) as GABAergic synaptic events, these latter being associated with glycinergic synaptic events after E13.5 (Scain et al., 2010). These synaptic events are likely to be initiated in Renshaw cells (Alvarez et al., 2013). A cholinergic and glutamatergic synaptic component has also been described as participating at the genesis of spontaneous activity in the spinal cord before E15.5 (Branchereau et al., 2002; Myers et al., 2005). Synaptogenesis then gradually occurs in spinal motor networks but precise mechanisms governing this ontogenic process remain elusive. Interestingly, it occurs concomitantly to the vascularization of the spinal cord (Rigato et al., 2011) leading to the hypothesis of common regulatory factors.

Angiogenesis is under the control of growth factors including the vascular endothelial growth factor (VEGF) family and more particularly VEGFA (Carmeliet et al., 1996; Chung and Ferrara, 2011). Deletion of VEGFA is lethal at early developmental stages and haploinsufficiency has been observed (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF has two classes of transmembrane receptors. The first class is composed of tyrosine kinase VEGF receptor 1, 2, and 3 (VEGFR1, 2, and 3). These receptors are essential for embryonic development and for VEGF-dependent signaling (Vieira et al., 2010). The second class of receptors includes neuropilin 1 and 2 (NRP1 and 2). On the contrary to VEGFRs, their cytoplasmic domain is not phosphorylated in response to VEGF and they are considered as coreceptors. Besides their role in VEGF signaling, NRPs are also receptors for semaphorines, which are involved in axonal guidance. It is important to note that all these receptors (VEGFRs and NRPs) can heteromerize, which leads to modulation of cell signaling (Cebe-Suarez et al., 2006).

The development and maintenance of the vascular and neuronal network are under control of several factors. However, it has been recognized in recent years that factors implicated in one network can also act on the other (Zachary, 2005; Rosenstein et al., 2010). In the nervous tissue, VEGF promotes angiogenesis as expected but has also a direct role on neurogenesis (Le Bras et al., 2006), dendritogenesis (Licht et al., 2010), axonal outgrowth, and guidance (Sondell et al., 1999; Ruiz de Almodovar et al., 2011; for review see Mackenzie and Ruhrberg, 2012). These results are derived from *in vitro* studies, as well as from studies conducted in model organisms for neurodegenerative disease pathologies such as amyotrophic lateral sclerosis or Alzheimer disease. Only a few studies have been conducted on the link between the vascular and neuronal network during ontogeny, which still remains largely elusive (Hogan et al., 2004; Nagase et al., 2005; Ruiz de Almodovar et al., 2011).

In this article, we studied the effect of VEGF on lumbar spinal MNs synaptic activity in the mouse embryo. We evidenced a modulatory role of VEGF on synaptic transmission that occurs specifically on GABA/glycinergic activity during a specific time window.

METHODS

Animal and Spinal Cord Preparation

Pregnant adult and newborn OF1 mice (Charles River Laboratories, St. Germain sur L'Arbresle, France) were maintained and killed according to protocols approved by the European Community Council and conforming to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Hb9-eGFP mice (Wichterle et al., 2002) were produced by animal facility of Bordeaux University. E0.5 corresponded to the day following the mating night (i.e., day 0.5 of gestation, confirmed by the observation of a vaginal plug), and postnatal day 0 (P0) corresponded to the date of birth. Experiments were performed on embryos at E11.5, E13.5, E15.5, and E17.5 and on newborn mice at P0. Embryos were removed surgically from pregnant mice killed by cervical dislocation. Newborn mice were decapitated. For immunohistochemistry, embryos and newborns were rinsed with Dulbecco's phosphate-buffered saline (PBS) at 6–8°C, and their spinal cords with dorsal root ganglia were dissected before fixation with 4% paraformaldehyde. To study GABA/glycine release, spinal cord was treated in presence of ALX-5407 (1 μM, Sigma), ALX-1393 (0.5 μM, Sigma), and nipecotic acid (200 μM, Sigma). For electrophysiology, brainstem-spinal cord was dissected out, in physiological liquid dorsally opened, and the meninges removed as described previously (Delpy et al., 2008). The neuraxis, in "open-book" configuration, was continuously perfused at 30°C (perfusion rate of 1.5 mL min^{-1}) with physiological solution equilibrated with 95% O_2-5% CO_2.

Electrophysiology

For current clamp recordings, the artificial cerebrospinal fluid (aCSF) contained the following (m*M*): 113 NaCl, 25 $NaHCO_3$, 11 D-glucose, 4.5 KCl, 2 $CaCl_2$ dihydrate, 1 $MgCl_2$ hexahydrate, and 1 NaH_2PO_4 monohydrate. Electrode tips were filled with a filtered patch solution

containing the following (m*M*): 130 K gluconate, 2 $CaCl_2$ dihydrate, 10 Hepes, 10 EGTA, and 2 MgATP, pH 7.4.

For voltage clamp recordings, extracellular medium was the same except for KCl 6 m*M* and NaCl 111.5 m*M*. Electrodes tips were filled with the following medium in m*M*: 130 CsCl; 4 $MgCl_2$ hexahydrate; 10 HEPES; 4 ATP_2Na, and 10 EGTA. Tetrodotoxin (TTX, 200 n*M*, Latoxan) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; inhibitor of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) glutamate receptor, 20 μM, Tocris) or Gabazine (2 μM, Tocris) and strychnine (1 μM, Sigma) were added to aCSF to detect GABA/glycine or glutamate miniature postsynaptic currents (mPSCs), respectively. E13.5 MNs were voltage-clamped at -55 mV, E15.5 at -60 mV, and E17.5 at -70 mV to match physiological membrane potential previously determined (Delpy et al., 2008). Junction potential (14 and 3.4 mV for current clamp and voltage clamp intracellular medium, respectively) was not corrected. We ensured that recordings were performed from MNs by identifying them through their morphology and location as well as through their input resistance and membrane capacitance (Delpy et al., 2008).

Patch pipettes had access resistance from 3 to 5 MΩ. Membrane capacitance and resistance, control interevent and amplitude of GABA and glycinergic mPSCs, were not statistically different between pools of data for the same developmental stage. For all protocols, experiment started with 20–30 min of control acquisition before acute treatment (10 min) was applied. Events were detected during a 4-min period just before and at the end of the acute drug treatment. These latter were: VEGF (50 ng/mL, Peprotech, ref 100-20), B20 (5 μg/mL, Genentech), MF1 (anti-mVEGFR1, 50 μg/mL, ImClone), DC101 (anti-mVEGFR2, 50 μg/mL, ImClone), mF4-31C1 (anti-mVEGFR3, 50 μg/mL, ImClone), NRP1b (anti-VEGF ligation site on NRP1, 25 μg/mL, Genentech), NRP2b (anti-VEGF ligation site on NRP2, 25 μg/mL, Genentech). aCSF indicated control experiment performed in the absence of VEGF or B20 neutralizing antibody.

Immunofluorescence

Whole spinal cords or slide of transversal sections of spinal cords were rinsed with PBS and incubated in primary antibody for 48 h at 4°C in 0.2 *M* PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100. The primary antibodies used in this study were as follows: rabbit polyclonal anti-GABA (A2052; 1:200; Sigma), rat anti-glycine (1:3000, ImmunoSolution, Jesmond, New South Wales, Australia), mouse anti-Islet-1/2 (39.4D5 and 40.2D6, 1:100, DSH Bank, Iowa), rat anti-CD31 (PECAM-1, 553370, 1:100, BD Biosciences), hamster anti-CD31 (MAB1398Z, 1:500, Chemicon International), mouse anti-VEGF (B20, 1:500, Genentech), mouse monoclonal anti-NeuN (MAB377; 1:500; Millipore), and rabbit anti-vesicular inhibitory amino acid transporter (VIAAT; 1:500; gift from B. Gasnier, Institut de Biologie physicochimique, France) which recognizes the common presynaptic vesicular transporter of glycine and GABA (Dumoulin et al., 1999). After rinsing, spinal cords were incubated at room temperature for 2 h with adapted secondary antibodies (1:500) coupled with Alexa 488 (green), Alexa 546 (red), and Alexa 647 (far red) diluted in same buffer as primary antibodies. After three extensive rinses in PBS, the preparations were mounted with Fluoromount G (SouthernBiotech).

Confocal Acquisitions

Preparations were observed with a BX51 Olympus (Olympus France, Rungis) Fluoview 500 or C2 Nikon (Nikon France, Champigny sur Marne) confocal microscope equipped with an argon laser light source (for Alexa 488 detection) and two neon helium lasers for Alexa 546 and 647 detection. For the quantitative analysis, serial optical sections at intervals of 1.2 μm were imaged with a 10× objective or 0.3 μm with a 60× oil objective. Unless otherwise indicated, images correspond to single optical section (0.3 μm in z). Image analysis was performed using ImageJ program (Wayne Rasband, NIH).

Statistics

All values were expressed as mean ± standard error of the mean (SEM). Statistical significance of difference was assessed, using GraphPad Prism software, by a nonparametric Kruskal–Wallis one-way ANOVA followed by a pairwise multiple comparisons *post hoc* test (Dunn's method), nonparametric paired Wilcoxon test, two-way ANOVA with Bonferroni post test, or by one-way ANOVA followed by Tukey's multiple comparison test. The level of significance was set at $p < 0.05$. p and n values are indicated for each developmental stage and/or condition.

RESULTS

Development of the Spinal Vascular Network

We first examined the ontogeny of the vascular network in the mouse spinal cord and its vicinity to MNs during the second part of the embryonic life. Vessel development was studied in Hb9-eGFP transgenic mice that express GFP in MNs (Wichterle et al., 2002). Blood vessels, stained with CD31, were progressively intermingled with group of $Hb9^+$ MNs [Fig. 1(A)]. A perineural vascular plexus surrounding the whole spinal cord was detected at E11.5 with capillaries invading the gray matter [Fig. 1(A1,A3)]. The capillaries density increased at later developmental stages with expanded vascular connections [arrowheads in Fig. 1(A4)]. Except for E11.5, vessel area appeared constant [$p < 0.0001$, Kruskal–Wallis

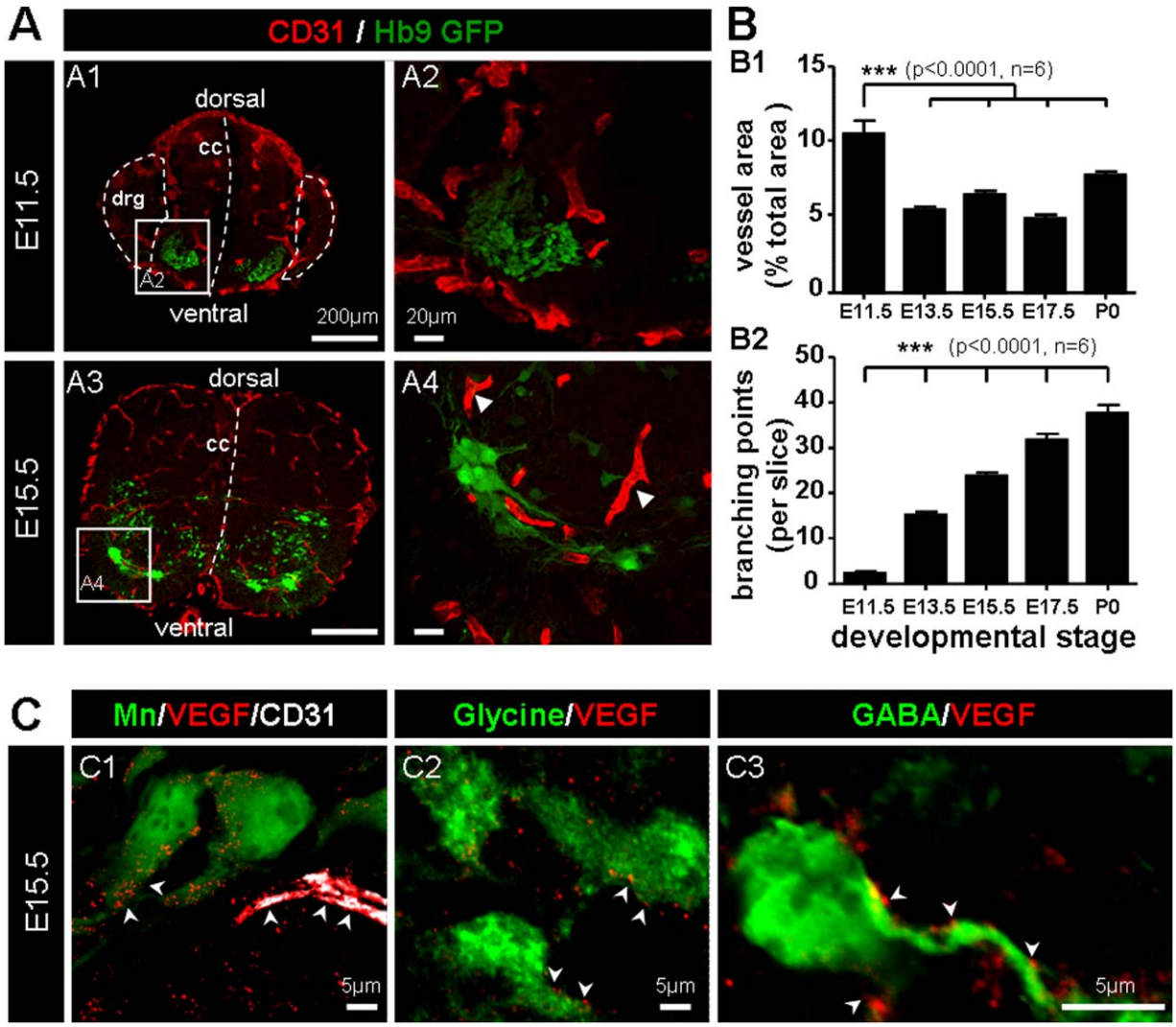

Figure 1 Development of vascular network in the mouse embryonic spinal cord. (A) CD31 staining (red) and Hb9-eGFP MNs (green) at E11.5 and E15.5. Arrowheads indicate branching points in blood vessels. (B1) Ratio vessel/spinal cord surface between E11.5 and birth. (B2) Number of branching points between E11.5 and P0. Kruskal–Wallis test followed by Dunn's post test. (C1) VEGFA immunolabeling (red) is detected at E15.5, in the cytoplasm of Hb9-eGFP (green) spinal lumbar MNs as well as in blood vessels (arrowheads). VEGFA (red) is also detected in the cytoplasm of glycine-immunoreactive (green, C2) and GABA-immunoreactive (green, C3) interneurons (arrowheads). cc: central canal, drg: dorsal root ganglia.

test, Fig. 1(B1)], whereas branching points increased with embryonic development [$p < 0.0001$, Kruskal–Wallis test, Fig. 1(B2)].

VEGFA immunoreactivity was detected in the gray matter of E15.5 mouse lumbar spinal cord, with a strong labeling in the cytoplasm of $Hb9^+$ MNs [Fig. 1(C1)] and, to a lesser extent, in glycinergic and GABAergic interneurons [Fig. 1(C2 and C3)]. The intensity of the VEGF immunolabeling in the ventral gray matter remained constant between E11.5 and birth (VEGF labeling in arbitrary unit: 6.7 ± 1.3, 4.2 ± 0.9, 5.5 ± 0.7, 4.9 ± 0.9, and 5.6 ± 0.9 at E11.5, E13.5, E15.5, E17.5, and P0, respectively), suggesting that spinal motor networks may release VEGF throughout the embryonic development.

Acute Application of VEGF Does Not Affect Spinal MNs Directly

We next investigated whether VEGF, besides its role in angiogenesis, exerts an effect on MN activity during the ontogeny of the spinal cord. To test this

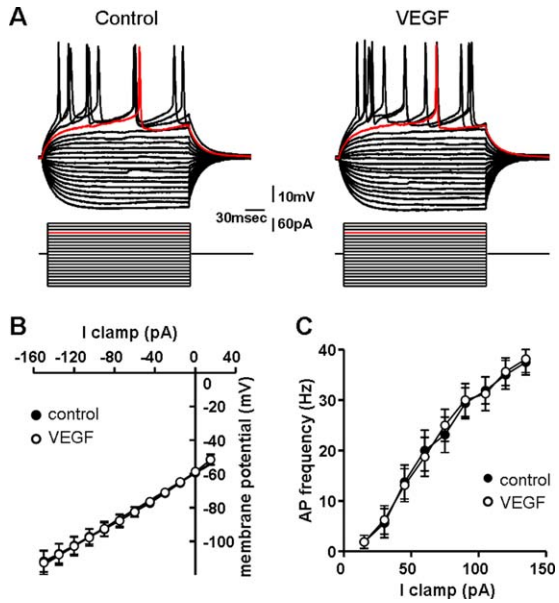

Figure 2 VEGF has no effect on MNs intrinsic properties at E15.5. (A) Voltage-current curve in control condition (left) and following VEGF treatment (right, 50 ng/mL, Peprotech, 10 min). Note that the MN fires an action potential (AP; red trace) for the same injection of positive current. (B) Mean voltage–current (*V/I*) relationship ($n = 8$) in control (filled circles) and VEGF (open circles) revealing the absence of VEGF effect on MN input resistance. (C) Average AP frequency ($n = 8$), plotted against current intensity. Note that the AP frequency is unchanged in the presence of VEGF ($p = 0.9334$, two-way ANOVA with Bonferroni post test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hypothesis, we performed whole-cell recordings from E15.5 MNs and, after a 30 min control period, VEGF was perfused for 10 min at the concentration of 50 ng/mL. This concentration was chosen because it induced a maximal proliferation of endothelial cells as tested in our laboratory (not shown). VEGF did not induce any modification of the MN membrane potential and voltage–current curves did not disclose any change in the MN input resistance [Fig. 2(A,B)]. The excitability was unaffected by VEGF as revealed by the number of action potentials elicited by depolarizing currents [$n = 8$, Two-way ANOVA, $p = 0.9334$, Fig. 2(C)]. Action potential properties (amplitude, spike threshold, duration, and after hyperpolarization) were also not modified by VEGF (Table 1). Taken together, these results indicate that acute application of VEGF had no effect on spinal MNs at E15.5.

Acute Application of VEGF Modulates Synaptic Activity

To test whether VEGF affects synaptic transmission, we first recorded mPSCs (with TTX 0.2 μM), in the presence of CNQX (20 μM) to specifically focus on GABA/glycinergic events. These mPSCs were abolished in the presence of Gabazine (2 μM)/strychnine [1 μM; Fig. 3(A)].

As illustrated on Figure 4(A,B) that depicts a representative experiment, exogenous VEGF significantly decreased the period between GABA/glycinergic mPSCs from 3.17 ± 0.28 s to 1.80 ± 0.15 s [Kolmogorov–Smirnov test, $p < 0.0001$; Fig. 4(B1)]. Control amplitude (38.97 ± 4.15 pA) was not affected by VEGF [38.89 ± 4.15 pA, Kolmogorov–Smirnov test, $p = 0.2361$; Fig. 4(B2)]. When collected from 11 MNs, interevent was significantly decreased by VEGF [−24.06 ± 9.19%, Wilcoxon test, $p = 0.0322$, $n = 11$; Fig. 4(C1)]. Variation of the amplitude (+11.8 ± 7.887%) was not statistically significant (Wilcoxon test, $p = 0.1748$). Interevent and amplitude were stable in control condition, when VEGF was omitted (−7.25 ± 7.596%, $p = 0.8438$ and +4.692 ± 7.261%, $p = 0.6406$, respectively, $n = 8$, not shown). To study the role of endogenous VEGF, we applied, under the same experimental conditions, a neutralizing antibody specific for murine VEGF (B20, Genentech, 5 μg/mL). We observed an increase of the period between GABA/glycinergic mPSCs [+49.74 23.15%, $p = 0.0134$, $n = 14$, Fig. 4(C1)]. No change was found for the amplitude of the same events [−6.995 ± 4.137%, $p = 0.1353$,

Table 1 VEGF has no Effect on MNs Intrinsic Properties

			AP Characteristics			
$n = 8$	Rm (MΩ)	Intercept (mV)	Amplitude (mV)	Threshold (mV)	Duration ½ spike (ms)	Ahp (mV)
Ctrl	369 ± 47	−58.73 ± 1.88	47.15 ± 2.36	−35.93 ± 1.33	2.00 ± 0.09	9.25 ± 1.17
VEGF	377 ± 49	−58.1 ± 1.83	45.76 ± 2.46	−36.38 ± 1.45	1.96 ± 0.09	10.17 ± 0.89
p value	0.4609	0.7422	0.0781	0.1094	0.5	0.1094

Input membrane resistance (Rm), voltage-current curve intercept and AP properties show that VEGF does not affect MNs. $n = 8$ cells on eight different spinal cords. *p* values from Wilcoxon paired tests.

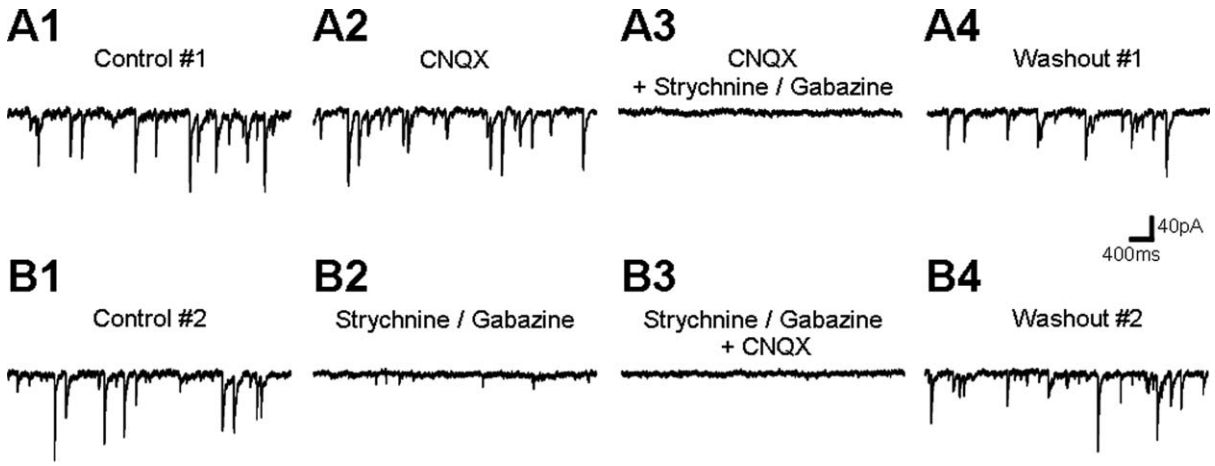

Figure 3 mPSCs recorded from E15.5 spinal MNs involve glutamatergic AMPA and GABA/glycine receptors. (A) Cumulative applications of 20 μM CNQX, and Gabazine (2 μM)/strychnine (1 μM) completely eliminate mPSCs. (B) On the same MN (after washout out all blockers), application of Gabazine (2 μM) / strychnine (1 μM) reveals AMPAR-related mPSCs (B2). Note that a subsequent application of CNQX (20 μM) again abolishes all mPSCs. Recording performed at Vh −60 mV in the presence of TTX 0.2 μM.

Fig. 4(C2)]. These results indicate that the exogenous VEGF and the endogenous VEGF blocker B20 lead to opposite effect: VEGF potentiates the GABA/glycinergic synaptic transmission, whereas B20 downregulates it.

To test whether VEGF also modulate the glutamatergic synaptic transmission, we performed, at E15.5, whole-cell recordings from mPSCs in the presence of Gabazine (2 μM)/strychnine (1 μM). These events were blocked by a further application of CNQX (20 μM), indicating that they involved AMPA/kaïnate receptors [Fig. 3(B)]. Interestingly, no significant changes of the interevent of the AMPA/kaïnate mPSCs were noticed in the presence of VEGF ($+12.23 \pm 10.93\%$, $p = 0.6149$, $n = 19$) or neutralizing antibody B20 [$+10.98 \pm 7.789\%$, $p = 0.2402$, $n = 11$; Fig. 4(D1)]. The amplitude of glutamatergic mPSCs also remained constant when VEGF ($-1.73 \pm 4.207\%$, $p = 0.2514$) or B20 ($+0.03 \pm 3.765\%$, $p = 0.5195$) was applied [Fig. 4(D2)]. Altogether, our data indicate that spinal VEGF exerts a direct effect on GABA/glycinergic synaptic release on E15.5 spinal MNs, without affecting the AMPA/kaïnate synaptic transmission. Using immunodetection of GABA and glycine, we checked whether spinal cords incubated with either VEGF or neutralizing antibody B20 could exhibit a modified level of GABA and glycine immunoreactivity [GABA-ir and Glycine-ir; Fig. 5(A)]. We found that a 10-min long application of exogenous VEGF reduced the GABA-ir level in the MN area compared to control [aCSF; Kruskal–Wallis test followed by Dunn's post test, $n = 5$, Fig. 5(B)]. On the contrary, the B20 incubation did not modify the level of GABA-ir compared to aCSF [Fig. 5(B)]. We also found that a 10-min long application of exogenous VEGF significantly reduced the glycine-ir level in the MN area compared to control [aCSF; [Kruskal–Wallis test with Dunn's post test, $n = 5$, Fig. 5(C)]. Again, as for GABA-ir, the B20 application did not increase this level compared to aCSF [$n = 5$, Fig. 5(C)].

VEGF Increase the VIAAT Density at the MN Membrane

To gain more insights into the molecular mechanisms, we performed additional immunostaining at E15.5 using WT mice and HB9-GFP mice. We used an antibody directed against the VIAAT. VIAAT is the common presynaptic vesicular transporter of glycine and GABA (Dumoulin et al., 1999). Staining was performed on control-, VEGF- or B20 antibody-treated spinal cords (10-min long treatment, $n = 3$). Our data show that the density of VIAAT-terminal on Hb9-eGFP MNs is increased by VEGF, whereas B20 has no obvious effect [Fig. 5(D,E)]. Thus, an acute application of VEGF changes the amount of a GABA/glycine presynaptic marker, in agreement with our electrophysiological data.

VEGF Affects the GABA/Glycinergic Synaptic Transmission Within a Specific Time-Window Effect

We next determined across various developmental stages if the effect of VEGF on GABA/glycinergic

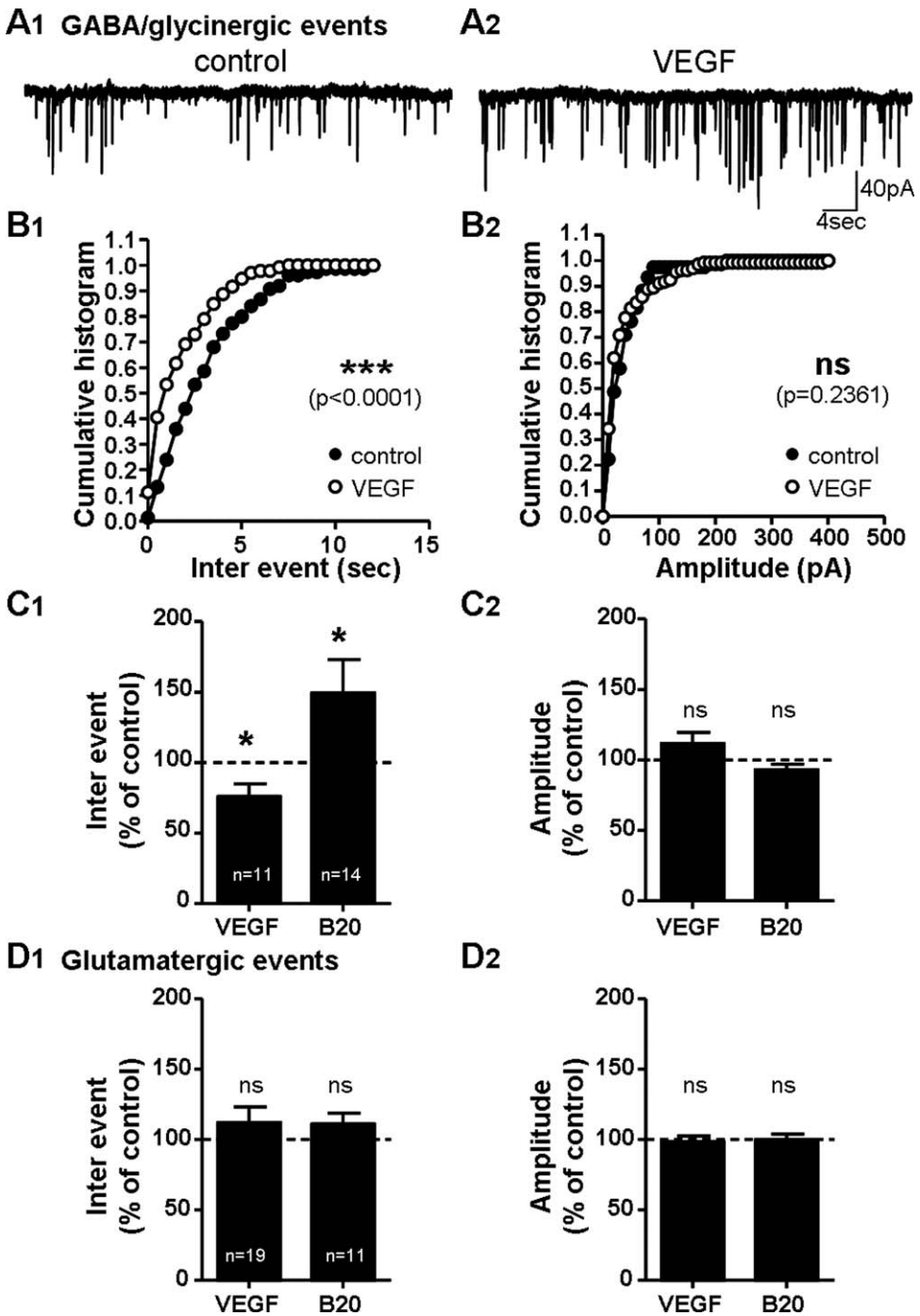

Figure 4 VEGF modulates interevent of GABA/glycinergic mPSCs without affecting AMPA mPSCs at E15.5. (A) GABA/glycinergic mPSCs (0.2 μM TTX) recorded in the presence of CNQX (1 μM) in control condition (A1) or with VEGF (A2, 50 ng/mL, Peprotech, 10 min) in the same spinal MN. Note the increase of the mPSCs frequency in VEGF. (B) Summary graphs of interevent (B1) and amplitude (B2) distributions of GABA/glycinergic mPSC recorded during a 4-min control (filled circles) and VEGF period (open circles). ***$p < 0.0001$, Kolmogorov–Smirnov test. (C) Interevent (C1) and amplitude (C2) variation of GABA/glycinergic mPSCs after VEGF or B20 (VEGF neutralizing antibody, Genentech, 5 μg/mL) treatment (10 min) from 11 and 14 experiments, respectively. * Wilcoxon paired test, for *p*-value see text. (D) Interevent (D1) and amplitude (D2) variation of glutamatergic mPSCs after VEGF or B20 treatment (10 min) from 19 and 11 experiments, respectively). * Wilcoxon paired test, for *p*-value see text.

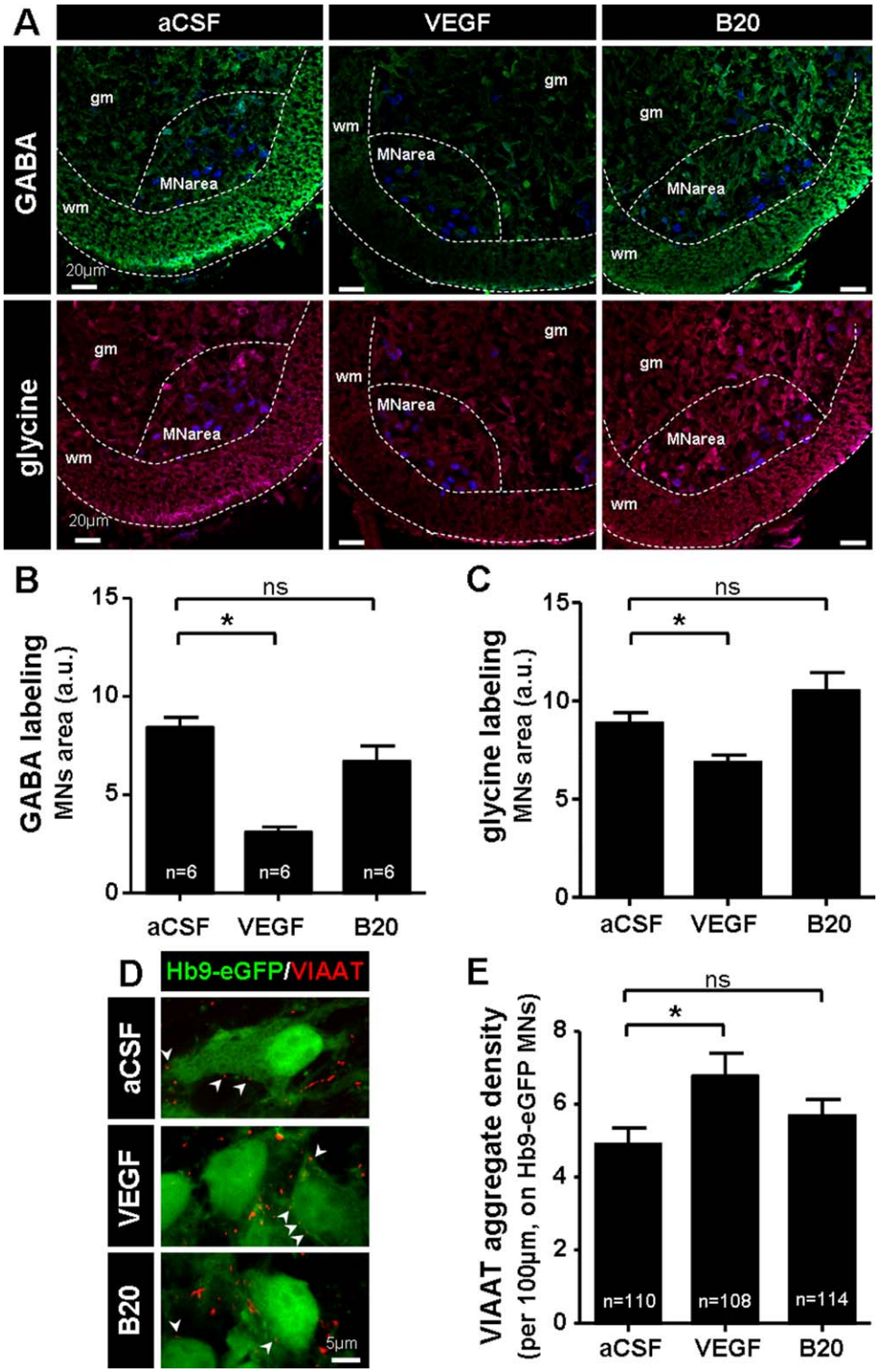

Figure 5 Increase of the GABA and glycine release in the presence of VEGF. Immunostaining of GABA (green) or glycine (red) and MNs (Islet-1/2 positive, Blue) were performed on spinal cord incubated with either control medium (aCSF) or VEGF or B20 during 10 min in presence of the glycine membrane transporter 1 (GlyT1) blocker ALX-5407 (1 μM), nonspecific GlyT blocker ALX-1393 (0.5 μM) and GABA membrane transporter (GAT) blocker nipecotic acid (200 μM). GABA (B) and glycine (C) labeling intensity was measured in the MN area in the three experimental conditions ($n = 6$). Kruskal–Wallis test followed by Dunn's post test. (D) Acute (10 min) VEGF treatment leads to an increase in the number of VIAAT immunoreactive terminals on $HB9^+$ MNs, whereas B20 treatment does not change the number of VIAAT aggregates. (E) Quantitative analysis (number of VIAAT aggregates per 100μm somatic membrane of $HB9^+$ MNs) in control (aCSF), VEGF, and B20 condition. Numbers in bars correspond to $HB9^+$ MNs cell bodies analyzed (from three experiments) from single 125-nm thick confocal sections. One-way ANOVA followed by Tukey's multiple comparison test ($*p < 0.05$).

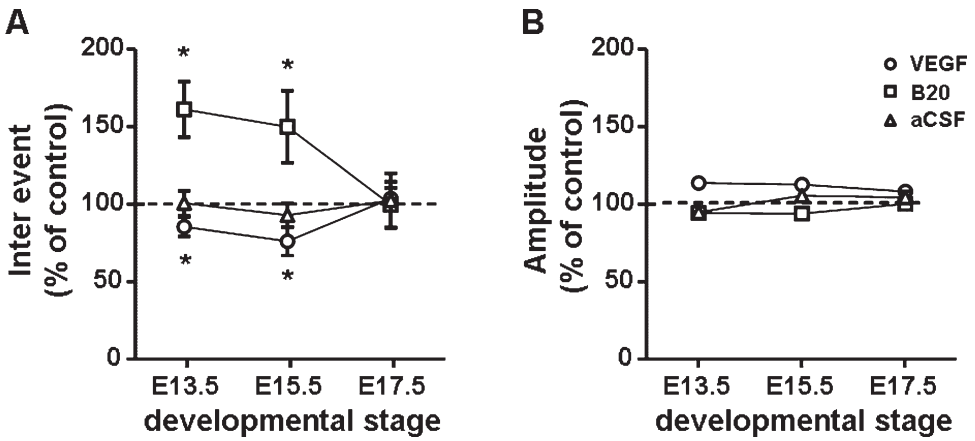

Figure 6 VEGF acts on GABA/glycinergic events at early developmental stages. Interevent (A) and amplitude (B) variation of GABA/glycinergic mPSCs after VEGF (open circle), B20 (open square), and control (aCSF, open triangle) treatment (10 min) at E13.5, E15.5, and E17.5. Note that VEGF and B20 do not affect GABA/glycinergic mPSCs at E17.5. * Wilcoxon paired test, see text for p values and number n of experiments.

mPSCs persists during the whole embryonic life. We used the same protocol at E13.5 and E17.5 as described above. MNs were voltage-clamped at values closed to the resting membrane potential determined at E13.5 and E17.5 (−55 and −70 mV, respectively; Delpy et al., 2008). We decided to focus on GABA/glycinergic mPSCs that were exclusively modulated by VEGF at the E15.5 developmental stage (Fig. 4).

At E13.5, we found that exogenous VEGF also significantly decreased the interevent of GABA/glycinergic mPSCs [14.82 ± 6.24%, Wilcoxon test, p = 0.0425, n = 12; Fig. 6(A)]. Blocking endogenous factor with B20 had opposite effect with an increase by 60.84 ± 17.83% [p = 0.0020, n = 10; Fig. 6(A)]. The GABA/glycinergic mPSCs amplitude was modified neither by VEGF (12.99 ± 7.444%, p = 0.1099) nor by B20 [−6.62 ± 4.177%, p = 0.1934; Fig. 6(B)].

At E17.5, on the contrary to E13.5 and E15.5 stages, the period of GABA/glycinergic mPSCs was unchanged by VEGF (+3.46 ± 7.005%, p = 0.6523, n = 9) or neutralizing antibody [−0.5454 ± 14.87%, p = 0.2412, n = 14; Fig. 6(A)]. The amplitude also remained stable when VEGF or B20 was perfused [+7.26 ± 7.772%, p = 0.4961 and −0.79 ± 6279%, p = 0.1040, respectively; Fig. 6(B)]. Control experiments in which GABA/glycinergic mPSCs were recorded from E13.5, E15.5, and E17.5 spinal MNs in the presence of aCSF devoid of exogenous VEGF or neutralizing B20 antibody revealed that the interevent and the amplitude were not modified during the recording session [Fig. 6(A,B)].

Individual VEGF Receptor Does Not Account for the Effect of VEGF on Synaptic Transmission

Our results demonstrate that VEGF potentiates the frequency of GABA/glycinergic mPSCs recorded from lumbar spinal MNs during a short time-window in embryonic life (E13.5–E15.5). We next aimed to determine which receptor(s) is(are) implicated in this effect. We focused our attention on E15.5 stage because synaptic activity is more pronounced at this stage than at E13.5, which allows an easier quantification of the modification induced by drug treatment. Specific neutralizing antibodies directed against murine receptors were used. We applied the antibodies in similar conditions as VEGF (i.e., 10 min). A neutralizing antibody specific for murine VEGFR1 (MF1, 50 μg/mL) was unable to change the interevent [Fig. 7(A)] and the amplitude [Fig. 7(B)] of GABA/glycinergic mPSCs (+4.438 ± 6.848%, p = 0.6848, and +1.248 ± 3.526%, p = 0.7354, respectively, n = 13). Inhibiting VEGFR2 (DC101, 50 μg/mL) also did not affect interevent or amplitude of mPSCs [+6.733 ± 8.658%, p = 0.4143 and +16.16 ± 8.361%, p = 0.1099, respectively, n = 13; Fig. 7(A,B)]. Neither interevent (+15.28 ± 14.17%, p = 0.6848, n = 13) nor amplitude [−7.159 ± 4.325%, p = 0.1465) was modulated when VEGFR3 was blocked (mF4-31C1, 50 μg/mL; Fig. 7(A,B)]. A neutralizing antibody specific for VEGF ligation site on NRP1 (NRP1b, 25 μg/mL) also did not affect the interevent and the amplitude of GABA/glycinergic mPSCs [+4.382 ± 7.602%, p = 0.5521, and +5.695 ± 4.680%, p = 0.3133, respectively, n = 16;

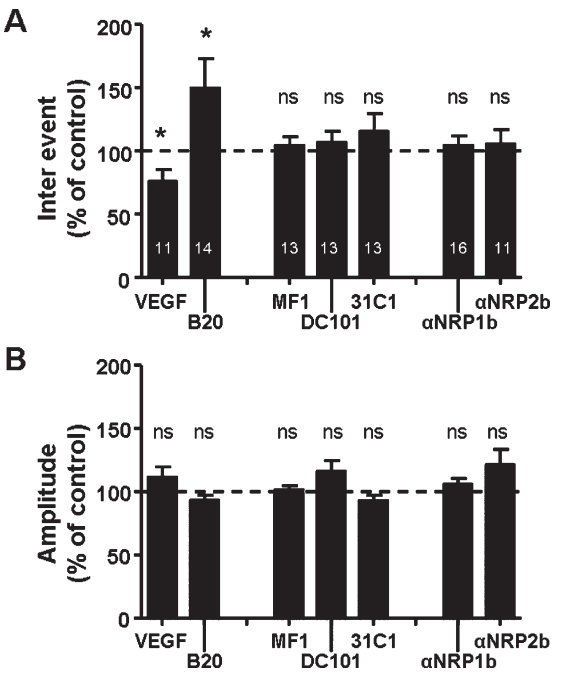

Figure 7 A Single VEGF receptor is not involved in modulating the GABA/glycinergic mPSCs at E15.5. Interevents (A) and amplitude (B) were not modified in the presence of the neutralizing antibody for VEGFR1 (MF1, 50 μg/mL, Imclone), VEGFR2 (DC101, 50 μg/mL Imclone), VEGFR3 (mF4-31C1, 50 μg/mL), NRP1 (NRP1b, 25 μg/mL, Genentech), and NRP2 (NRP2b, 25 μg/mL). * Wilcoxon paired tests, for p values see text.

Fig. 7(A,B)]. Finally, inhibiting NRP2 (NRP2b antibody, 25 μg/mL) did not alter interevent or amplitude of mPSCs [+5.207 ± 11.55%, $p = 0.7002$ and +21.03 ± 12.37%, $p = 0.0830$, respectively, $n = 8$; Fig. 7(A,B)]. Altogether, our results indicate that VEGF does not exert its action *via* a single conventional VEGF receptor (VEGFR1, VEGFR2, VEGFR3, NRP1, and NRP2). It may indicate that several VEGF receptors are involved at the same time and that receptor cooperation is necessary.

DISCUSSION

The mouse spinal cord is progressively vascularized during the second part of the embryonic life. This is in good agreement with other studies describing the ontogeny of the lumbar spinal vasculature using an antibody directed against the von Willebrand Factor (Rigato et al., 2011). Spinal cord angiogenesis is VEGF-dependent which highlights the key role of this factor during ontogeny. Because of the vicinity of the vasculature with MNs in the spinal cord during ontogeny, we thought that VEGF may regulate the development of MN activity as well. Here, we provide evidence that this is indeed the case and that MN activity is regulated by VEGF at specific developmental stages.

We detected VEGF mainly in MNs at E15.5. This observation is in agreement with a recent study reporting a large amount of VEGF mRNA and VEGF in the motoneuronal area at E11.5 (Ruiz de Almodovar et al., 2011). VEGF was also detected in GABA/glycinergic interneurons surrounding MNs. This may indicate that VEGF participates in two ways in the modulation of GABA/glycine synaptic transmission, either as VEGF from MNs or interneurons acting as paracrine or autocrine signals.

Trophic factors such as brain-derived neurotrophic factor (BDNF; Bouvier et al., 2008) and fibroblast growth factor (FGF; Terauchi et al., 2010) have been described as modulating synaptic activity during embryonic development. However, the role of VEGF has never been explored during the crucial developmental period when synaptogenesis occurs. VEGF has been described as acting on the synaptic activity in adult rat motor neurons (McCloskey et al., 2008) and hippocampal neurons (McCloskey et al., 2005). In this study, we focused on the spinal MNs from E13.5, that is, when they start receiving GABA/glycinergic synaptic activity (E13.5; Scain et al., 2010) to E17.5, that is, when GABA/glycinergic and glutamatergic synaptic events coexist. Our data demonstrate that VEGF does not modify the resting membrane potential and the input resistance of MNs. The absence of acute VEGF treatment on intrinsic membrane properties of spinal MNs is also described on adult rat hippocampal pyramidal neurons (McCloskey et al., 2005, 2008), whereas other studies report that, VEGF, briefly applied (10 min), may elicit a rise in cytosolic Ca^{2+}, likely triggering membrane depolarization in hippocampal neurons (Kim et al., 2008). In our study, if VEGF does not induce any postsynaptic changes, it enhances GABA/glycinergic mPSCs, recorded from MNs, without affecting glutamatergic ones at E15.5. This VEGF upregulating effect contrasts with downregulating effect reported in adult neurons (McCloskey et al., 2005, 2008). The selective effect of VEGF on GABA/glycinergic but not glutamatergic synaptic activity is reminiscent of the effect of FGF22 and FGF7 on glutamatergic and GABAergic differentiation of presynaptic terminals, respectively (Terauchi et al., 2010). Interestingly, the modulation of NMDA receptors activity in cerebellar granule cells by VEGF was reported before synapse formation (Meissirel et al., 2011).

We found that the spontaneous synaptic release of GABA/glycine in the vicinity of spinal MNs is increased in frequency when exogenous VEGF is applied. This is evidenced by changes in interevent of GABA/glycinergic mPSCS but not in amplitude. This indicates that VEGF acts on presynaptic terminals to release GABA and/or glycine. Blocking the endogenous VEGF with B20 leads to opposite effects. This shows that spinal network activity is continuously modulated by VEGF. This observation is important because GABA is known to play an important role as trophic factor (Lauder et al., 1998). Thus, VEGF, by modulating synaptic GABA release, could indirectly control the maturation of the spinal network. Using immunohistochemistry, we were able to detect changes in the global amount of GABA- and glycine-immunoreactivity in the presence of exogenous VEGF (10 min application), suggesting a release of these inhibitory amino acids. We can only speculate about the absence of an effect of the B20 antibody. Immunostaining coupled to standard confocal imaging may be not a sufficiently sensitive to reveal differences with the blockade of endogenous VEGF is performed. Interestingly, VIAAT-immunoreactivity detected in the vicinity of HB9+ MNs, was increased by the acute VEGF treatment implying an accumulation of GABA/glycine vesicles in terminals, in line with the increase of the GABA/glycine release by presynaptic terminals. A study also reported an accumulation of the vesicular GABA transporter in terminals contacting hippocampal pyramidal neurons maintained in primary cultures, due to BDNF released from astrocytes (Elmariah et al., 2005).

VEGF could act indirectly through blood vessels and activate VEGFR2, which then releases factor(s) that modulate the synaptic transmission. Even though short (10 min) VEGF applications are able to potentiate the synaptic activity, we cannot exclude such an indirect effect of VEGF on GABA/glycine terminal. In this study, we performed experiments with blocking antibodies for murine VEGFRs and NRPs at E15.5 because synaptic activity is more developed at this stage than at E13.5. For these experiments, we used exactly the same conditions as for VEGF and the B20 antibody. It should be noted that experiments using neutralizing antibodies were performed to inhibit a single receptor. It is possible that combinations of different receptors forming heteromeric complexes are required to modulate synaptic transmission. Indeed, VEGF receptor heteromers have been described for VEGFR2-VEGFR3 (Nilsson et al., 2010) or NRPs and VEGFRs (Vieira et al., 2010; Zachary, 2011). Another less-likely explanation would be the interaction with a new not yet identified atypical VEGF receptor that is specifically expressed in the nervous system. This would be in frame with the intriguing observation that VEGFR2 expressed in neurons was only detected with antibodies that recognize neuronal but not vessel-associated VEGFR2 (Ruiz de Almodovar et al., 2011). It would be of course important to validate the results of the antibody blockade further by other approaches such as *in vivo* gene knock-down or transgenic mice. For example, specific ablation of VEGFRs on GABA/Glycine interneurons using the VIAAT-Cre mouse crossed with the VEGFRs flox/+ mice for each VEGFR and neuropillins would be an interesting approach.

VEGF potentiates GABA/glycinergic miniature events during a short embryonic time-window (between E13.5 and E15.5). Interestingly, this effect occurs when GABA and glycine act as excitatory neurotransmitters (Delpy et al., 2008). This excitatory effect of GABA/glycine is strongly involved in the genesis of spontaneous activity occurring in the immature spinal cord until E15.5 (Branchereau et al., 2002; Yvert et al., 2004; Scain et al., 2010). Spontaneous activity plays important roles during the development of the CNS and contributes to the maturation of local neuronal networks (Moody and Bosma, 2005; Mohajerani and Cherubini, 2006; Spitzer, 2006). VEGF may also be involved in the establishment of the inhibitory synaptic transmission after E15.5 in motor networks (Delpy et al., 2008). Therefore, we may speculate that an impairment in VEGF expression or VEGF activity, during development, may lead to pathological conditions that originate from a wrong balance between the excitatory and inhibitory GABA/glycine synaptic transmission (Allain et al., 2011). Interestingly, a time-dependent modulation of the GABAergic synaptic activity by allopregnanolone, a metabolite of progesterone, has been reported at postnatal stages of development in the locus coeruleus neurons in the context of Rett syndrome (Jin et al., 2013). A presynaptic NMDA receptor modulation of GABA release onto neocortical pyramidal cells has also been reported as being present at postnatal P12-15 but not anymore at P21-25 (Mathew and Hablitz, 2011). Hence, by potentiating excitatory GABA/glycinergic synaptic events, VEGF may thus be considered as a modulator of maturation of the motor neuron network.

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REFERENCES

Allain AE, Le Corronc H, Delpy A, Cazenave W, Meyrand P, Legendre P, Branchereau P. 2011. Maturation of the GABAergic transmission in normal and pathologic motoneurons. Neural Plast 2011:905624.

Alvarez FJ, Benito-Gonzalez A, Siembab VC. 2013. Principles of interneuron development learned from Renshaw cells and the motoneuron recurrent inhibitory circuit. Ann N Y Acad Sci 1279:22–31.

Bouvier J, Autran S, Dehorter N, Katz DM, Champagnat J, Fortin G, Thoby-Brisson M. 2008. Brain-derived neurotrophic factor enhances fetal respiratory rhythm frequency in the mouse preBotzinger complex in vitro. Eur J Neurosci 28:510–520.

Branchereau P, Chapron J, Meyrand P. 2002. Descending 5-hydroxytryptamine raphe inputs repress the expression of serotonergic neurons and slow the maturation of inhibitory systems in mouse embryonic spinal cord. J Neurosci 22:2598–2606.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, et al. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439.

Cebe-Suarez S, Zehnder-Fjallman A, Ballmer-Hofer K. 2006. The role of VEGF receptors in angiogenesis; complex partnerships. Cell Mol Life Sci 63:601–615.

Chung AS, Ferrara N. 2011. Developmental and pathological angiogenesis. Annu Rev Cell Dev Biol 27:563–584.

Delpy A, Allain AE, Meyrand P, Branchereau P. 2008. NKCC1 cotransporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. J Physiol 586:1059–1075.

Dumoulin A, Rostaing P, Bedet C, Levi S, Isambert MF, Henry JP, et al. 1999. Presence of the vesicular inhibitory amino acid transporter in GABAergic and glycinergic synaptic terminal boutons. J Cell Sci 112 (Pt 6):811–823.

Elmariah SB, Oh EJ, Hughes EG, Balice-Gordon RJ. 2005. Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABAA receptors. J Neurosci 25:3638–3650.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, et al. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442.

Hogan KA, Ambler CA, Chapman DL, Bautch VL. 2004. The neural tube patterns vessels developmentally using the VEGF signaling pathway. Development 131:1503–1513.

Jin X, Zhong W, Jiang C. 2013. Time-dependent modulation of GABA(A)-ergic synaptic transmission by allopregnanolone in locus coeruleus neurons of Mecp2-null mice. Am J Physiol Cell Physiol 305:C1151–1160.

Kim BW, Choi M, Kim YS, Park H, Lee HR, Yun CO, Kim EJ, et al. 2008. Vascular endothelial growth factor (VEGF) signaling regulates hippocampal neurons by elevation of intracellular calcium and activation of calcium/calmodulin protein kinase II and mammalian target of rapamycin. Cell Signal 20:714–725.

Lauder JM, Liu J, Devaud L, Morrow AL. 1998. GABA as a trophic factor for developing monoamine neurons. Perspect Deve Neurobiol 5:247–259.

Le Bras B, Barallobre MJ, Homman-Ludiye J, Ny A, Wyns S, Tammela T, Haiko P, et al. 2006. VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. Nat Neurosci 9:340–348.

Licht T, Eavri R, Goshen I, Shlomai Y, Mizrahi A, Keshet E. 2010. VEGF is required for dendritogenesis of newly born olfactory bulb interneurons. Development 137:261–271.

Mackenzie F, Ruhrberg C. 2012. Diverse roles for VEGF-A in the nervous system. Development 139:1371–1380.

Mathew SS, Hablitz JJ. 2011. Presynaptic NMDA receptors mediate IPSC potentiation at GABAergic synapses in developing rat neocortex. PLoS One 6:e17311.

McCloskey DP, Croll SD, Scharfman HE. 2005. Depression of synaptic transmission by vascular endothelial growth factor in adult rat hippocampus and evidence for increased efficacy after chronic seizures. J Neurosci 25: 8889–8897.

McCloskey DP, Hintz TM, Scharfman HE. 2008. Modulation of vascular endothelial growth factor (VEGF) expression in motor neurons and its electrophysiological effects. Brain Res Bull 76:36–44.

Meissirel C, Ruiz de Almodovar C, Knevels E, Coulon C, Chounlamountri N, Segura I, de Rossi P, et al. 2011. VEGF modulates NMDA receptors activity in cerebellar granule cells through Src-family kinases before synapse formation. Proc Natl Acad Sci USA 108:13782–13787.

Mohajerani MH, Cherubini E. 2006. Role of giant depolarizing potentials in shaping synaptic currents in the developing hippocampus. Crit Rev Neurobiol 18:13–23.

Moody WJ, Bosma MM. 2005. Ion channel development, spontaneous activity, and activity-dependent development in nerve and muscle cells. Physiol Rev 85:883–941.

Myers CP, Lewcock JW, Hanson MG, Gosgnach S, Aimone JB, Gage FH, Lee KF, et al. 2005. Cholinergic input is required during embryonic development to mediate proper assembly of spinal locomotor circuits. Neuron 46:37–49.

Nagase T, Nagase M, Yoshimura K, Fujita T, Koshima I. 2005. Angiogenesis within the developing mouse neural tube is dependent on sonic hedgehog signaling: possible roles of motor neurons. Genes Cells 10:595–604.

Nilsson I, Bahram F, Li X, Gualandi L, Koch S, Jarvius M, Soderberg O, et al. 2010. VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. Embo J 29:1377–1388.

Rigato C, Buckinx R, Le-Corronc H, Rigo JM, Legendre P. 2011. Pattern of invasion of the embryonic mouse spinal cord by microglial cells at the time of the onset of functional neuronal networks. Glia 59:675–695.

Rosenstein JM, Krum JM, Ruhrberg C. 2010. VEGF in the nervous system. Organogenesis 6:107–114.

Ruiz de Almodovar C, Fabre PJ, Knevels E, Coulon C, Segura I, Haddick PC, Aerts L, et al. 2011. VEGF mediates commissural axon chemoattraction through its receptor Flk1. Neuron 70:966–978.

Scain AL, Le Corronc H, Allain AE, Muller E, Rigo JM, Meyrand P, Branchereau P, et al. 2010. Glycine release from radial cells modulates the spontaneous activity and its propagation during early spinal cord development. J Neurosci 30:390–403.

Sondell M, Lundborg G, Kanje M. 1999. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 19:5731–5740.

Spitzer NC. 2006. Electrical activity in early neuronal development. Nature 444:707–712.

Terauchi A, Johnson-Venkatesh EM, Toth AB, Javed D, Sutton MA, Umemori H. 2010. Distinct FGFs promote differentiation of excitatory and inhibitory synapses. Nature 465:783–787.

Vieira JM, Ruhrberg C, Schwarz Q. 2010. VEGF receptor signaling in vertebrate development. Organogenesis 6: 97–106.

Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385–397.

Yvert B, Branchereau P, Meyrand P. 2004. Multiple spontaneous rhythmic activity patterns generated by the embryonic mouse spinal cord occur within a specific developmental time window. J Neurophysiol 91:2101–2109.

Zachary I. 2005. Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. Neurosignals 14:207–221.

Zachary IC. 2011. How neuropilin-1 regulates receptor tyrosine kinase signalling: the knowns and known unknowns. Biochem Soc Trans 39:1583–1591.