

N-cadherin regulates beta-catenin signal and its misexpression perturbs commissural axon projection in the developing chicken spinal cord

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Abstract N-cadherin is a calcium-sensitive cell adhesion molecule that plays an important role in the formation of the neural circuit and the development of the nervous system. In the present study, we investigated the function of N-cadherin in cell–cell connection in vitro with HEK293T cells, and in commissural axon projections in the developing chicken spinal cord using in ovo electroporation. Cell–cell connections increased with N-cadherin overexpression in HEK293T cells, while cell contacts disappeared after co-transfection with an N-cadherin-shRNA plasmid. The knockdown of N-cadherin caused the accumulation of β -catenin in the nucleus, supporting the notion that N-cadherin regulates β -catenin signaling in vitro. Furthermore, N-cadherin misexpression perturbed commissural axon projections in the spinal cord. The overexpression of N-cadherin reduced the number of axons that projected alongside the contralateral margin of the floor plate, and formed intermediate longitudinal commissural axons. In contrast, the knockdown of N-cadherin

perturbed commissural axon projections significantly, affecting the projections alongside the contralateral margin of the floor plate, but did not affect intermediate longitudinal commissural axons. Taken together, these findings suggest that N-cadherin regulates commissural axon projections in the developing chicken spinal cord.

Keywords N-cadherin · Cell connection · Beta-catenin signaling · Axon projection · In vivo electroporation

Introduction

Cell–cell adhesion, which provides structural connections between cells, is essential for morphological changes and rearrangement of cells (Harris and Tepass 2010; Baum and Georgiou 2011). Many molecules, including cadherin, catenin, and integrin, play an important role in cell–cell adhesion (Pieters and van Roy 2014). Cadherins are a

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family of transmembrane proteins that mediate calcium-dependent homophilic cell–cell contacts (Flannery and Bruses 2012). In humans, the cadherin superfamily of adhesion molecules consists of more than 100 members (Lammens et al. 2012). These molecules play a crucial role in embryonic morphogenesis by affecting cell shape, differentiation, migration, and proliferation (Tepass et al. 2000; Sotomayor et al. 2014).

Cadherins consists of an extracellular, transmembrane, and cytoplasmic component. The extracellular component consists of several cadherin repeats (EC) of approximately 110 amino acids, while the cytoplasmic component of cadherin links to a multitude of other molecules, such as the p120 catenin, β -catenin, and α -catenin, which are possible regulators of cadherin function (Derycke and Bracke 2004). The four cadherin sub-families conserved between *Caenorhabditis elegans*, *Drosophila*, and humans are classic cadherins, fat-like cadherins, seven-pass transmembrane cadherins, and protocadherins (Wang et al. 2014). Classic cadherins can be classified as type I and type II (Takeichi 1995; Lin et al. 2014). Vertebrate type I classic cadherins include epithelial cadherin, placental cadherin, neural cadherin, and retinal-cadherin.

N-cadherin is a type I cadherin that is localized at adheren junctions (Hatta and Takeichi 1986). It mediates calcium sensitive homotypic binding between apposed cell membranes, and regulates cytoskeletal dynamics through interactions with p120 catenin, β -catenin, and α -catenin (Hatta et al. 1988; Nollet et al. 2000; Derycke and Bracke 2004; Gumbiner 2005; Pokutta and Weis 2007; Ouyang et al. 2013). β -catenin can regulate the cadherin/catenin complex by linking the complete complex to the actin network via α -catenin (Derycke and Bracke 2004). Although the canonical function of N-cadherin is to establish cell–cell adhesion, the presence of N-cadherin also induces pro-migratory signaling (Cui and Yamada 2013). In addition to its adhesive function during embryonic development, N-cadherin plays an important role in spine morphology, synaptic remodeling, synapse stabilization, plasticity, and long-term potentiation (Bozdagi et al. 2000; Togashi et al. 2002; Okamura et al. 2004; Jungling et al. 2006; Tai et al. 2007; Arikath 2010; Mendez et al. 2010).

N-cadherin is also crucial for the migration of the neural cells during early embryonic development, and plays an important role in the development of the cortex (Gartner et al. 2012). A recent report shows the effects N-cadherin has on commissural projections (Sakai et al. 2012). Commissural axonal projections are present at all axial levels, and span the midline of the central nervous system (CNS) integrating information from both sides of the body (Sandoval-Minero and Varela-Echavarría 2008). The

establishment of these commissural projections, which starts soon after neural tube closure, is one of the earliest events in the wiring of the CNS (Colamarino and Tessier-Lavigne 1995). However, the role N-cadherin plays in commissural axon projections is still unknown.

In the present study, we used *in vivo* electroporation in combination with shRNA to specifically knockdown or overexpress N-cadherin, in the developing chicken spinal cord, to examine its role in axonal growth. Furthermore, we also used human embryonic kidney (HEK) 293T cells to identify the role of N-cadherin *in vitro*.

Materials and methods

Embryo and tissue preparation

Fertilized eggs of Sea blue brownhad, obtained from a local farm were incubated (HWS-150, JingHong, China) at 37.8 °C and 65 % humidity. The Hamburger and Hamilton (1992) system was used to stage the embryos. Embryos were studied at stage 18 (E3) to stage 29 (E6), with at least three embryos at each stage. Depending on its size, embryos were fixed in 4 % formaldehyde solution for 6–24 h for *in situ* hybridization and immunohistochemistry. After fixation, the tissue was immersed in 18 % sucrose solution, embedded in Tissue-Tec O.C.T. compound (Sakura Finetek, USA), frozen in liquid nitrogen, and stored at -80 °C until required. Samples were sectioned on a cryotome (Leica 1850, Germany) and the 20- μ m thick sections were mounted on Poly-L-lysine coated slides.

Cell culture

HEK293T cells were cultured in low-glucose Dulbecco's Modified Eagles Medium (DMEM; Gibco, USA) supplemented with 10 % fetal bovine serum (FBS; Hyclone, USA). Cells were trypsinized and replated in 12-well-plastic tissue culture plates and transfected with pCAGGS-GFP (Redies laboratory gift) 24 h later, which expresses green fluorescent protein and contains the CAG promoter that contains a cytomegalovirus (CMV) immediate early enhancer promoter and a chicken β -actin promoter. Cells were transfected with pCAGGS-N-Cadherin (Redies laboratory gift), pCAGGS-N-Cadherin and pGPU6-GFP-neo-N-Cadherin-shRNA vectors (ABGENT, China), pCAGGS-N-Cadherin, and pGPU6-GFP-neo vectors (ABGENT, China), using lipofectamine 2000 transfection reagent (Invitrogen, USA). Cells were collected 48 h after transfection, and analyzed using immunohistochemistry and western blot.

Immunohistochemistry

For immunohistochemistry, cultured cells were placed on ice, washed with ice-cold phosphate buffered saline (PBS) three times, fixed with 4 % paraformaldehyde in PBS for 30 min at 25 °C. Following another PBS wash, the blocking solution (2 % sheep serum, 4 % bovine serum albumin, 0.3 % Triton X-100, and 0.1 % sodium azide in Tris-buffered saline, TBS, sheep serum and bovine serum albumin; Beijing Dingguo co. LTD, China) was applied to cells or tissue sections, for 1 h at room temperature. The primary antibodies were then applied overnight at 4 °C. The primary antibodies used in the present study were rabbit anti chicken N-Cadherin (1:300 dilution), mouse anti α -catenin monoclonal antibody (ZSGB-BIO, China; 1:100 dilution), and mouse anti β -catenin monoclonal antibody (ZSGB-BIO, China; 1:100 dilution). Next, the appropriate goat-anti-rabbit Cy3-labeled (Jackson Immuno Research, Europe Ltd) or FITC-labeled (ZSGB-BIO, China; 1:100 dilution) secondary antibody against species IgG was applied for 2 h at 25 °C. A similar process was employed for double staining. Finally, DAPI (4',6-diamidino-2-phenylindole, DAPI, Roche, Germany) was used to stain all cell nuclei. A fluorescence microscope (Nikon ECLIPSE 80i, Japan), equipped with a digital camera (LEICA DFC300FX, Germany), was used to visualize and photograph fluorescence.

Western blot analysis

For Western blot analysis, cells grown in plastic tissue culture dishes were placed on ice, rinsed with ice cold PBS, and lysed with RIPA Buffer (50 mM Tris, 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS; Beyotime, China) through pipetting and sonication on ice. Cell lysates were centrifuged at 18,000g at 4 °C. The supernatants were transferred to a clean tube and protein concentration was determined using a Nanodrop 2000 (Thermo Scientific, USA). Equal amounts of total proteins from each sample were separated by 10 % SDS-PAGE, and proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Beyotime, China). A blocking solution (2 % sheep serum, 4 % bovine serum albumin, 0.3 % Triton X-100, and 0.1 % sodium azide in Tris-buffered saline, TBS), which inhibits unspecific binding sites, was applied for 30 min at 25 °C. The PVDF membranes were immunoblotted with primary antibodies overnight at 4 °C, washed, and incubated with secondary antibodies for 1 h at room temperature. After several washes with TBS, ECL reagent (Beyotime, China) and exposure to X-rays (Kodak Company, China) were used to develop the membranes. A rabbit anti-chicken N-Cadherin primary antibody (Redies laboratory gift, Germany, 1:500)

was used. All secondary antibodies were HRP-conjugated (Beyotime, China).

In ovo electroporation

The N-Cadherin shRNA plasmid was synthesized by ABGENT, China. The target sequences were GCAAATGAAGGTGAAGCTAAC, GCACATGCAGTGGATGTAAAT, and GGTAGCATGTTGACAACCTTTC, which were inserted in a pGPU6/GFP/Neo shRNA plasmid. All used plasmids were extracted with a kit (Cwbio, China) and diluted in water.

The in ovo electroporation protocol was modified from (Luo et al. 2006; Lin et al. 2016; Yang et al. 2015). A stereomicroscope was used to assist with all the steps. In brief, fertilized eggs were incubated until stage 18 (E3); then, 3–4 ml of albumin was removed from the egg without disrupting the yolk. Further, the shell was cut carefully with a pair of curved scissors to get one 1–2-cm diameter window without touching the embryo. The plasmid, which contained 2 $\mu\text{g}/\mu\text{l}$ of pCAGGS-N-Cadherin, pGPU6-GFP-neo, or pGPU6-GFP-neo-N-Cadherin-shRNA, and 0.25 $\mu\text{g}/\mu\text{l}$ of pCAGGS-GFP plasmid, pCAGGS-GFP plasmid, or pCAGGS-GFP plasmid, respectively, and Fast Green dye (0.01 %), were injected and loaded into the neural tube lumen with a mouth pipette until the dye filled the entire space. The electrodes were then immediately placed on both sides of the embryonic neural tube in parallel. A total of six 18-volt pulses, which lasted for 60 ms, with a pause of 100 ms between each pulse, were performed. Bubbles near the electrodes indicated a successful technique. After electroporation (CUY-21, Nepa Gene, Japan), the electrodes were carefully removed and the egg was sealed with tape. The operated eggs were then placed back in the incubator until they reached the desired stage for sample collection, fixation, and analysis.

Spinal cord open-book preparation

The spinal cord open-book preparation was performed according to the protocol outlined by Avraham et al. (2010). Briefly, the electroporated embryo was removed at stage 29 (E6) from the egg and placed in a petri dish containing PBS and coated with paraffin. Once the membranes were cut, the embryo was stretched and pinned on the ventral side. A longitudinal incision was made along the roof plate, from the hindbrain down to the tail with a sharp micro-needle. Two additional longitudinal incisions were made at both sides of the spinal cord, detaching the dorsal root ganglia (DRGs) from the spinal cord. The floor plate was detached from the tissue starting from the tail to the hindbrain, leaving the spinal cord intact. The spinal cord was then cut in transverse at the hindbrain, and

separated from the body. The isolated spinal cord was pinned in a new petri dish, with the hindbrain to the tail producing a flat-mount preparation. PBS was replaced with 4 % paraformaldehyde in PBS and samples were incubated for 1 h at 25 °C. The fixed spinal cord was transferred into a 6-well-plastic tissue culture plates containing DAPI (Roche, Germany) in PBS. Following three 5-min PBS washes, the spinal cord was mounted and coverslipped. A fluorescence microscope (Nikon ECLIPSE 80i, Japan), equipped with a digital camera (LEICA DFC300FX, Germany), was used to visualize and photograph the fluorescence signal.

cRNA probe synthesis and in situ hybridization

Digoxigenin-labeled sense and antisense cRNA probes were transcribed in vitro using purified PBS-SK plasmids containing the full-length of N-Cadherin according to the manufacturer's instruction (Roche, Germany). Sense cRNA probes were used as negative controls for in situ hybridization.

For in situ hybridization, 20 µm thick cryosections were fixed with 4 % formaldehyde in PBS and pretreated with proteinase K and acetic anhydride. Sections were hybridized overnight with cRNA probe at a concentration of about 3 ng/µl at 70 °C in hybridization solution (50 % formamide, 3 × SSC, 10 mM EDTA, 10 % dextran sulfate, 1 × Denhardt's solution, 42 µg/ml yeast transfer RNA and 42 µg/ml salmon sperm DNA; Roche, Germany). Sections were washed to remove unbound cRNA by RNase reaction, and then incubated with alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Roche, Germany) at 4 °C overnight. For the visualization of the labeled mRNA, a substrate solution of nitroblue tetrazolium salt (NBT, Fermentas, Lithuania) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Fermentas, Lithuania) were added. The sections were viewed under a microscope (Nikon ECLIPSE 80i, Japan), which were equipped with a digital camera (LEICA DFC300FX, Germany).

Statistical analysis

The average optical density and area of fluorescence intensity were calculated using Plus Image-Pro 6 software (Media Cybernetics, USA), and the data were analyzed by Statistics 17.0 SPSS software (IBM, USA). All analytic data were presented as a mean ± standard deviation (S.D.), of at least three independent experiments. Significance of differences among different transfection groups were determined using ANOVA, where *P* value <0.05 was significant.

Results

Temporospatial expression pattern of N-cadherin in the developing chicken spinal cord

The spatial and temporal expression pattern of N-cadherin has been reported in early chicken embryos (Hatta et al. 1987), and the mRNA distribution of N-cadherin has been also analyzed during chicken spinal cord development (Lin et al. 2014). In the present study, we analyzed the protein expression pattern of N-cadherin in the spinal cord during chicken embryonic development at stage 18–36 (E3–E10). In the early chicken spinal cord, N-cadherin shows wide expression at stage 18 (E3) in the roof plate (rp), the floor plate (fp), the notochord (nc), and the myotome (mt; Fig. 1a). At stage 24 (E4, Fig. 1b), stage 26 (E5, Fig. 1c), and stage 29 (E6, Fig. 1d) N-cadherin expression is very prominent in the white matter (wm) and in the dorsal root ganglion (drg). However, it is weak in the gray matter (gm), and almost negative in the lateral motor column (lm; Fig. 1c, d). At stage 34 (E8, Fig. 1e) and stage 36 (E10, Fig. 1f), the expression of N-cadherin in the gm becomes weak (Fig. 1e, f). The expression pattern weakened after stage 36 (E10), with little expression in spinal cord until adults (data not shown). There are some differences between the spatial distributions of N-cadherin mRNA and protein. For example, there is no N-cadherin mRNA expression in white matter, which suggests that N-cadherin protein is strongly expressed in neural axons, but their temporal expression pattern is same (Lin et al. 2014).

Knockdown or overexpression of N-cadherin in vitro

The HEK293T cell line was used to validate N-cadherin overexpression and shRNA plasmid efficacy, and determine how this can regulate cell–cell connections. Given the cross-reaction of the anti-mouse N-cadherin antibody, the anti-chicken N-cadherin antibody was used for western blot and immunohistochemistry. HEK293T cells were transfected with pCAGGS-GFP, pCAGGS-N-cadherin, or pCAGGS-N-Cadherin + pGPU6-GFP-neo-N-Cadherin-shRNA. The N-cadherin overexpression or knockdown was validated with western blot. There was an increase in N-cadherin in the group transfected with pCAGGS-N-cadherin (Fig. 2a), in comparison to others (Fig. 2b–d). Among the three shRNA groups, where pCAGGS-N-Cadherin was co-transfected with pGPU6-GFP-neo-N-Cadherin-shRNA1, 2, and 3, respectively, there was less N-cadherin in pCAGGS-N-Cadherin + shRNA1, in comparison to the other two (Fig. 2b–d). Since shRNA1 of

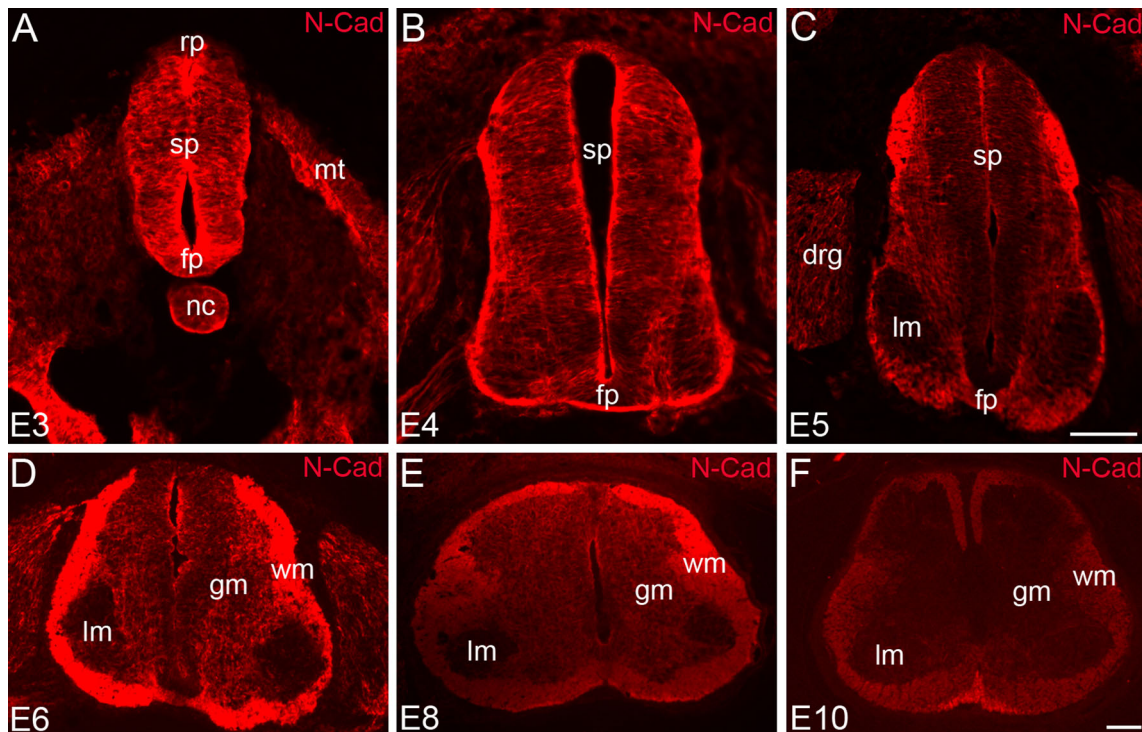


Fig. 1 N-cadherin protein distribution in the developing chicken spinal cord. N-cadherin protein expression in the spinal cord at different developing stages. **a** At stage 18 (E3), **b** at stage 24 (E4), **c** at stage 27 (E5), **d** at stage 29 (E6), **e** at stage 34 (E8), and **f** at stage 36

(E10). *drg* dorsal root ganglia, *fp* floor plate, *gm* grey matter, *lm* lateral motor column, *mt* myotome, *nc* notochord, *rp* roof plate, *sp* spinal cord, *wm* white matter. Scale bar, 100 μ m in **f** for **a–f**

N-cadherin worked well in vitro, it was selected for interference in later experiments.

Next, we checked if N-cadherin affects cell–cell connection in vitro. Our results showed that N-cadherin positive cells in the overexpression group have obvious pseudopodium and form cell–cell connection (Fig. 3a, b), compared to the control group (Fig. 3g, h), corroborating previous results (Shih and Yamada 2012). However, in cells co-transfected with pCAGGS-N-Cadherin and pGPU6-GFP-neo-N-Cadherin-shRNA, N-cadherin expression was knocked down, and so, no pseudopodium and cell–cell connection could not be detected (Fig. 3m, n). To further analyze its function and examine directly whether N-cadherin regulates the β -catenin signaling pathway in vitro, we investigated the expression of β -catenin and α -catenin in different transfected HEK293T cells. Results showed that N-cadherin regulates endogenous β -catenin expression in HEK293T. In contrast to the control group (Fig. 3i, j), we observed that N-cadherin overexpression increases the expression level of endogenous β -catenin (Fig. 3c, d), while N-cadherin knockdown resulted in the nuclear accumulation of β -catenin (Fig. 3o, p). Although β -catenin reportedly regulates the expression of α -catenin, which is affected by N-cadherin expression (Doherty and Walsh 1996), the expression of α -catenin increased after

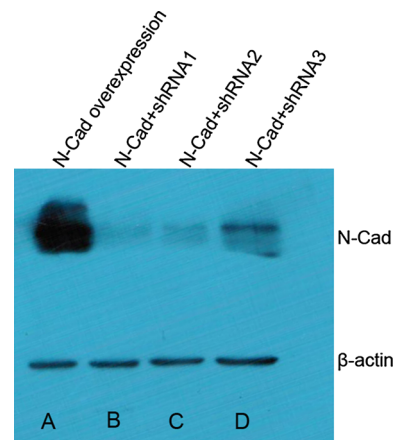


Fig. 2 N-cadherin overexpression and knockdown in HEK293T cells, analyzed using Western blotting. **a** N-cadherin overexpression from pCAGGS-N-Cadherin plasmid transfection. **b** pCAGGS-N-Cadherin and pGPU6-GFP-neo-N-Cadherin-shRNA1 plasmid co-transfection to knockdown N-cadherin overexpression. **c** pCAGGS-N-Cadherin and pGPU6-GFP-neo-N-Cadherin-shRNA2 plasmid co-transfection to knockdown N-cadherin overexpression. **d** pCAGGS-N-Cadherin and pGPU6-GFP-neo-N-Cadherin-shRNA3 plasmid co-transfection to knockdown N-cadherin overexpression. β -actin served as an internal control

N-cadherin misexpression (overexpression in Fig. 3e, f, and knockdown in Fig. 3q, r). However, there was no nuclear accumulation of α -catenin after N-cadherin

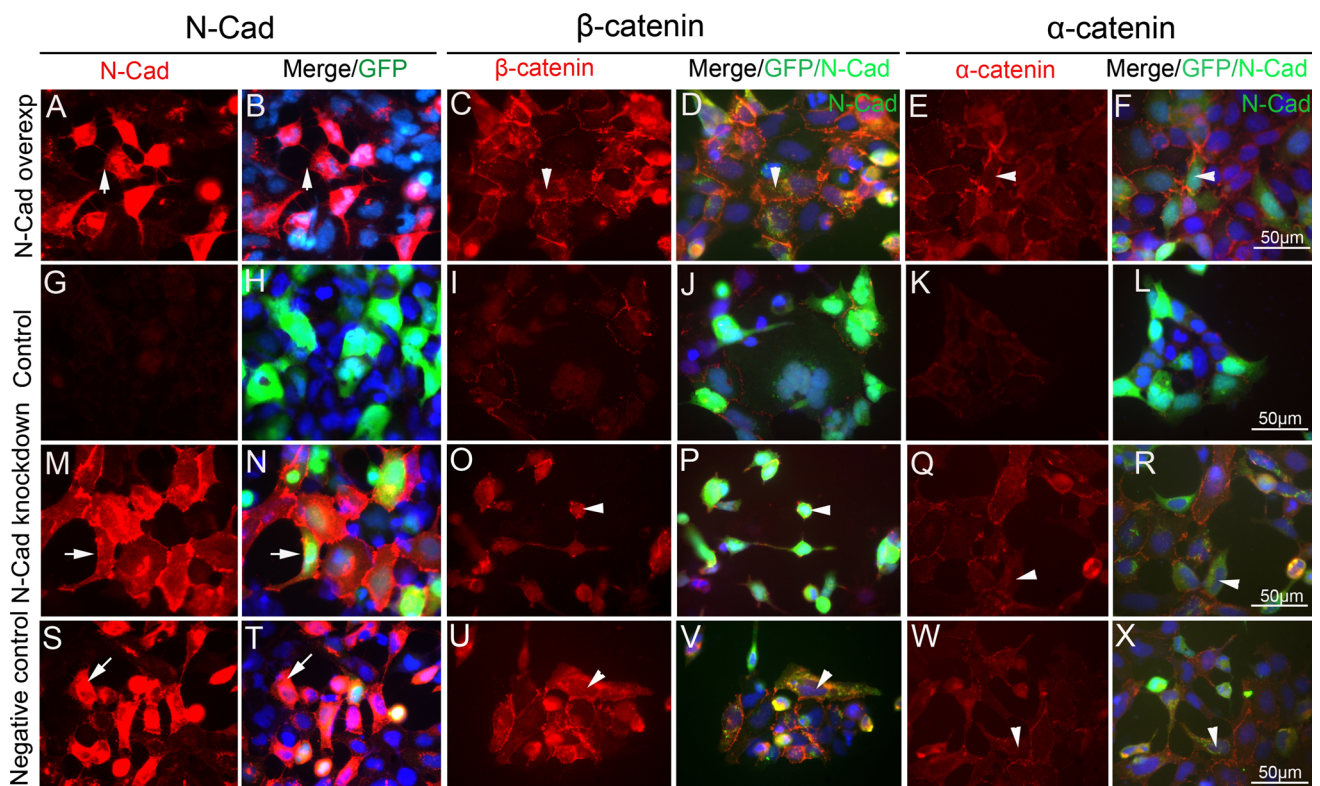


Fig. 3 The effect of N-cadherin on cell–cell connection and catenin signaling in HEK293T cells. **a–f** N-cadherin overexpression with pCAGGS-N-Cadherin plasmid transfection. **g–l** Transfection of pCAGGS-GFP plasmid alone, which served as a control. **m–r** pCAGGS-N-Cadherin and pGPU6-GFP-neo-N-Cadherin-shRNA plasmid co-transfection to knockdown N-cadherin overexpression. **s–x** pCAGGS-N-Cadherin and pGPU6-GFP-neo plasmid co-transfection as a negative control. **a, b** Cultured HEK293T cells imaged using an antibody against chicken N-cadherin; exogenous N-cadherin expression is shown in red. Arrows in **a, b** highlight cell pseudopodium and cell–cell connection. **c, d** Endogenous β -catenin expression. Arrowheads in **c, d** demonstrate the increase in β -catenin expression due to N-cadherin overexpression. **e, f** Endogenous α -catenin expression. Arrowheads show α -catenin positive cells. **g–l** control group showing no exogenous N-cadherin expression (in **g** and **h**), but low endogenous β -catenin (in **i** and **j**) and α -catenin

expression (in **k** and **l**). **m, n** Cultured HEK293T cells imaged with antibody against N-cadherin, showing exogenous N-cadherin expression (red) and shRNA expression (green). Arrows indicate the expression of both N-cadherin and interference sequence in which cell–cell connections are not obvious. **o, p** Endogenous β -catenin expression after N-cadherin knockdown. Arrowheads highlight β -catenin accumulation in the cytoplasm and nucleus in N-cadherin shRNA knockdown cells. Cells with shRNA plasmids are seen in green. **q, r** Endogenous α -catenin expression did not change after N-cadherin knockdown. **s, t** Cultured HEK293T cells analyzed with the antibody against chicken N-cadherin, with exogenous N-cadherin expression (red). Arrows show cell pseudopodium and cell–cell connections. **u, v** Endogenous β -catenin expression. Arrowheads indicate increased β -catenin expression with N-cadherin overexpression. **w, x**: Endogenous α -catenin expression results. Scale bar 50 μ m in **f, l, r**, and **x** for **a–x**. (Color figure online)

knockdown. Taken together, these findings suggested that N-cadherin is involved in the regulation of β -catenin signaling pathway in vitro.

Finally, the results from the negative control group and N-cadherin overexpression group were similar, illustrating that the pGPU6-GFP-neo vector of shRNA did not affect the results (Fig. 3s–x). Thus, a negative control group was not used in subsequent experiments.

N-cadherin misexpression in the chicken developing spinal cord

In ovo electroporation, where the plasmid can be unilaterally electroporated, was done to examine the role of

N-cadherin in the developing spinal cord. Three experimental groups were designed as follows: (1) pGPU6-GFP-neo (2 μ g/ μ l) + pCAGGS-GFP (0.25 μ g/ μ l) co-electroporation served as a control, (2) pCAGGS-N-cadherin (2 μ g/ μ l) + pCAGGS-GFP (0.25 μ g/ μ l) co-electroporation, and (3) pGPU6-GFP-neo-N-Cadherin-shRNA (2 μ g/ μ l) + pCAGGS-GFP (0.25 μ g/ μ l) co-electroporation. Electroporation was performed on the chicken embryonic spinal cord at stage 18 (E3). After 3 days of electroporation, GFP positive embryos were collected at stage 26 (E6). After a 24-h transfection, the N-cadherin overexpression was clearly observed through immunohistochemistry and in situ hybridization (Fig. 4c, e). After a 72-h transfection, although it was difficult to observe N-cadherin

overexpression or knockdown through immunohistochemistry (Fig. 4h, m, r), this could be done via in situ hybridization (Fig. 4j, o, t). Perhaps, at the later stage, the N-cadherin protein is mainly located in nerve fibers (Fig. 4h, m, r), while the mRNA is mainly distributed in the cytoplasm (Fig. 4j, o, t). In order to further test the misexpression of N-cadherin, the average optical density of different groups in fluorescence immunohistochemistry and in situ hybridization were analyzed. To control for individual differences, data from the same spinal cord were matched, where the transfected and untransfected sides served as experiment and control, respectively. The average optical density ratio between the experimental side and the control side was greater than 1 in the overexpression group, approximately 1 in the control group, and less than 1 in the knockdown group. Although there were no significant differences ($p > 0.05$) among the three groups (Fig. 4u) at protein level, significant differences were present at the mRNA level ($p < 0.01$; Fig. 4u).

N-cadherin misexpression perturbs commissural axon projecting in the chicken developing spinal cord

Interestingly, the projected commissural axons almost disappeared through the contralateral floor plate (fp) after N-cadherin knockdown (Fig. 4p–s). However, N-cadherin overexpression also seemed to reduce commissural axons projecting contralaterally through the floor plate (fp) (Fig. 4f–i). GFP expression in the commissural axon projecting to the contralateral side was compared between the two experimental groups and control to analyze the effect of N-cadherin expression on commissural axon projection. The commissural axon projecting to the contralateral side was significantly ($p < 0.01$) reduced after N-cadherin overexpression and knockdown, in comparison to control (Fig. 4v), indicating that N-cadherin misexpression perturbs commissural axon projection in the developing spinal cord.

It is difficult to define the reduction in axonal projection reduction from the transfer sections. Therefore, we used an open-book method to further test if N-cadherin overexpression/knockdown perturbs commissural axonal projection in the developing chick spinal cord, from stage 18–29 (Fig. 5j). The control group was co-electroporated with pGPU6-GFP-neo and pCAGGS-GFP. GFP-labeled commissural axons turned into the longitudinal plane and projected alongside the floor plate as a fiber tract (called the medial longitudinal commissural, mlc), with some projections extending from the ventral midline (VM) into the intermediate region of the spinal cord (called intermediate longitudinal commissural, ilc; Fig. 5d–f; Reeber et al. 2008). However, in the N-cadherin overexpression group, most GFP-labeled commissural axons turned into

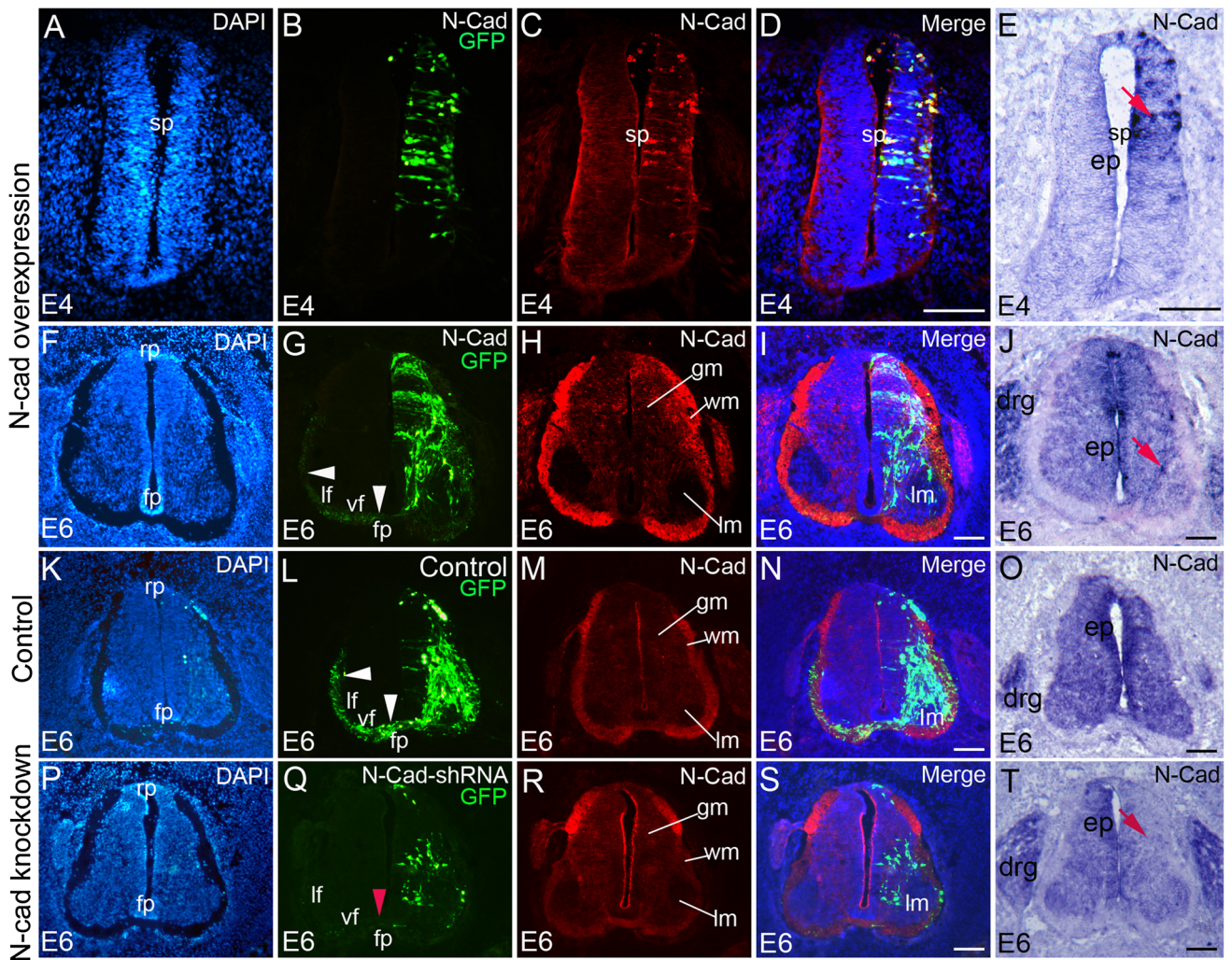
the longitudinal plane and projected alongside the floor plate as the mlc, with just a few axons extending from the VM into the ilc (Fig. 5a–c). This indicated that N-cadherin overexpression partially affected the commissural axonal projection, with a reduction in commissural axons crossing the floor plate in both in the mlc and the ilc, in comparison to control (Fig. 5a–e). Further almost none of the GFP-labeled axons extended away from the VM, after N-cadherin was knocked down with a specific shRNA, (Fig. 5g–i), which suggested that N-cadherin knockdown significantly perturbs commissural axonal projection. The projected axons alongside the contralateral margin of the floor plate only form as the mlc, rather than both the mlc and ilc (Fig. 5g–i). Additionally, the percentage of commissural axon projection to the contralateral side in the N-cadherin misexpression group was significantly lower, in comparison to the control (Fig. 5k, $p < 0.01$), and was more obvious in the N-cadherin knockdown group (Fig. 5k).

It has been reported that N-cadherin inhibition mediated by Robo protein controls spinocerebellar tract formation (Sakai et al. 2012). Our in vitro experiments also showed that N-cadherin regulates β -catenin signaling in HEK293T cells (see above). Therefore, next we investigated catenin expression in the developing spinal cord after N-cadherin misexpression.

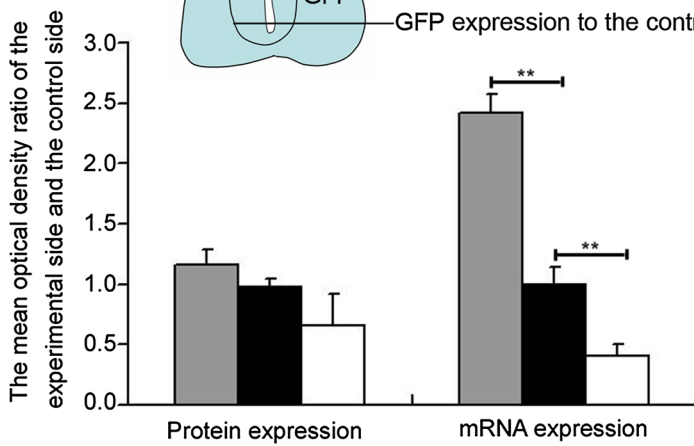
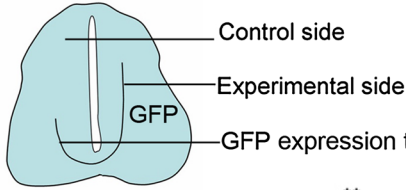
N-cadherin misexpression affects catenin expression in spinal cord

To investigate directly whether N-cadherin regulates catenin signaling in vivo, we overexpressed or knocked down N-cadherin by in vivo electroporation in chick spinal cord (Fig. 6). We found that N-cadherin overexpression increased β -catenin expression (arrows in Fig. 6c–e) while N-cadherin knockdown decreased β -catenin expression, in both white and gray matter (Fig. 6k–o) in the spinal cord. Thus, N-cadherin plays an important role in maintaining β -catenin signaling during spinal cord development in vivo. Recent studies suggested that N-cadherin regulates β -catenin signaling through Wnt and AKT (Zhang et al. 2013). β -catenin itself binds tightly to the cytoplasmic domain of type I cadherins, playing an essential role in the structural organization and function of cadherins by linking cadherins through α -catenin to the actin cytoskeleton. Wnts, which are secreted lipid-modified signaling proteins, influence multiple processes in animal development. Conventional Wnt signaling causes β -catenin to combine with the transcription factor TCF/LEF, thus, regulating target gene expression (Nelson and Nusse 2004).

In the current study, we also examined the expression of α -catenin (Fig. 7). However, α -catenin expression did not differ significantly between the N-cadherin overexpression (Fig. 7a–e), N-cadherin knock down (Fig. 7k–o), and



U



V

N-cad overexpression group
 Control group
 N-cad knockdown group

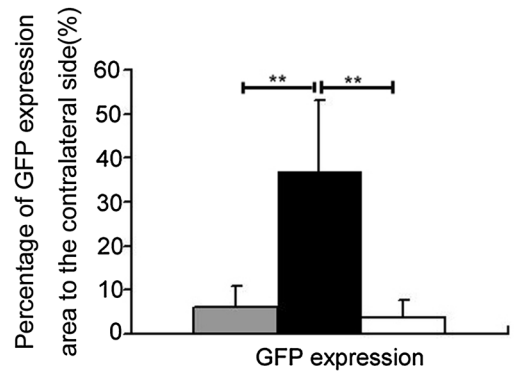


Fig. 4 Section analysis of N-cadherin misexpression in the chicken spinal cord. **a–j** N-cadherin overexpression group with pCAGGS-N-Cadherin and pCAGGS-GFP plasmid co-transfection. **k–o** Control group with pGPU6-GFP-neo and pCAGGS-GFP plasmid co-transfection. **p–t** N-cadherin knockdown group with pCAGGS-GFP and pGPU6-GFP-neo-N-Cadherin-shRNA plasmids co-transfection. **a–j** N-cadherin overexpression following pCAGGS-N-Cadherin and pCAGGS-GFP co-transfection for 24 h (**a–e**) and 72 h (**f–j**). DAPI nuclear staining (**a, f**), GFP expression (**b, g**), N-cadherin expression (**c, h; red**), and merged images are shown. N-cadherin mRNA distribution was based on in situ hybridization. **k–o** Control group after pGPU6-GFP-neo and pCAGGS-GFP co-transfection for 72 h, showing DAPI nuclear stain (**k**), GFP expression (**l**), N-cadherin expression result (*red*; **m**), and the merged image (**n**). N-cadherin expression based on in situ hybridization (**o**). **p–t** N-cadherin knockdown after pGPU6-GFP-neo-N-Cadherin-shRNA and pCAGGS-GFP co-transfection for 72 h. **p** nucleus staining with DAPI. **q** GFP expression result. **r** N-cadherin expression result (*red*). **s** the merge of **p–r**. **t** N-cadherin expression from in situ hybridization. **u** mean optical density ratio between the experimental and control sides. **v** the percentage of GFP expression area to the contralateral side. Data are presented as mean \pm SD. $**p < 0.01$. *drg* dorsal root ganglion, *gm* grey matter, *lf* lateral funiculus, *lm* lateral motor column, *rp* roof plate, *sp* spinal cord, *vf* ventral funiculus, *wm* white matter. Scale bar 100 μ m in **e** for **a–e**, in **t** for **f–t**. (Color figure online)

control groups (Fig. 7f–j). β -catenin phosphorylation by casein kinase II positively regulates the integrity of the cadherin-catenin complex. Changes in the phosphorylation state of β -catenin affect cell–cell connection, cell migration, and the level of β -catenin signaling (Nelson and Nusse 2004). Together, these findings suggest that N-cadherin regulates β -catenin signaling activity, which in turn can perturb the projection of commissural axons in the spinal cord.

Discussion

Cell migration is an essential process during embryonic development, throughout adult life, and in some pathological conditions. Cadherins, especially the neural cell adhesion molecule N-cadherin, play an important role in cell migration (Derycke and Bracke 2004; Hirata et al. 2014). N-cadherin affects not only the cell migration but also the projection of commissural axons (Sakai et al. 2012). In the vertebrate spinal cord, most commissural axons form the mlc or ilc trajectories, projecting alongside or a significant distance away from the floor plate, respectively (Kadison and Kaprielian 2004). In this study, we unilaterally exploited in ovo electroporation combined with open-book technology to label developing chicken mlc and ilc axons in the spinal cord reproducibly, to assess the effect of N-cadherin overexpression or knockdown on axonal projection. Since N-cadherin knockout mice died at day 10 of gestation (Radice et al. 1997), we applied

combined in ovo electroporation and shRNA technology unilaterally to achieve N-cadherin knockdown in the chicken spinal cord. In order to obtain the direct evidence of N-cadherin overexpression or knockdown result, we chose HEK293T cells transfected with plasmids to analyze the N-cadherin function in vitro.

N-cadherin promotes cell–cell connections and regulates the β -catenin signal pathway in the HEK293T cells

Our results showed that N-cadherin promotes cell processes and the formation of cell–cell connection, and affects the β -catenin signal pathway in the HEK293T cells. Although the overexpression of N-cadherin in HEK293T cells did not promote synaptic growth, its expression in CHO cells was sufficient to induce axon growth in cholinergic axons, in comparison with cells that did not express N-cadherin (Flannery and Bruses 2012). Previous reports indicated that N-cadherin contributed to synapse development of glutamatergic neurons by recruiting synaptogenic molecules to the nascent synaptic contact (Stan et al. 2010). However, the overexpression of N-cadherin in non-neuronal cell lines did not promote presynaptic terminal differentiation that was found in glutamatergic neurons. In this study, we found that N-cadherin overexpression promotes pseudopodium formation and increases endogenous β -catenin expression levels, while N-cadherin knockdown results in the accumulation of β -catenin in the nucleus in vitro. The level of endogenous N-cadherin remained unchanged under the conditions of exogenous N-cadherin misexpression in this experiment. Thus, we speculated that high concentrations of N-cadherin increased the β -catenin concentration in the cell membrane, while the low concentration of N-cadherin decreased β -catenin near the cell membrane, causing it to accumulate in the nucleus. A recent study provided evidence that N-cadherin mainly regulates endogenous β -catenin signaling activity in neural precursors (Zhang et al. 2010). Furthermore, our findings showed that the nuclear accumulation of β -catenin in the N-cadherin knockdown may perturb β -catenin signaling pathway. Zhang et al. (2010) demonstrated that N-cadherin regulates β -catenin signaling via both Wnt and AKT. Nuclear β -catenin interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) affecting gene transcription. When cells receive Wnt signals, the degradation pathway is inhibited, and β -catenin consequently accumulates in the cytoplasm and nucleus (Logan and Nusse 2004). Thus, the regulation of downstream molecules involved in β -catenin signaling pathway by N-cadherin requires further investigation.

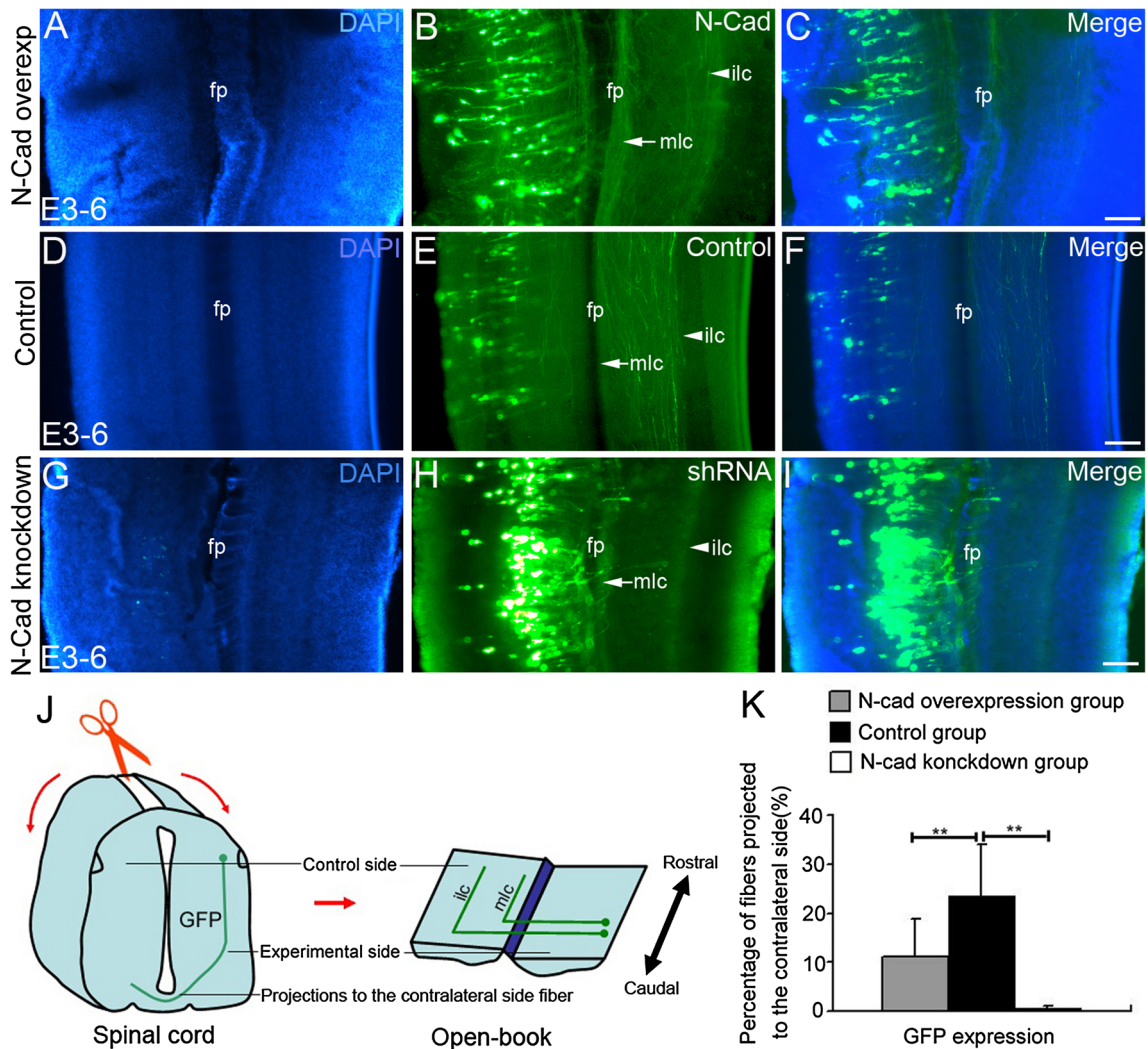


Fig. 5 N-cadherin misexpression perturbs commissural axon projection in the chicken spinal cord. **a–c** N-cadherin overexpression group with pCAGGS-N-Cadherin and pCAGGS-GFP plasmid co-transfection. **d–f** Control group with pGPU6-GFP-neo and pCAGGS-GFP plasmid co-transfection. **g–i** N-cadherin knockdown group with pCAGGS-GFP and pGPU6-GFP-neo-N-Cadherin-shRNA plasmid

co-transfection. DAPI nuclear staining is shown in *blue* and GFP expression is seen in *green*. **j** the model of open-book process. **k** the percentage of fibers projected to the contralateral side; data are presented as mean \pm SD. $**p < 0.01$. *fp* floor plate, *ilc* intermediate longitudinal commissural, *mlc* medial longitudinal commissural. Scale bar 100 μ m in **c**, **f** and **i** for **a–i**. (Color figure online)

In ovo electroporation

To investigate the function of N-cadherin on commissural neuron axon projection in vivo, we performed in ovo electroporation in the developing chicken spinal cord at E3. In the chick spinal cord, neurogenesis of neuronal dII-6 populations occurs during a narrow time window, which is initiated at Hamburger and Hamilton stage 18 (30–36 somites, day 3 of development), with 50 % of dII-6 neurons being generated by HH23 (>45 somites, day 4 of development; Le Dréau et al. 2012). Thus, we chose to conduct in ovo electroporation at stage 18 (E3). The pCAGGs plasmid expressed the target genes 4 h after they entered the cells (data not shown). However, N-cadherin

protein expression significantly decreased after 3 days. Following 3 days of in ovo electroporation, an obvious difference between both sides of the spinal cord, and in comparison with control, was evident by in situ hybridization (Fig. 4o, t). These results demonstrated the knockdown of N-cadherin expression in vivo with a specific shRNA.

N-cadherin regulates axon projection in the chicken spinal cord

Cadherin-mediated interactions form as a network, which polarizes the axon growth cone, determining its targeting specificity (Schwabe et al. 2013). N-cadherin is localized at

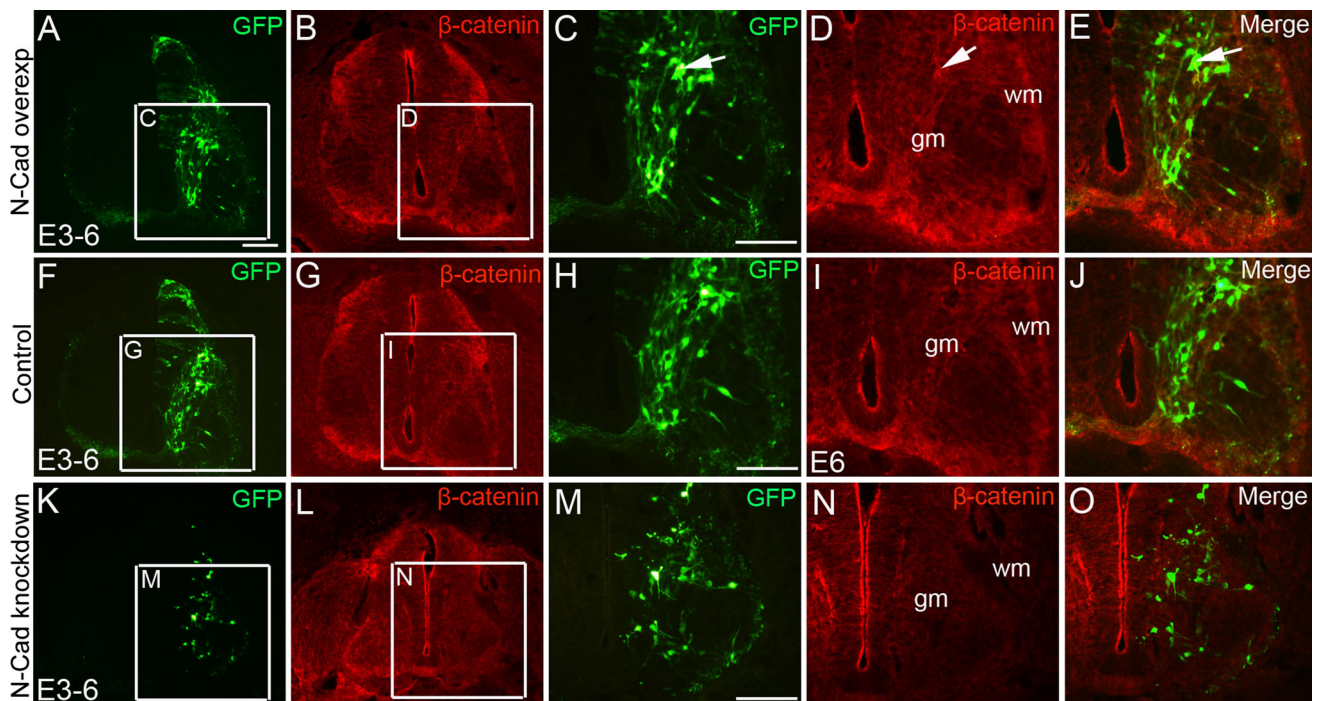


Fig. 6 The effect of N-cadherin misexpression on β -catenin signal in the chicken spinal cord. **a–e** N-cadherin overexpression group with pCAGGS-N-Cadherin and pCAGGS-GFP plasmid co-transfection, showing GFP (**a**, higher magnification in **c**) and β -catenin (**b**, higher magnification in **d**) expression. **f–j** The control group with pGPU6-GFP-neo and pCAGGS-GFP plasmids co-transfection, showing GFP (**f**, higher magnification in **h**) and β -catenin (**g**, higher magnification

in **i**) expression **k–o** N-cadherin knockdown group pCAGGS-GFP and pGPU6-GFP-neo-N-Cadherin-shRNA plasmid co-transfection, showing GFP (**k**, higher magnification in **m**) and β -catenin (**l**, higher magnification in **n**) expression. *White arrows* indicate the co-expression of GFP and β -catenin. *gm* grey matter, *wm* white matter. *Scale bars* 100 μ m in **a**, **b**, **f**, **g**, **k**, **l**, 100 μ m in **c**, **h**, and **m** for **c–e**, **h–j**, and **m–o**, respectively

glutamatergic and cholinergic synaptic contacts from early stages of synapse development in the developing nervous system, suggesting its role in synapse formation (Flannery and Bruses 2012). N-cadherin regulates β -catenin signaling and neuronal differentiation through Wnt and AKT during cortical development (Zhang et al. 2013). In the current study, we also found that N-cadherin misexpression perturbs axon projection in the chicken spinal cord. N-cadherin overexpression affected one part of the commissural axon projection, with other parts of that projection traveling along the contralateral margin of the floor plate, forming both the mlc and ilc (Fig. 5). This is consistent with previous reports that the overexpression of N-cadherin results in the reduction of ilc axons within the lateral funiculus (Reeber et al. 2008; Sakai et al. 2012). In comparison to its overexpression, N-cadherin knockdown led to axonal projection alongside the contralateral margin of the floor plate in the mlc alone. Although, other researchers report that the loss of N-cadherin function, with specific morpholinos, did not strongly perturb the projection of postcrossing commissural axons (Sakai et al. 2012), we suggest that inhibition of N-cadherin expression do have an effect.

N-cadherin regulates axon projection through β -catenin signaling pathway in the chicken spinal cord

β -catenin is a cytoplasmic adaptor protein involved in the Cadherin, Wnt, and other developmental signaling pathways (Wiley et al. 2008). In the cadherin pathway, β -catenin couples cadherins to the actin cytoskeleton through interaction with α -catenin (Gooding et al. 2004). Although it is well known that N-Cadherin is required for axon outgrowth (Riehl et al. 1996), its mechanism of action remains unclear. A large number of studies indicate that β -catenin relates to the formation of axons, through the promotion of synaptogenesis and neurite growth via the Wnt/ β -catenin signaling pathway in vitro (Yang et al. 2013), and affecting the terminal ventral optic axonal projection in vivo (Wiley et al. 2008). In this study, we found that N-cadherin misexpression affects β -catenin expression in vitro; a high concentration of N-cadherin increased β -catenin expression in the cell membrane, while a low N-cadherin concentration induced nuclear β -catenin aggregation. β -catenin expression was also affected by N-cadherin misexpression in vivo. Thus, we speculated that in the developing chicken spinal cord, N-cadherin

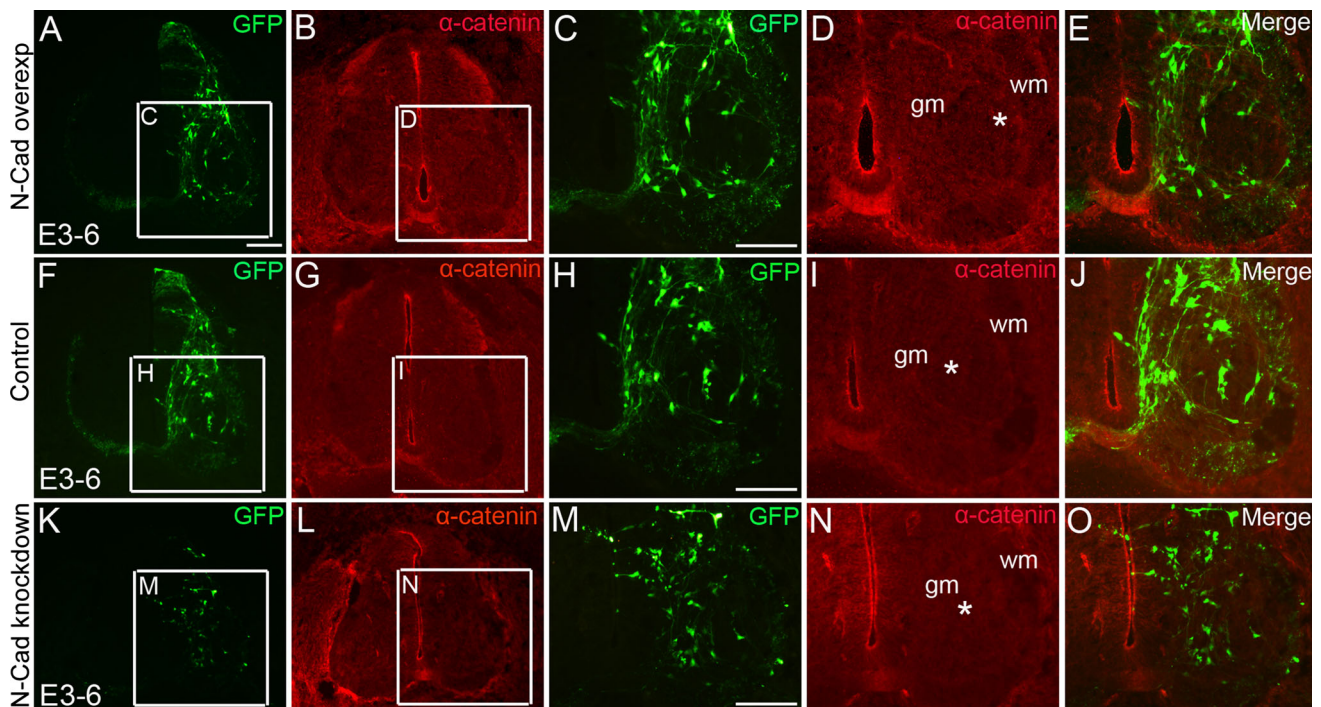


Fig. 7 The effect of N-cadherin misexpression on α -catenin in the chicken spinal cord. **a–e** N-cadherin overexpression group with pCAGGS-N-Cadherin and pCAGGS-GFP plasmid co-transfection, showing GFP (**a**, higher magnification in **c**) and α -catenin (**b**, higher magnification in **d**) expression. **f–j** Control group with pGPU6-GFP-neo and pCAGGS-GFP plasmid co-transfection, showing GFP (**f**, higher magnification in **h**) and α -catenin (**g**, higher magnification in

i) expression. **k–o** N-cadherin knockdown group with pCAGGS-GFP and pGPU6-GFP-neo-N-Cadherin-shRNA plasmid co-transfection, showing GFP (**k**, higher magnification in **m**) and α -catenin (**l**, higher magnification in **n**) expression. *gm* grey matter, *wm* white matter. Asterisk denotes similar areas. Scale bars 100 μ m in **a**, **b**, **f**, **g**, **k**, and **l**. 100 μ m in **c**, **h**, and **m** for **c–e**, **h–j**, and **m–o**, respectively

misexpression perturbs axon projection through its effect on β -catenin, which in turn may acts via the Wnt/ β -catenin signaling pathway. However, the role N-cadherin plays in commissural axon projection requires further investigation.

The current study provided evidence that N-cadherin function on cell–cell connection regulates endogenous β -catenin signaling in vitro. The misexpression of N-cadherin in vivo perturbs commissural axon projection in the chicken spinal cord, which may be attributed to the disturbance of β -catenin signaling pathway.

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Author contributions Conceived and designed the experiments: Zhikun Guo, Shanting Zhao, Juntang Lin. Performed the experiments: Ciqing Yang, Xiaoying Li, Congrui Wang Sulei Fu, Han Li. Analyzed the data: Ciqing Yang. Wrote the paper: Ciqing Yang.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no known conflicts of interest associated with this publication.

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