VEGF was initially discovered due to its angiogenic activity and therefore named “vascular endothelial growth factor.” However, its more recently discovered neurotrophic activity may be evolutionarily more ancient. Our previous work showed that all the changes produced by axotomy on the firing activity and synaptic inputs of abducens motoneurons were completely restored after VEGF administration. Therefore, we hypothesized that the lack of VEGF delivered by retrograde transport from the periphery should also affect the physiology of otherwise intact abducens motoneurons. For VEGF retrograde blockade, we chronically applied a neutralizing VEGF antibody to the lateral rectus muscle. Recordings of extracellular single-unit activity and eye movements were made in alert cats before and after the application of the neutralizing antibody. Our data revealed that intact, noninjured abducens motoneurons retrogradely deprived of VEGF exhibited noticeable changes in their firing pattern. There is a general decrease in firing rate and a significant reduction in eye position and eye velocity sensitivity (i.e., a decrease in the tonic and phasic components of their discharge, respectively). Moreover, by means of confocal immunocytochemistry, motoneurons under VEGF blockade showed a marked reduction in the density of afferent synaptic terminals contacting with their cell bodies. Altogether, the present findings demonstrate that the lack of retrogradely delivered VEGF renders abducens motoneurons into an axotomy-like state. This indicates that VEGF is an essential retrograde factor for motoneuronal synaptic drive and discharge activity.

VEGF plays an important role as a neurotrophic factor for motoneurons. If VEGF were an essential factor for motoneurons, it would be expected that a lack in its availability should lead to physiological alterations. Different sources of VEGF availability could operate. These sources could be retrograde, anterograde, paracrine (from neural and glial cells), or autocrine. Moreover, recent experiments have revealed that the major source of VEGF for extraocular motoneurons is retrogradely derived from their target muscles (21). We decided to test the retrograde hypothesis by using the oculomotor system as our experimental model. In particular, the work was carried out in abducens motoneurons, whose axons travel through the VIth nerve to innervate the ipsilateral extraocular lateral rectus muscle. Abducens motoneurons offer several advantages: their discharge pattern is well characterized, and both their afferents and the signals they carry have been described in detail (22–25). Therefore, we aimed at reducing the availability of VEGF at the muscular level by applying a neutralizing antibody that sequesters the muscle-derived VEGF, making it unavailable for uptake and retrograde transport toward the motoneuron somata. It has been previously reported that muscle fibers are enriched with VEGF (26), and specifically we have demonstrated that the lateral rectus muscle, the target of abducens motoneurons, contains this factor (5).

We have used the alert chronic preparation to evaluate the effects of VEGF blockade in intact, nonlesioned, abducens motoneurons, which offers the advantage of allowing the study of motoneuron firing under normal physiological conditions, while simultaneously recording eye movement behavior for a prolonged period of time. The physiological study showed a marked reduction in the density of afferent synaptic terminals contacting with their cell bodies. Altogether, the present findings demonstrate that the lack of retrogradely delivered VEGF renders abducens motoneurons into an axotomy-like state. This indicates that VEGF is an essential retrograde factor for motoneuronal synaptic drive and discharge activity.

Vascular endothelial growth factor (VEGF) is well-known for its vasculogenic and angiogenic activity (1–3). In addition, this molecule also has powerful neuroprotective effects after different types of insult in the CNS, such as mechanical lesions (4–7), excitotoxic injury (8), oxidative stress (9), ischemia (10, 11), epilepsy (12, 13), and neurological diseases (14–16).

It has also been demonstrated that low levels of VEGF in mutant mice lead to muscle weakness and motoneuronal degeneration resembling the symptoms of amyotrophic lateral sclerosis (ALS) (17). VEGF also protects spinal motoneurons against excitotoxic degeneration (8, 18, 19). In human postmortem studies of ALS patients, the levels of VEGF and its receptors in spinal motoneurons are decreased (20). In addition, we have demonstrated that the firing and synaptic alterations found in axotomized abducens motoneurons are prevented and reversed after VEGF application (5). Altogether, these data indicate that VEGF plays an important role as a neurotrophic factor for motoneurons.

Significance

Vascular endothelial growth factor (VEGF) plays a critical role in motoneurons. Low levels of VEGF lead to motoneuron degeneration in mutant mice, which can be alleviated by VEGF delivery. We have recently shown that axotomy-induced alterations in abducens motoneurons recover completely after VEGF administration. Therefore, we tested, in vivo, whether retrograde VEGF blockade could affect the physiology of intact, uninjured abducens motoneurons. By means of extracellular single-unit recordings and immunocytochemistry, we have found that VEGF blocking produces a marked reduction in motoneuronal firing and eye-movement-related sensitivities, as well as a profound synaptic stripping. Altogether, our findings point to VEGF as an essential retrograde neurotrophic factor for extraocular motoneurons.

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was complemented with immunocytochemistry at the confocal level to assess likely alterations in the density of synaptic boutons impinging upon the treated motoneurons. In parallel, we also studied the firing and synaptic inputs of abducens internuclear neurons, since they lie intermingled with the motoneurons within the abducens nucleus and share common afferents, but do not receive VEGF from the periphery (22, 27–30).

Results

Behavior of Abducens Motoneurons during Spontaneous Eye Movements after Retrograde VEGF Blockade. Antidromically identified motoneurons normally show a tonic-phasic firing pattern during spontaneous eye movements. During fixations, they exhibit a stable firing rate that correlates with eye position, so that firing increases progressively as fixations stand more laterally (on-direction). For rapid eye movements or saccades, abducens motoneurons exhibit a burst of spikes of high frequency for on-directed saccades and a pause or an abrupt decay in firing for off-directed saccades (23). An example of a control (left) abducens motoneuron is shown in Fig. 1A, illustrating its phasic-tonic firing. Axotomized motoneurons show an overall reduction in firing frequency, during both fixations and saccades, as can be observed in Fig. 1B (5, 6, 24). After blocking VEGF retrograde uptake, the firing pattern of abducens motoneurons markedly changed. They showed a general decrease in firing rate that affected both the tonic and phasic components of their discharge (Fig. 1C and D). These alterations were observed throughout the study period (21 d). This is illustrated in Fig. 1C for motoneurons recorded after a short period (8 d) and in Fig. 1D for motoneurons recorded after a long period (20 d). During fixations, the slope of the regression line between firing rate and eye position represents the neuronal eye position sensitivity ($k_r$, in spikes per second per degree). Comparison of the rate-position plots for the four motoneurons in Fig. 1 A–D shows the axotomized one has a substantially lower slope than the normal one, while the slopes for the control motoneurons are also substantially decreased at both time points (Fig. 1E). The same occurred for the neuronal eye velocity sensitivity ($\tau_v$, in spikes per second per degree per second) calculated as the slope of the regression line between firing rate (after subtraction of the eye position sensitivity) and saccadic eye velocity (Fig. 1F). The representation of $k_r$ and $\tau_v$ parameters for the entire population of control recorded motoneurons ($n = 46$), and for those recorded during the period of administration of the VEGF neutralizing antibody ($n = 67$) clearly revealed a segregation between control and treated cells (Fig. 1G for $k_r$ and Fig. 1H for $\tau_v$), with control motoneurons showing higher $k_r$ and $\tau_v$ than treated cells.

Abducens internuclear neurons, cells found within the abducens nucleus, were also recorded before and during the muscle treatment. They are intermingled with the motoneurons in the abducens nucleus, their firing pattern is similar to that of motoneurons (24, 31), and they share common afferents (22, 27–30). They were recorded to check whether anti-VEGF antibody treatment could produce any effects on neighboring cells. The discharge of abducens internuclear neurons in control and during VEGF antibody administration were not different, as can be observed by comparing the behavior of the two cells shown in Fig. 1I (control) and Fig. 1J (VEGF antibody administration). The neuronal eye position ($k_e$) and velocity ($\tau_v$) sensitivities of these two abducens internuclear neurons displayed similar values (Fig. 1K and L, respectively). For the whole population of recorded abducens internuclear neurons ($n = 11$ in control and 12 during treatment) the rate-position plots (Fig. 1M) and rate-velocity plots (Fig. 1N) overlapped, in contrast to the segregation observed between control and treated motoneurons (Fig. 1G and H, respectively).

Quantitative Analysis of Discharge Characteristics. We first grouped the motoneurons recorded throughout the treatment period into three pools, depending on whether they were recorded during the first ($n = 19$), the second ($n = 29$), or the third week ($n = 19$) of the anti-VEGF antibody administration. Then, a statistical comparison of neuronal eye position ($k_r$) and velocity ($\tau_v$) sensitivities was made between these three groups and controls. There was no significant difference in mean $k_r$ between the data corresponding to the three different weeks, but these three groups all showed significantly lower $k_r$ values than control [one-way ANOVA followed by Holm–Sidak method for all pairwise multiple comparisons, $F_{3, 169} = 7.545; P < 0.001$ for comparison between control and the second or third week, $P = 0.002$ for comparison control-first week; Cohen’s $d = 0.978$ (Fig. 2A)]. Similar findings were obtained for the comparison of $r_v$ between the four groups: that is, no difference in the mean $r_v$ between each of the three different week groups, but each of these three groups showed significantly lower $r_v$ values than control population [one-way ANOVA followed by Holm–Sidak method, $F_{3, 169} = 8.339; P < 0.001$ for comparisons control-second and control-third week and $P = 0.008$ for comparison control-first week; Cohen’s $d = 1.075$ (Fig. 2B)]. We further studied the decay of $k_r$ and $r_v$ during the first week by grouping the data at 2, 4, and 6 d after the first dose of the VEGF antibody. Results indicated no differences in mean $k_r$ [one-way ANOVA, $F_{2, 16} = 1.509$, $P = 0.251$] or median $r_v$ (Kruskal–Wallis one-way ANOVA on ranks, $H = 1.042$, $P = 0.594$) at the three data points selected. Since no difference was found either in $k_r$ or $r_v$ throughout the study period, we proceeded to pool the data obtained during the three experimental weeks into a single group.

Consequently, we next compared $k_r$ and $r_v$ values between the populations of control motoneurons ($n = 46$) and motoneurons recorded during the administration of the anti-VEGF antibody ($n = 67$). To check whether the observed reduction in $k_r$ and $r_v$ after treatment was similar to axotomy ($n = 57$), we also included in the statistical comparison the axotomy data from our previous studies, corresponding to recordings during a time interval of 21 d postaxotomy (5, 6). Results are illustrated in Fig. 2C (for $k_r$) and F (for $r_v$). Mean $k_r$ ± SEM was 5.51 ± 0.28 for control, 4.08 ± 0.12 for axotomy (a 26% decrease), and 4.09 ± 0.15 for the treated group (a 25.8% decrease) (in spikes per second per degree). Mean $k_r$ was similar for the axotomized and treated motoneurons and both groups showed a significantly lower $k_r$ than control motoneurons [one-way ANOVA test followed by the Holm–Sidak method, $F_{2, 167} = 18.067$, $P < 0.001$ for both control-axotomy and control-antibody treatment comparisons; Cohen’s $d = 1.041$] (Fig. 2C). The same happened for $r_v$. Axotomy and antibody treatment groups yielded similar mean $r_v$ values (0.60 ± 0.03 and 0.57 ± 0.02 spikes per second per degree, respectively), whereas each of these two groups showed significantly lower $r_v$ values (15.5% and 19.7% decrease, respectively) than control (0.71 ± 0.03 spikes per second per degree), as can be observed in Fig. 2F [one-way ANOVA test followed by Holm–Sidak method, $F_{2, 167} = 7.667; P < 0.001$ for control-antibody treatment and $P = 0.005$ for control-axotomy comparisons; Cohen’s $d = 0.735$]. The other two parameters analyzed during fixations were $F_0$
Fig. 1. Effects of anti-VEGF neutralizing antibody treatment on the spontaneous eye movement-related discharge characteristics of abducens neurons. (A–D) Firing rate of a control (A), an axotomized (B), and two treated motoneurons (Mn) recorded 8 d (C) and 20 d (D) after the first application of the antibody (VEGF ab) during spontaneous eye movements. Traces illustrate (left) horizontal EP (in degrees) and FR (in spikes per second) of (left) abducens units. The double arrow in A indicates leftward (L) and rightward (R) eye movements. (E) Linear regression lines between FR and EP for the four neurons illustrated in A–D. The slope of these lines represents the neuronal eye position sensitivity (ks, in spikes per second per degree). (F) Linear regression lines between FR minus the position component (FR − F0 − ks × EP) versus eye velocity (EV). The slope of these lines corresponds to the neuronal eye velocity component (rs, in spikes per second per degree per second). (G and H) Same as E and F, respectively, but for the whole population of abducens motoneurons recorded in control and during treatment. (I and J) Firing rate of two abducens internuclear neurons (Int) recorded in control (I) and during treatment (J). (K and L) Regression lines of FR versus EP (K) or EV (L) of the two internuclear neurons illustrated in I and J following the same procedure as for the motoneurons in E and F. (M and N) Rate-position (M) and rate-velocity (N) plots for the whole population of abducens internuclear neurons recorded in control and during the antibody administration period.
Fig. 2. Analysis of the discharge activity of abducens motoneurons and internuclear neurons during spontaneous eye movements, in controls and after the administration of VEGF neutralizing antibody (VEGF ab). (A and B) Neuronal eye sensitivity to eye position (in A: ks, in spikes per second per degree) and eye velocity (in B: rs, in spikes per second per degree per second) in control motoneurons (Mns) and motoneurons treated during the first week (w), second week, and third week after the application of the first dose of the antibody (ab). Bars illustrate mean ± SEM. No significant differences were found between each of the three 1-wk periods. However, data from each of the 3 wk were significantly different (* in A and B) from control data (for ks in A: P < 0.001 for control-week 2 and control-week 3, and P = 0.002 for control-week 1 comparisons; for rs in B: P < 0.001 for control-week 2, P = 0.001 for control-week 3, and P = 0.008 for control-week 1 comparisons). For A and B, the one-way ANOVA test followed by Holm–Sidak method was used and n = 46, 19, 29, and 19 motoneurons for control, first, second, and third weeks, respectively. (C–F) Bar charts illustrating the mean ± SEM of ks (in C), F0 (in D), threshold (Th, in E), and rs (in F) for the three populations of motoneurons (Ax, axotomy; C, control; and VEGF ab) and during the 3 wk of administration of the VEGF antibody, VEGF ab). Axotomy and treated motoneurons showed similar ks and rs values, but both groups showed ks and rs values (C and F, respectively) significantly lower than controls (* P < 0.001, one-way ANOVA followed by Holm–Sidak method). F0 showed significantly lower values in the VEGF antibody group as compared with control and axotomy groups (* P < 0.01, one-way ANOVA followed by Holm–Sidak method; in D). There was absence of significant differences in threshold between the three groups (one-way ANOVA; in E). (G–J) Comparison of ks, rs, F0, and threshold for abducens internuclear neurons (Ints) between the control (n = 11) and treatment (n = 12) situations. No significant differences were found in any of these parameters (Mann–Whitney rank sum test). Quantitative data are represented with whisker box plots indicating the median and Q1, Q3 quartiles.
(the firing rate at straight-ahead gaze) and eye position threshold. For $F_0$, treated motoneurons showed a significantly lower mean value than axotomy and control (one-way ANOVA test followed by Holm–Sidak method, $F_{(2,167)} = 4.780; P = 0.011$ for control-antibody treatment and $P = 0.008$ for axotomy-antibody treatment comparisons; Cohen’s $d = 0.49$). These values were $F_0 = 21.16 \pm 5.27$, 20.93 $\pm$ 2.56, and 7.77 $\pm$ 3.24 spikes per second for control, axotomy, and treated motoneurons, respectively (Fig. 2D). Threshold for recruitment in the three groups of motoneurons demonstrated no significant difference between them (one-way ANOVA test, $F_{(2,167)} = 2.562, P = 0.08$). Mean threshold for the three motoneuron groups were $-5.23 \pm 1.15$, $-5.36 \pm 0.67$, and $-2.93 \pm 0.86$ degrees, respectively (Fig. 2E).

Abducens internuclear neurons recorded during the period of administration of the VEGF antibody showed no significant changes as compared with controls in any of the parameters analyzed in motoneurons. Thus, when the statistical comparison for abducens internuclear neurons between control and treated cells was made by means of Mann–Whitney rank sum test for $k_v$ (Fig. 2G), $r_v$ (Fig. 2H), $F_0$ (Fig. 2I), and recruitment threshold (Fig. 2J), in all cases $P > 0.05$.

**Discharge Activity during Vestibular Eye Movements.** For vestibularly induced eye movements, the firing rates of abducens motoneurons were analyzed during the slow phases of this reflex. It has been previously described that abducens motoneurons show sensitivity for both eye position and eye velocity during the slow phases of the vestibulo-ocular reflex, as can be observed in the discharge activity of the control motoneuron illustrated in Fig. 3A. As previously reported, axotomy dramatically diminished the discharge activity of abducens motoneurons during vestibularly induced eye movements (Fig. 3B) (5, 6, 32). In contrast to spontaneous eye movements, when unlesioned abducens motoneurons were provided with the VEGF neutralizing antibody, their behavior during vestibular eye movements was similar to that of control motoneurons (Fig. 3C). Neuronal eye position ($k_v$) and eye velocity ($r_v$) during vestibular eye movements were calculated by multiple regression fitting during the slow phases.

For the whole population of abducens motoneurons recorded during vestibular eye movements, we compared $k_v$ and $r_v$ between motoneurons of the control ($n = 16$) and the antibody treatment ($n = 17$) periods, and also included in this comparison the data from axotomized motoneurons ($n = 13$) taken from our previous publications (5, 6). For $k_v$, the statistical comparison revealed a significantly lower $k_v$ value for the axotomized population than for the control and treated pools of motoneuron ($P = 0.009$), whereas there was absence of significant difference

**Fig. 3.** Discharge characteristics of abducens motoneurons during vestibularly induced eye movements before and after the administration of the VEGF antibody. (A–C) FR of a control (A), an axotomized (B), and a treated (C) motoneuron recorded 12 d after the onset of treatment during vestibular eye movements. EP stands for (left) eye position in the horizontal plane. The double arrow in A indicates leftward (L) and rightward (R) eye movements. Recordings were carried out in the left abducens nucleus. (D and E) Comparison between neuronal eye position sensitivity ($k_v$) and neuronal eye velocity sensitivity ($r_v$) during vestibular eye movements, respectively, between control motoneurons (C, $n = 16$), axotomized motoneurons (Ax, $n = 13$), and motoneurons recorded during the VEGF antibody administration period (VEGF ab, $n = 17$). A significant difference was found in $k_v$ (D) and $r_v$ (E) between the axotomy group, as compared with both the control and treated groups (Kruskal–Wallis one-way ANOVA on ranks followed by Dunn’s method; for $k_v$, $P = 0.009$; for $r_v$, $P = 0.006$), as indicated by the asterisks. However, there were no significant differences between the control and the antibody administration situations with regard to both $k_v$ and $r_v$. 

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between the two latter groups (Kruskal–Wallis one-way ANOVA on ranks, \(H = 9.500\); Cohen’s \(d = 0.919\) (Fig. 3D). Similar findings were obtained for \(r_c\): that is, axotomized motoneurons showed a significantly lower \(r_c\) in comparison with control and antibody-treated cells (\(P = 0.006\)), which, in turn, showed similarity between them (Kruskal–Wallis one-way ANOVA on ranks, \(H = 10.299\); Cohen’s \(d = 0.978\) (Fig. 3E)).

**Synaptic and Astroglial Coverage of Treated Abducens Motoneurons.** To determine whether the low firing and sensitivities of motoneurons recorded during the administration of the VEGF neutralizing antibody could correlate with a loss in afferent synaptic endings contacting motoneurons, we performed double immunofluorescence at the confocal level against synaptophysin, a general marker of synaptic boutons, and ChAT to label motoneurons. Motoneurons from treated animals (21 d after the onset of the antibody administration) generally appeared to have a lower density of synaptophysin-immunoreactive boutons in apposition to their cell bodies (Fig. 4C) than control motoneurons (Fig. 4A). However, axotomized motoneurons (21 d postaxotomy) showed a massive loss of synaptic boutons contacting with their perikarya (Fig. 4B). For this reason, we compared the three situations: control (\(n = 84\) motoneurons), axotomy (\(n = 54\)), and antibody treatment (\(n = 94\)). The results are illustrated in Fig. 4D. The one-way ANOVA test followed by the Holm–Sidak method revealed that the three groups showed statistical differences between them in all pairwise comparisons performed (Fig. 4D) \(F(2, 229) = 98.159, P < 0.001;\) Cohen’s \(d = 2.419\). Thus, axotomized motoneurons presented the lowest synaptic coverage around their somatic perimeter (measured as the percent of the perimeter contacted by synaptophysin-positive boutons; \(18.12 \pm 0.97\%\)), as compared with both control and treated motoneurons. In turn, treated motoneurons presented a significantly \((P < 0.001)\) lower synaptic coverage \((35.30 \pm 1.11\%)\) than control motoneurons \((41.99 \pm 1.13\%)\). With respect to the controls, the decrease in mean somatic synaptic coverage was of \(56.9\%\) in axotomized motoneurons and of \(15.9\%\) in treated motoneurons. Therefore, motoneurons of the anti-VEGF antibody situation showed a synaptic coverage intermediate between control and axotomy motoneurons. This means that synaptic stripping took place, but at a lower magnitude than in axotomy.

In association with synaptic stripping, there is an increase in glial profiles surrounding axotomized motoneurons (33). In the present experiments, we observed that treated motoneurons appeared with a higher density of GFAP-immunoreactive profiles around their somata (labeled by ChAT) (Fig. 5C) than control motoneurons (Fig. 5A). By comparison, axotomized motoneurons (Fig. 5B) were characterized by the vast number of GFAP+ profiles surrounding their cell bodies. A comparison of GFAP somatic coverage (percent of the somatic perimeter surrounded by GFAP+ profiles) was made between treated (\(n = 84\)), axotomized (\(n = 55\)), and control (\(n = 80\)) motoneurons. Results were similar to those found for synaptic coverage. Thus, there were significant differences in all pairwise comparisons between the three groups [one-way ANOVA test followed by Holm–Sidak method; \(F(2, 216) = 66.837, P < 0.001;\) Cohen’s \(d = 2.012\) (Fig. 5D). Axotomized motoneurons showed the highest percentage of GFAP+ profiles in apposition to their cell bodies.

**Fig. 4.** Synaptic coverage of abducens neurons after anti-VEGF antibody treatment. (A) Confocal microscopy image showing synaptophysin (SYN)-immunoreactive boutons in apposition to the cell bodies of control abducens motoneurons identified by ChAT labeling. (B and C) Same as A, but for axotomized (B) and VEGF antibody-treated motoneurons after the 21-d study period (C). (Scale bars, 20 μm in C, for A–C) (D) Bar charts comparing synaptic coverage around the soma of abducens motoneurons (Mns) in the three situations: control (\(C, n = 84\) motoneurons), axotomy (Ax, \(n = 54\)), and after the anti-VEGF antibody treatment (VEGF ab, \(n = 94\)). The asterisk indicates significant differences with control (\(*P < 0.001\) ). The hashtag indicates significant difference between axotomy and VEGF antibody groups (\(\#P < 0.001\) ). One-way ANOVA test followed by Holm–Sidak method. (E and F) Confocal microscopy images showing synaptophysin-labeled boutons around the cell bodies of abducens internuclear neurons, identified as calretinin (CR)-immunoreactive, in control (E, CR pseudocolored in light blue) and 21 d after VEGF antibody treatment (F). (Scale bars, 20 μm in F, for E and F) (G) Comparison of synaptic coverage around the soma of abducens internuclear neurons (Ints) in control (\(C, n = 44\)) and after VEGF antibody treatment (VEGF ab, \(n = 37\)) by means of the Student’s \(t\) test. No significant differences were found between the two groups. Data in D and G show mean ± SEM.
The present results show that blockade of retrograde VEGF signaling following the administration of VEGF neutralizing antibody into the lateral rectus muscle renders abducens motoneurons into an operating mode that in some ways resembles the axotomy state. Thus, their discharge activity during fixations and spontaneous eye movements exhibited an overall decrease in firing rate, with a significant drop in neuronal eye position and velocity sensitivities, except during vestibular eye movements. In congruence with the physiological data, the immunocytochemical study revealed a concomitant loss of synaptic terminals. Altogether, the present data indicate that VEGF is an essential factor required by abducens motoneurons to display normal phasic-tonic firing behavior related to spontaneous eye movements and to preserve their synaptic inputs.

**Discussion**

The present results show that blockade of retrograde VEGF signaling following the administration of VEGF neutralizing antibody into the lateral rectus muscle renders abducens motoneurons into an operating mode that in some ways resembles the axotomy state. Thus, their discharge activity during fixations and spontaneous eye movements exhibited an overall decrease in firing rate, with a significant drop in neuronal eye position and velocity sensitivities, except during vestibular eye movements. In congruence with the physiological data, the immunocytochemical study revealed a concomitant loss of synaptic terminals. Altogether, the present data indicate that VEGF is an essential factor required by abducens motoneurons to display normal phasic-tonic firing behavior related to spontaneous eye movements and to preserve their synaptic inputs.

**VEGF and Motoneuronal Firing Pattern.** After the reduced availability in retrogradely delivered VEGF arising from the muscle due to the anti-VEGF antibody treatment, notable alterations in motoneuronal firing patterns were observed during the study period (21 d). Thus, abducens motoneurons exhibited an overall reduction in firing frequency that affected both the tonic and phasic components of their discharge (Fig. 6). Motoneuronal eye position and velocity sensitivities during spontaneous eye movements fell to values that were significantly lower than in controls and were similar to those found in axotomy.

The signals displayed by abducens motoneurons are provided primarily by three sources of afferents (reviewed in ref. 22). First, the tonic discharge is mainly due to input from prepositus hypoglossi neurons (25, 30). Second, reticular excitatory and inhibitory burst neurons are the principal inputs responsible for the motoneuronal phasic discharge during on- and off-directed eye movements.
saccades, respectively (28, 35, 36). And third, vestibular signals are channeled mostly through neurons located in the medial vestibular nucleus (25, 29, 37, 38). Therefore, the loss of eye-related signals in treated motoneurons during spontaneous eye movements was likely the result of a lower efficacy in synaptic transmission from prepositus and reticular neurons, in agreement with this, our immunocytochemical results showed significantly reduced synaptic coverage on motoneuron cell bodies, as compared to controls. Vestibular signals driving abducens motoneurons were not affected by the antibody treatment. Similarly, in our previous work (5), the inhibition of the VEGF receptor 2, which is the main receptor mediating the neurotrophic action of VEGF (19), altered spontaneous, but not vestibular, signals in motoneuron firing. This indicates that the vestibular input onto abducens motoneurons is likely to depend on a different neurotrophic factor. This is not unlikely, in view of previous findings for our model, indicating that different neurotrophic factors may act on different synaptic afferents. In particular, in axotomized abducens motoneurons, the exogenous administration of brain-derived neurotrophic factor (BDNF) rescues only the eye position signal (mainly coming from the premotor prepositus hypoglossi neurons), whereas neurotrophin-3 (NT-3) treatment selectively recovers the eye velocity signal (derived from afferent reticular burst neurons) (39). Not surprisingly, the neurotrophic hypothesis of synaptic connections relies upon the versatility of the signaling routes and the diversity of trophic factors, so no trophic factor is universal within the brain or within a particular circuit.

Interestingly, the behavior of abducens motoneurons, deprived of their retrograde source of VEGF, resembled that of axotomy, even though they were unlesioned. Thus, axotomized motoneurons also show a general reduction in firing frequency and a significant decrease in eye position and velocity sensitivities (5, 32, 39, 40). These results are in agreement with our previous findings showing that exogenous VEGF rescues the firing of axotomized abducens motoneurons, highlighting the relevance of this neurotrophic factor for the normal function of these motoneurons (5, 6). In contrast, we have shown previously that axotomized abducens motoneurons supplied with BDNF, NT-3, or nerve growth factor recover only partially from the axotomy-induced firing alterations (39, 40). Altogether these findings reinforce the crucial role of VEGF as a retrograde neurotrophic factor for oculomotor motoneurons. It should be pointed out that this in vivo study, in a behaving animal, demonstrating the relevance of VEGF as a neurotrophic factor is unique. To our knowledge, only one previous study, performed in hippocampal slices, has shown the effects of VEGF blockade. It demonstrated impairment in synaptic plasticity and reductions in dendritic spine number and length (41).

**VEGF and Motoneuronal Degeneration.** Low levels of VEGF in mutant mice (VEGF<sup>−/−</sup>) lead to muscle weakness and spinal motoneuronal degeneration that resembles the neuropathology of ALS (17). Moreover, when mutant mice that overexpress VEGF are crossed with a well-known model of murine ALS (SOD1), the double transgenic animals show a lower degree of motoneuronal degeneration than the single transgenic SOD1 mutant mice (42). Similar results have been found when the mutant mice SOD1 are treated with VEGF using viral vectors, demonstrating the ability of VEGF to prevent, at least in part, spinal motoneuronal death (43, 44). Other authors have proven that the intrathecal delivery of VEGF rescues degenerating motoneurons from cell death induced by excitotoxicity (8, 18). All of those findings demonstrate that VEGF is a powerful neuroprotective agent. In the same line, our data indicating that motoneurons deprived of retrograde VEGF, by reducing the availability of this trophic factor, lose synaptic inputs and resemble an axotomy-like state, reinforce the role of VEGF as a relevant neurotrophic factor for motoneurons. It is important to clarify that the neurotrophic effects of VEGF are due to a direct action on the neurons themselves, and not to its angiogenic activity, as previously described in various lesion models, including axotomized extraocular motoneurons (6, 8, 43, 45–47). It should also be considered that our methodology does not ensure a full blockade of the muscle production of VEGF, but likely a reduction in its availability. However, this method renders effects that were equivalent to those found by a plain axotomy. Devastating effects on motoneurons are also obtained by solely reducing the levels of VEGF in genetic murine models (17).

**Loss of Synapses and Astrocytic Reaction Induced by VEGF Neutralizing Antibody.** We found a significant decrease in synaptic boutons in apposition to motoneuron cell bodies in the affected side in comparison to the control side. These results indicated that retrograde VEGF is required by abducens motoneurons to maintain their normal synaptic coverage and those losses are on congruence with our physiological data (Fig. 6). However, synaptic removal was not as intense as after axotomy, which could also correlate with our findings of a normal discharge activity during vestibular eye movements. For example, vestibular inputs were retained and therefore treated motoneurons showed an intermediate value in synaptic coverage between control and axotomy. Synaptic stripping is a general feature of axotomized neurons (5, 32, 33, 48–52). Target-derived factors

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**Fig. 6.** Schematic diagram summarizing the major results of the present work. (A) Graphic representation of a control motoneuron contacting with its target muscle and receiving appropriate levels of retrograde VEGF. The motoneuron displayed a normal tonic-phasic firing, illustrated as single-unit extracellularly recorded action potentials (AP), that increases for on-directed fixations and saccades (EP, eye position). Moreover, it received a normal density of synaptic boutons detected by synaptophysin immunoreactivity (SYN<sup>+</sup>), as well as a low GFAP<sup>+</sup> astrocytic coverage (GFAP<sup>+</sup>). (B) In contrast, motoneurons treated with the anti-VEGF antibody (VEGF ab) lacked the retrograde source of VEGF from the muscle and showed a reduced firing of AP with regard to EP, and a smaller burst of spikes during on-directed saccades. Motoneurons with retrograde VEGF blockade also suffered synaptic stripping as presented a reduced density in synaptophysin-positive boutons contacting their soma. In parallel, GFAP-immunoreactive profiles were more abundant around their cell body than in control.
seem to be required for the maintenance of afferent synapses onto the parent neurons, since either target reinnervation or the administration of neurotrophic factors produce recovery of the synaptic loss (5, 6, 39, 40, 53, 54). Altogether, we conclude that the reduction in the somatic synaptic coverage of abducens motoneurons found in the present work was due to the lack of the target-derived VEGF caused by the neutralizing antibody.

Astrogliosis was also observed around motoneurons treated with the VEGF antibody, even though these neurons were not axotomized. Thus, the density of GFAP-immunoreactive processes surrounding the perikarya of treated motoneurons was significantly higher than that of control. Astrogliosis was also present in the neuropil, but it was not analyzed because it contains dendrites of both types of abducens neurons. The hypertrophy of astrocytes and the concomitant rise in GFAP is a typical response around the soma of axotomized neurons, and there is evidence suggesting that astrocytic processes play a role in the shedding of synapses from axotomized motoneurons (33, 55–57). In our experiments, the increase in GFAP linear density around axotomized motoneurons was not as powerful as in axotomy, giving an intermediate value significantly different from both control and axotomized motoneurons. We suggest two rationales for these data. First, the maintenance of vestibular inputs would lead to a lesser astrocytic reaction around motoneuron cell bodies. Second, the axotomy damages the neuron and prevents all muscle produced trophic factors from accessing its soma, so it is more severe than VEGF antibody treatment.

The selective retraction of certain synapses on the motoneurons (e.g., those driving spontaneous fixations and saccades), but the maintenance of others (like those derived from vestibular inputs) is a remarkable finding of the present work. Likely, VEGF triggers a molecular cascade that involves signaling between the pre- and the postsynaptic element, which is disrupted by the VEGF neutralizing antibody only at certain synapses. The synaptotrophic effect of VEGF has only been reported, to the best of our knowledge, by our previous work (5, 6). However, at present, the molecular pathways initiated by VEGF have been partly elucidated in neuroprotection studies and involve the activation of PI3-K and the inhibition of p38MAPK (8, 44). Moreover, intercellular signaling pathways are also likely to occur between the pre- or postsynaptic element and the glia cells since VEGF abolishes the astrocytic reaction in several models of motoneuron insult (5, 6, 8, 44, 58). The interaction between motoneurons and glia also extends to microglial cells, since VEGF is able to attenuate the expression of classically activated toxic (M1) microglial cells and enhance the expression of alternatively activated neuroprotective (M2) microglial cells (44). The molecular mechanisms of this motoneuron–glia communication are not fully understood, but elements of the classic complement cascade are involved in microglial activation and also in the modulation, but not initiation, of bouton removal (reviewed in ref. 33). On the other hand, astrocytes activated through the JAK/STAT3 pathway prevent reinnervation when the regenerating neuron does not contact its target. Signals such as ephrin up-regulate in axotomized motoneurons and activate their cognate tyrosin kinase receptors coupled to the JAK/STAT3 pathway in astrocytes that hypertrophy in response (59).

An alternative mechanism that could mediate the synaptic remodeling found in the present work might be that VEGF is transported and secreted from the motoneurons onto presynaptic nerve terminals containing the receptors for VEGF. Thereafter, the VEGF-receptor complex could be internalized by endocytosis, leading to transducing activity at the presynaptic level that would maintain the structure and function of the afferent synapses. This mechanism has been described for several neurotrophic factors, especially for BDNF, a neurotrophin associated with synaptic plasticity (60, 61). If this assumption holds, then the treatment with VEGF antibody could block this mechanism, leading to synaptic withdrawal of VEGF-dependent presynaptic inputs. Other cellular partners of the abducens nucleus, the abducens internuclear neurons, although sharing common synaptic inputs with the motoneurons, were not exposed to the VEGF neutralizing antibody, and therefore, this could explain why synapses were not retracted from these cells and why they did not show any astroglial reaction around their cell bodies.

Abducens Internuclear Neurons Are Not Affected by VEGF Blockade. A striking finding of the present study was that firing alterations and synaptic withdrawal occurred selectively in abducens motoneurons. Thus, neighboring abducens internuclear neurons showed a normal discharge pattern and a normal density of synaptic terminals despite receiving the same synaptic inputs as motoneurons (22, 27, 28, 30) and likely sharing common afferent fibers (29). This selective retraction of presynaptic boutons could imply that a single afferent axon that branches into the abducens nucleus would retract only those terminals contacting the treated motoneurons, while those that contact abducens internuclear neurons would remain attached. Consequently, synaptic shedding appears to be a fairly selective and controlled process.

In conclusion, retrograde VEGF blockade produced firing and synaptic alterations in abducens motoneurons that resemble the axotomy state and are consistent with a crucial role of this neurotrophic factor for the normal operating mode of these motoneurons. As stated above, VEGF also plays an important role in preventing the degeneration of spinal motoneurons. Altogether, we propose that VEGF is an essential neurotrophic factor for motoneurons.

Materials and Methods

Animals and Surgical Procedures. Experiments were performed on adult female cats weighing 2.0 to 2.5 kg obtained from authorized suppliers (Universidad de Córdoba, Spain). All procedures were performed in accordance with the guidelines of the European Union (2010/63/EU) and the Spanish legislation (R.D. 53/2013, BOE 34/11370-421) for the use and care of laboratory animals and were approved by the institutional ethics committee. All efforts were made to minimize the number of animals used and their suffering during experiments.

A total of six female cats were used for the present work. Three of them were prepared for chronic electrophysiological recordings and the other three for the morphological study. Axotomy data were obtained from our previous works (5, 6). Cats planned for recordings were prepared as previously described (5, 6, 40). Briefly, after the injection of atropine sulfate (0.5 mg/kg, intramuscularly) to reduce vagal reflexes, animals were anesthetized with ketamine hydrochloride (15 mg/kg, intramuscularly), mixed with xylazine (1 mg/kg, intramuscularly), and placed in a stereotaxic frame. During surgery, animals were implanted with stimulating electrodes, scleral coils, and the recording chamber. Two bipolar stimulating electrodes were implanted intracranially, in the left and right Vth nerves (at their exit from the brainstem) for the antidromic activation of abducens motoneurons. Coils, made up of two turns of Teflon-insulated, stainless-steel wire, were implanted in the sclera of both eyes for the recording of eye movements. For access to the brainstem for recordings, a square window (5 × 5 mm) was drilled in the occipital bone. A restraining system was also constructed to immobilize the head during the recordings. Pre- and postoperative care was provided daily, as needed.

Chronic Extracellular Recording. Recordings started after a postoperative period of 10 d. The animal was gently restrained inside a fabric bag, wrapped with elastic bandages, and placed in a Perspex box (with its head immobilized),
which was located inside the magnetic field for eye movement recordings (62). Single-unit extracellular recordings were carried out with glass micropipettes filled with 2 M NaCl, and fixed to a three-axis micromanipulator, which descended through the intact cerebellum to reach the brainstem. The abducens nucleus was located by recording the antidromic field potential induced following the electrical stimulation to the VIIth nerve. When a motoneuron was isolated, we systematically performed a collision test to ensure that the recorded orthodromic unit was indeed the one that was antidromically activated from the nerve. Once identified, we proceeded to record the extracellular action potentials of the motoneurons during the performance of spontaneous eye movements under alert conditions. In some cells, it was also possible to record the motoneuron discharge during vestibular eye movements. Vestibular stimulation in the horizontal plane was achieved by a servo-controlled motor attached to a turntable. Some of the units that were recorded in the abducens nucleus (identified by its antidromic field potential) were not antidromically activated from the VIIth nerve. These correspond to the so-called abducens internuclear neurons, which lie intermingled with the motoneurons within the same nucleus (31). Neuronal activity was amplified and filtered for display and digitization purposes.

Data Storage and Analysis. Neuronal activity along with the horizontal eye position of both eyes were digitally stored for off-line analysis (Power 1401, Cambridge Electronic Design). We used computer programs written in Matlab 7.5 for selecting the data of instantaneous firing frequency (calculated as the reciprocal of the interspike intervals) recorded simultaneously with the corresponding horizontal position and velocity of both eyes.

Firing rates (FR) of abducens motoneurons (and internuclear neurons) correlate with both eye position (EP) and eye velocity (EV) according to the equation $F_R = F_0 + k_{EP} \times r + r_{EV}$, where $k$ and $r$ represent the position and velocity neuronal sensitivities, respectively, and $F_0$ is the firing rate at straight-ahead gaze (i.e., when $EP = 0^\circ$) (23, 63).

During eye fixations, since $EV = 0$, the former equation can be expressed as $F_R = F_0 + k_{EP} \times r$, and thus motoneuron FR can be fitted to eye position by a linear regression line, whose slope represents the neuronal eye position sensitivity during spontaneous fixations, named $k_E$ (in spikes per second per degree). During spontaneous rapid eye movements, or saccades, we calculated $r_E$ (neuronal eye velocity sensitivity during spontaneous eye movements, in spikes per second per degree per second) by linear regression analysis after subtraction of the position component ($k_{EP} \times r_0$) obtained from the previously known sensitivity to EP. Thus, the equation used was $F_R = k_{EP} \times r + r_0 \times EV$.

During vestibular stimulation, the equation used was $F_R = F_0 + k_{EP} \times r + r_{EV}$, and the neuronal sensitivities $k_E$ (in spikes per second per degree) and $r_E$ (in spikes per second per degree per second) were obtained by multiple linear regression analysis, selecting only the slow phases of the nystagmus.

Administration of VEGF Neutralizing Antibody. To block the retrograde signaling of VEGF to abducens motoneurons, a neutralizing antibody (monoclonal mouse IgG2b, clone # 26503, R&D Systems) was applied unilaterally to the lateral rectus muscle. A dose of $10^5$ neutralizing antibody. Recording was restarted at 2 d after the intramuscular implantation of the tube. A dose of $10^5$ neutralizing antibody prepared in $500 \mu l$ of saline followed by the fixative consisting in 4% paraformaldehyde prepared in 0.1 M sodium phosphate buffer, pH 7.4 (2 L). The brainstem was removed and cut into coronal sections on a vibrotome to a thickness of 50 $\mu m$.

The synaptic morphological study was carried out in abducens motoneurons and in abducens internuclear neurons somata (uniquely identified by the corresponding immunohistochemical marker) to check the specificity of the neutralizing antibody treatment on the motoneurons, due to the fact that both cell types have a similar firing pattern and receive the same afferents (24, 31). The neuropil was not analyzed because it contains the dendrites of both motoneurons and abducens internuclear neurons. Abducens internuclear neurons were specifically immunostained with calretinin, a marker that does not label the motoneurons (34).

Sections were first washed in PBS containing 0.1% Triton X-100 (PBS/TX) and then blocked with normal donkey serum (1:10 in PBS/TX). Washes were carried out when needed using PBS/TX, and in all cases the blocking solution was prepared with 10% normal donkey serum in PBS-TX. The primary antibodies used were the following: 1) goat polyclonal antibody against ChaT (1:200; Millipore) as a marker of motoneurons; 2) rabbit polyclonal antibody against calretinin (1:1,000; Swant) to specifically label abducens internuclear neurons (34); 3) mouse monoclonal antibody against synaptophysin (1:1,000; Millipore) as a marker of synaptic boutons; and 4) mouse monoclonal antibody against GFAP (to label astrocytes and their processes; 1:1,000; Sigma-Aldrich). Double immunofluorescence was carried out to combine ChaT or calretinin with either synaptophysin or GFAP labeling.

Secondary antibodies (Jackson Immunoresearch), were used at 1:50 in PBS/TX and were the following: 1) donkey anti-goat IgG coupled to TRITC, for ChaT or calretinin detection; and 2) donkey anti-mouse IgG coupled to FITC to reveal synaptophysin or GFAP. Finally, sections were rinsed in PBS, mounted on glass slides, and coverslipped with Dako fluorescence mounting medium (Dako).

Confocal microscopy (Zeiss LSM 7 DUO) was used to capture images by means of different filters using the Zeiss microscope software ZEN. Gray scales were adjusted to expand the maximum dynamic range of the image. Images were then analyzed using the program ImageJ (NIH); as previously described (5, 6, 39, 40). Briefly, we measured synaptic coverage of abducens motoneurons and internuclear neurons (i.e., linear density) as the percent of the total somatic perimeter that was contacted by synaptophysin-positive boutons (in percent). The same procedure was used to measure astrocytic processes surrounding the cell body.

Statistics. Comparisons between groups were carried out by the Student’s t test, one-way ANOVA test, or the Mann–Whitney rank sum test, at a level of significance of $P < 0.05$ by means of the statistic program SigmaPlot v11 (Systat Software). When a comparison resulted significant, we indicated the size of the effect by the Cohen’s d. Data are expressed as mean ± SEM. All regression analysis used in the present study were significant ($P < 0.05$).

Data Availability. Data are available at the Open Science Framework (OSF), https://osf.io/pm4t6/ (65). All study data are included in the main text. Previously published data were used for this work (6).

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