Nerve growth factor regulates axial rotation during early stages of chick embryo development

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Nerve growth factor (NGF) was discovered because of its neurotrophic actions on sympathetic and sensory neurons in the developing chicken embryo. NGF was subsequently found to influence and regulate the function of many neuronal and non neuronal cells in adult organisms. Little is known, however, about the possible actions of NGF during early embryonic stages. However, mRNAs encoding for NGF and its receptors TrkA and p75^{NTR} are expressed at very early stages of avian embryo development, before the nervous system is formed. The question, therefore, arises as to what might be the functions of NGF in early chicken embryo development, before its well-established actions on the developing sympathetic and sensory neurons. To investigate possible roles of NGF in the earliest stages of development, stage HH 11–12 chicken embryos were injected with an anti-NGF antibody (mAb α D11) that binds mature NGF with high affinity. Treatment with anti-NGF, but not with a control antibody, led to a dose-dependent inversion of the direction of axial rotation. This effect of altered rotation after anti NGF injection was associated with an increased cell death in somites. Concurrently, a microarray mRNA expression analysis revealed that NGF neutralization affects the expression of genes linked to the regulation of development or cell proliferation. These results reveal a role for NGF in early chicken embryo development and, in particular, in the regulation of somite survival and axial rotation, a crucial developmental process linked to left–right asymmetry specification.

neurotrophins | rotatin | csk | furin | proNGF

Nerve growth factor (NGF) was discovered for its “vital role[...] in the life of its target cells” (1), namely sympathetic and sensory neurons, during embryonic development (2). NGF was subsequently found to influence the function of neuronal and non neuronal cells throughout adulthood (3). NGF is first translated as a prepro-protein (4) and then processed into the mature form that exerts its biological activities through its signaling receptors TrkA and/or p75^{NTR} (5–7).

During chicken embryo development, NGF is required for the development and maintenance of specific populations of peripheral sympathetic and sensory neurons that start responding to NGF at embryonic stage 20 and 33–40 respectively, according to the Hamburger–Hamilton (HH) classification (8). However, in chicken embryos mRNAs for NGF, p75^{NTR} and TrkA are already expressed well before sympathetic or sensory neurons are specified. NGF mRNA expression was reported initially at HH 3–5 (9), reaching a peak at HH 33–34 (10). p75^{NTR} mRNA expression was found in chicken embryos already at HH 10 (11), whereas TrkA mRNA expression could be first visualized by in situ hybridization at HH 15 (11); when analyzed by RT PCR, it was found as early as HH 1 (9). The majority of available expression data for NGF, TrkA, and p75^{NTR} in the chicken embryo were obtained at the mRNA level; however, at the protein level, very few data are available for NGF and p75^{NTR} (12, 13) and no data are available for TrkA.

The striking disparity between the temporal expression pattern of the mRNAs encoding for the NGF ligand–receptor system and the embryonic stage at which the sympathetic and sensory target neurons are generated and start responding to NGF prompted the question whether NGF might display some previously unrecognized activity during earlier chicken embryo developmental stages. To ad-

dress this question, HH 11–12 chicken embryos were injected in ovo with anti-NGF antibody.

This work was first introduced by R.L.-M. at the International NGF 2008 Meeting (14).

Results

Early Embryonic Expression of NGF, proNGF, TrkA, and p75^{NTR} Proteins. Because the majority of available expression data for NGF, p75^{NTR}, and TrkA in the chicken embryo were obtained at the mRNA level, we first characterized their expression at the protein level.

Analysis of total NGF (mature NGF plus proNGF) by ELISA showed that they are present already at HH 19–20 (10 ng/g embryo extract) and increase to 200 ng/g embryo by HH 30–31 (Fig. 1*J*). To obtain spatial information, an immunohistochemical analysis, therefore, was undertaken.

At HH 3, anti-NGF antibody recognizing both NGF and proNGF stained cells throughout the area pellucida and the initial primitive streak (Fig. 1*A*), whereas at HH 4, NGF-immunoreactive cells congregated around the primitive groove, the primitive pit and Hensen's node (Fig. 1*B* and *E*). A similar staining pattern also persisted at later stages (HH 5–7) around the notochord, Hensen's node (Fig. 1*C* and *D*), and the head fold (Fig. 1*F*). A few immunoreactive cells persisted at the caudal part of the primitive streak until HH 8 (Fig. 1*H* and *I*), but at HH 7 and 8, more intense staining appeared in somites (Fig. 1*D*, *G*, and *I*), mostly in the region of the prospective dermomyotome (especially where the myotome will take place), consistently with the NGF mRNA expression described at later stages (11).

Nothing is known about the expression and distribution of unprocessed proNGF protein in the chicken embryo. Anti-proNGF-specific immunohistochemistry revealed a significant number of proNGF-immunoreactive cells, between HH 4 and 7 (Fig. 2*A–D*, *F*, and *G*), although their number was lower than that of total NGF-immunoreactive cells. These proNGF-immunoreactive cells, surrounded by a diffuse specific immunoreactivity, were identified around the cranial portion of the primitive groove and in correspondence to the Hensen's node (Fig. 2*A–D*, *F*, and *G*). At HH 8, proNGF immunoreactive cells were identified mainly in the lateral part of the head fold (Fig. 2*E* and *I*), whereas a diffuse immunoreactivity was observed at the level of somites, with a significant staining of their midline as well (Fig. 2*E* and *H*). Thus, NGF and proNGF proteins are expressed at early stages of chicken embryo development, well before the onset of NGF known actions on developing sensory and sympathetic neurons. The NGF expression data were consistent with those of a previous study, showing its expression at HH 3–4 (12). The proNGF

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Fig. 1. A–I and L–R, Whole-mount immunohistochemistry to detect total NGF (A–I) and TrkA (L–R) in chicken embryos at different stages (HH) of development. A–D, I, and K–N, Low magnification. E–H and O–R, High magnification focusing on Hensen's node (arrow), the head (asterisk), or somites (S). J, ELISA to detect total NGF in chicken embryos from 3 to 7 d of development. Inset in J, Scheme of the assay. K, Control embryo.

distribution appears not to overlap fully with that of mature NGF, such as in the prospective dermomyotome.

p75^{NTR}-immunoreactive cells were found as early as HH 3–4, widely distributed in the area pellucida (Fig. 2K, L, and P). At HH 7+ and 8, p75^{NTR}-immunoreactive cells were mainly found in correspondence to the lateral part of the head fold (Fig. 2M, N, Q, and S) and in correspondence to the caudal part of the embryonic body (Fig. 2M). A marked p75^{NTR} immunoreactivity was also observed in correspondence to somites (Fig. 2M, N, R, and T).

TrkA-immunoreactive cells and TrkA diffuse immunoreactivity were observed from HH 4–6 alongside the anterior part of the primitive groove and around Hensen's node (Fig. 1L, M, and O). At HH 7 and 8 (Fig. 1N), TrkA-immunoreactive cells were identified mainly in the lateral part of the head fold (HH 8) (Fig. 1N and R), some alongside the caudal part of the embryonic body (Fig. 1P), and a diffuse intense immunoreactivity was observed in the prospective dermomyotome in the somites (Fig. 1N and Q) that appears different and more intense than that observed for p75^{NTR}, showing that the two NGF receptors are not always coexpressed in these non neuronal cells.

No NGF, proNGF, p75^{NTR} and TrkA immunoreactivity was observed in embryos when the primary, secondary antibody or both were omitted (Figs. 1K and 2J and O).

Neutralizing NGF Activity with Anti-NGF Antibody Determines an Altered Axial Rotation. The early expression of NGF and its receptors suggested a potential developmental function in the

Fig. 2. Whole-mount immunohistochemistry to detect proNGF (A–I) and p75^{NTR} (K–N and P–T) in chicken embryos at different stages (HH) of development. A–E and K–O, Low magnification. F–J and P–T, High magnification focusing on Hensen's node (arrow), the head (asterisk), or somites (S). J and O, Control embryos.

chicken embryo. To address this question, HH 11–12 chicken embryos were injected in ovo with the anti-NGF monoclonal antibody (mAb) α D11 (15–17). This antibody has a much higher affinity for mature NGF than for unprocessed proNGF and specifically neutralizes NGF activity in vitro and in vivo by preventing binding to both TrkA and p75^{NTR} receptors. Different amounts of α D11 were injected in a 1- μ L volume (0.1, 1, and 5.5 μ g), and embryos were fixed 24 or 48 h after the injection. Control embryos were injected with saline or with equivalent doses of a control antibody [mAb 9E10 directed against c-myc (18)]. Embryo sections were stained with hematoxylin and eosin for morphological analysis. By 24 h after the injection, we found no effect on the gross overall morphology, organ morphology, or position or in left–right (L–R) asymmetry. On the other hand, a visible alteration in the whole embryo morphology was found in embryos fixed 48 h after anti-NGF injection, which showed a different orientation of the caudal part of the body with respect to the midline body axis. This was suggestive of a defect in the axial rotation of the embryo. In normal conditions (Fig. S1), from HH 12–13 onwards, the head of the chicken embryo begins to rotate such that it comes to lie on its left side. At this stage, the trunk has not yet turned and still lies on top of the yolk sac with its ventral side facing downward, but gradually the rotation progresses along the body until, at approximately HH 20, the entire embryo has rotated (8, 19). Thus, when viewed from the dorsal side, normal embryos rotate toward their right side. The extent of rotation was determined as described in *Materials and Methods*. The results showed that 48 h after the injection, the extent of rotation, as determined by measurement of the distal angle (Fig. 3A), was similar in chicken embryos injected with saline ($n = 19$; Fig. 3B and I), with 9E10 [$n = 11$, 10 and 14, respectively, for the doses of 0.1 μ g (Fig. 3C and I), 1 μ g (Fig. 3E and I), and 5.5 μ g (Fig. 3G and I)], and with α D11 at the doses of 0.1 μ g ($n = 11$; Fig. 3D and I) and 5.5 μ g ($n = 18$; Fig. 3H and I). In sharp contrast, at the 1- μ g dose (Fig. 3F and I), α D11 induced a striking change in the direction of rotation toward the left side of the body axis ($n = 4$; $P = 0.007$).

A second measure of axial rotation was performed more proximally, in sections where the notochord is linear (Fig. 3J). We found that the extent of rotation was similar in chicken embryos injected with saline ($n = 19$; Fig. 3K and R), with 9E10 [$n = 11$, 10 and 14, respectively, for the doses of 0.1 μ g (Fig. 3L

Fig. 3. Altered axial rotation in embryos injected with anti-NGF antibody. (A and J) drawings of frontal whole horizontal histological section of the chicken embryo, showing the distal (A) and proximal angle (J), calculated at the point of intersection between the midsagittal plane of the body (blue line) and the line symmetrically dividing the posterior portion of the neural tube (green line for embryos injected with 9E10 or saline; red line for embryos injected with α D11). (B–I and K–R) sections showing the distal (B–I) or proximal (K–R) angle 48 h after the injection of saline (B and K), 9E10 at the doses of 0.1 μ g (C and L), 1 μ g (E and M), 5.5 μ g (G and O) and α D11 at the same doses (respectively D, M, H, O, J, and P). Although altered, the difference in rotation is not statistically significant in the distal angle (I), but there is a statistically significant altered rotation in the proximal angle (R). Letters in the graphs correspond to the pictures.

and R), 1 μ g (Fig. 3 N and R), and 5.5 μ g (Fig. 3 P and R)], and with α D11 at the dose of 5.5 μ g ($n = 18$; Fig. 3 Q and R). On the contrary, at the doses of 0.1 μ g ($n = 11$; $P > 0.05$; Fig. 3 M and R) and 1 μ g ($n = 4$; $P = 0.0008$; Fig. 3 O and R), α D11 inverted the direction of the rotation toward the left side of the body axis.

Thus, the injection of α D11 provoked a dose-dependent (in the range between 0.1 and 1 μ g) alteration of axial rotation, and all embryos injected with 1 μ g of anti-NGF antibody showed an axial rotation in the opposite direction with respect to the normal rotation. Of note, the rotation defect was not observed in embryos injected with the higher dose of anti-NGF (5.5 μ g).

Axial rotation has been linked to neural tube and somitic development (20–22), and morphological and functional alterations of the notochord have been observed in mice mutants that fail to turn. Global L–R patterning requires insulation of the left and right sides by an intact notochord (23) and buffering signals that maintain the bilateral symmetry of somites (24). A morphological comparison of the notochord of control and anti-NGF-injected embryos showed no difference. On the other hand, 48 h after the injection of 1 μ g of α D11, we found an increased cell death at level of somites, in the proximal and distal region of the trunk (Fig. 4A and Fig. S2). Thus, the altered rotation induced by anti-NGF antibody might be linked to a regulation of cell death in the somitic compartment.

Early Neutralization of NGF Affects the Expression of Development-Related and Proliferation Genes. As a first step toward the elucidation of the mechanisms responsible for the altered rotation induced by anti-NGF injection in the chicken embryo, we looked by real-time PCR at the expression of candidate genes, likely involved in the process. In chicken embryos, studies to investigate defects in axial rotation have not been previously reported and, consequently, the mechanisms responsible for this phenomenon are unknown. On the other hand, in the mouse embryo, defects characterized by a lack of rotation occur in the natural “no turning” mutant (25) and in experimentally induced genetic knockout mutants for the *Csk* (26, 27), *rotatin* (28, 29), and *furin* (30, 31) genes. Real-time PCR shows that *Csk* mRNA expression is down-regulated 30 min after injection (Fig. 4B), when the rotation phenotype is not yet evident, whereas it is up-regulated 48 h after the injection. *Rotatin* is specifically required for axial rotation in mice (28) but has not been studied in avian embryos. Real-time PCR showed that *rotatin* is indeed expressed in chicken embryos and that NGF neutralization reduced its mRNA levels by 67% after 30 min. By contrast, similarly to *Csk*, a 60% increase of *rotatin* mRNA was seen after 48 h (Fig. 4C). For *furin*, real-time PCR shows that 30 min after anti-NGF injection, its

expression is only slightly down-regulated, with respect to control embryos, to become slightly up-regulated 48 h after the injection (Fig. 4D). Thus, *Csk*, *rotatin* and (to a lesser extent) *furin* mRNAs all display a biphasic regulation by anti-NGF, with an early down-regulation before the onset of the rotation defect.

To further investigate a mechanistic link between the anti-NGF injection and the observed rotation defect, we performed an unbiased microarray analysis of differential mRNA expression 48 h (HH 18) after the injection of 1 μ g of anti-NGF (time and dose at which the rotation phenotype is more evident). Noteworthy, only relatively few mRNAs are differentially regulated in anti-NGF-injected embryos with respect to controls (Table 1), possibly also because expression changes induced by anti-NGF antibody in a limited portion of embryonic tissue were diluted in the RNA sample from the total embryo. Thus, these may represent the subset of mRNAs subjected to the greatest regulation by anti-NGF. *Rotatin* and *furin* mRNAs did not appear in the microarray analysis, whereas *Csk* is up-regulated at this time point, as shown independently by real-time PCR. Table 1 shows the complete unselected list of the mRNA species selectively up- or down-regulated (in red and in green, respectively) in anti-NGF embryos. Among these, *CDCA1* (involved in cell cycle regulation), *AQP5*, *MALL*, and *PLAGL1* (also involved in regulation of cell proliferation) mRNAs show the greatest difference between anti-NGF- and control-injected embryos. Interestingly, a significant proportion of the anti-NGF-regulated mRNA species are either linked to developmental processes or to cell cycle regulation and proliferation. For example, *CDCA1*, *CDERMO-1*, *CCND2*, *CUBN*, *WNT11*, *S100A11*, *RGS2*, *CSK*, and *PLAGL1* are known to be involved in processes of proliferation or cell movements during embryonic development. Some of these have been previously shown to be regulated by Sonic hedgehog (Shh) and to be involved in notochord and somite differentiation or development. Altogether this analysis shows a remarkably consistent picture, identifying a small number of mRNAs as candidate targets for regulation by NGF during early phases of chicken embryo development, the dysregulation of which might contribute to the defect in the axial rotation process. It is noteworthy that the endothelin receptor B (*Ednrb*) mRNA is down-regulated in anti-NGF-injected embryos (Table 1), since mouse mutants *Ednrb* s-1Acr, with a large chromosomal deletion, centered around the *Ednrb* gene show abnormal or incomplete embryonic turning (32).

The direction of axial rotation in vertebrates is specified by global L–R-patterning cues. However, mRNA encoding such L–R determinants, including *Shh*, *lefty*, *nodal* and *Pitx2* were strikingly absent in the list of anti-NGF regulated genes (Table 1). This, together with the observed absence of gross visceral topographical anomalies

Fig. 4. NGF influence on the developing somites. (A) Different extent of cell death in the myomer, in horizontal sections from embryos fixed 48 h after the injection of 1 μ g of 9E10 or α D11 and noninjected embryos (N.I.), at the level of the distal and proximal angle. (B–D) Real-time PCR for CSK (B), *RTTN* (C), and *FURIN* (D), 30 min (white columns) and 48 h (grey columns) after injecting 1 μ g of α D11 or 9E10 or saline. *Inset*, Differential expression of mRNAs in embryos injected with α D11 with respect to 9E10. Values are in logarithmic scale. Error bars are computed according to the standard error of the mean and error propagation. (E) Diagram modified from Faisst et al. (28), showing the postulated role of NGF/proNGF signalling in the pathways involved in axial rotation. Accordingly, NGF might influence the developing somites, modulating gene expression and their survival/death, regulating axial rotation. [Figure 4E: Reprinted from Mechanisms of Development, 113, Anja M. Faisst, Gonzalo Alvarez-Bolado, Dieter Treichel, Peter Gruss, *Rotatinis* a novel gene required for axial rotation and left-right specification in mouse embryos. 15–23. Copyright (2002) with permission from Elsevier.]

or asymmetries, or defective heart looping, suggests that the signaling pathways for L-R specification and axial rotation separate into distinct regulatory cascades. Our results suggest a model whereby endogenous NGF might act downstream of Shh actions on somite cells and would regulate steps downstream of the separation of these pathways (Fig. 4E). NGF itself is regulated by Shh (11), which could maintain NGF expression and the ensuing p75^{NTR} and TrkA signaling and somite cell death in a physiological window (Fig. 4E). Whether alterations in the expression of any of the identified genes contributes, downstream of NGF, to the perturbed axial rotation in anti-NGF-injected embryos remains to be determined.

Discussion

This study first confirmed the early embryonic expression of NGF, proNGF, TrkA, and p75^{NTR} at the protein level, already at the time of gastrulation, mainly in correspondence to the Hensen's node and somites, well before the NGF target neurons and the nervous system are formed. This suggests the possible involvement of NGF in developmental processes, such as neural tube formation, L-R patterning, and axial rotation, responsible for the shaping of body plan and of the topographical relationships between the various organs.

To investigate possible roles of NGF in the earliest stages of development, HH 11–12 chicken embryos were injected with a high-affinity anti-NGF antibody (mAb α D11), preferentially binding mature NGF with respect to unprocessed proNGF and the effects of NGF blockade were investigated 48 h later (HH 18). The injection of α D11 provoked a dose-dependent (in the range between 0.1 and 1 μ g) alteration of axial rotation and an increase in cell death at the level of

somites that still persisted 48 h after the injection. The high specificity of mAb α D11 for NGF (15, 16) with respect to other neurotrophins ensures the NGF dependence of the phenotype observed.

Little is known about the molecular mechanisms underlying the axial rotation process. In mouse embryos, the neural tube and the somites have been proposed to be involved in turning or axial rotation. The neural tube grows faster than the underlying endodermal structures (20, 21), and differential mitotic rates occur between cells at each side of the neural tube (22), suggesting that a regulation of cell proliferation, migration, or survival might underlie the process of embryonic turning. The localization of NGF and its receptors in somites provides a direct link to these structures with the process of axial rotation.

Absence of axial rotation was observed in mice in which the *rotatin* gene, highly expressed in neural tube, somites, and notochord, was knocked out (28, 29). Although the *rotatin* gene has been isolated in chicken (GeneID no. 421023, GenBank), its expression or function has not been previously studied in this species. In this study, we showed that *rotatin* mRNA is expressed in early stages of chicken embryonic development and that it is rapidly and transiently affected by NGF neutralization, suggesting that if it plays a role in the axial rotation defect induced by anti-NGF, it does so only early after the injection.

Embryonic turning in mice involves a regulatory circuit comprising the Csk protein (26, 27). Csk, together with its tyrosine kinase ligands Src, Fyn, and Yes, is also implicated in gastrulation movements (33). Csk-deficient mouse embryos are developmentally arrested at the 10–12 somite stage, exhibiting impaired formation of neural tube and a failure of embryonic

Gene symbol	9E10	9E10	α D11	α D11	$\log_2 \alpha$ D11/9E10
<i>CDCA1</i>	9,64	11,90	0,50	0,49	-4,46
<i>EDNRB</i>	1,21	1,08	0,44	0,75	-0,94
<i>AKAP7</i>	1,00	0,93	0,44	0,79	-0,65
<i>CDERMO-1</i>	1,01	1,25	0,61	0,55	-0,97
<i>CCND2</i>	0,83	0,91	0,60	0,59	-0,55
<i>CUBN</i>	1,03	1,44	0,56	0,75	-0,92
<i>WNT11</i>	1,10	1,14	0,56	0,77	-0,75
<i>S100A11</i>	0,84	0,90	0,65	0,67	-0,41
<i>GSTK1</i>	0,85	0,97	0,65	0,70	-0,44
<i>AQP5</i>	1,53	1,62	0,60	0,77	-1,20
<i>MALL</i>	1,41	1,85	0,64	0,76	-1,22
<i>RGS2</i>	0,99	0,93	0,66	0,84	-0,36
<i>PPA2</i>	0,92	1,04	1,32	1,73	0,64
<i>KCNAB2</i>	0,91	1,07	1,70	1,36	0,62
<i>CSK</i>	1,44	1,87	2,18	2,59	0,53
<i>PLAGL1</i>	1,17	1,17	2,32	2,46	1,03
<i>MRPL39</i>	1,39	1,59	2,28	2,55	0,69
<i>SNF8</i>	2,03	0,88	4,04	4,82	1,60

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the different treatments, embryos were first checked for the absence of general gross abnormalities (such as spina bifida), the presence of which would make them excluded. Then, using a 2× objective and the image analysis program NIS (Nikon), the angle formed by the intersection between the midsagittal plane of the body (blue line in Fig. 3 A and J) and the line symmetrically dividing the posterior portion of the neural tube (red or green line in Fig. 3 A and J) was measured. The criterion was chosen to consider as negative those angles directed to the left of the body axis of the embryo.

Measurements were performed at two levels to minimize variability attributable to possible artifacts in the histological preparation. Further details are provided in *SI Materials and Methods*.

RNA Isolation. RNA was isolated from chick embryos 30 min and 48 h after the injection of 1 µg of αD11 or 9E10 as described in *SI Material and Methods*.

Microarray. A whole-genome microarray analysis (Agilent platform) was performed on RNA extracted from embryos injected with 1 µg of αD11 or

9E10 and from noninjected reference embryos, following the two-color protocol as described in *SI Materials and Methods*.

Only genes that were either up-regulated (fold change, >1.25) or down-regulated [fold change (fc) in the interval $0.00 < fc < 1/1.25$] for each group (αD11 and 9E10) and that were differentially expressed between the two groups were considered.

Real-Time PCR. Real-Time PCR for CSK, RTTN, and FURIN was performed as described in *SI Material and Methods* in chick embryos 30 min and 48 h after the injection of 1 µg of αD11 or 9E10.

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