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Transmembrane Protein GDE2 Induces Motor Neuron Differentiation in Vivo

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During neural development, coordinate regulation of cell-cycle exit and differentiation is essential for cell-fate specification, cell survival, and proper wiring of neuronal circuits. However, the molecules that direct these events remain poorly defined. In the developing spinal cord, the differentiation of motor neuron progenitors into postmitotic motor neurons is regulated by retinoid signaling. Here, we identify a retinoid-inducible gene, *GDE2* (glycerophosphodiester phosphodiesterase 2), encoding a six-transmembrane protein that is necessary and sufficient to drive spinal motor neuron differentiation in vivo. A single amino acid mutation in the extracellular catalytic domain abolishes protein function. This reveals a critical role for glycerophosphodiester metabolism in motor neuron differentiation.

During development of the nervous system, cell-cycle exit is coupled to cellular differentiation programs to ensure that correct numbers of neuronal subtypes are generated to construct functional neural circuits (*1*). This complex process involves the synchronized decrease in expression of progenitor determinants, the increase of cell-cycle inhibitors, and the implementation of defined cell-fate specification programs. The molecular mechanisms that coordinate and regulate these pathways remain unclear.

Spinal motor neuron generation in the chick requires the integration of three different extrinsic signals: sonic hedgehog, fibroblast growth factors, and retinoic acid (RA) (*2*, *3*). All three signaling pathways have been implicated in initial dorsal-ventral patterning of progenitor domains in the spinal cord (Fig. 1A). However, RA signaling is also necessary for the induction of oligodendrocyte transcription factor 2 (Olig2) in progenitors and their subsequent differentiation into postmitotic motor neurons (Fig. 1A) (*2*). When motor neuron progenitors differentiate, they decrease expression of Olig2 as they migrate out of the ventricular zone (VZ) and increase expression of postmitotic motor neuron markers such as islet1 and islet2 (Fig. 1A) (*4*). Olig2 has a pivotal role in motor neuron differentiation. It is required for the maintenance of a motor neuron progenitor state, and its down-regulation is essential for the implementation of neurogenic and motor neuron specification pathways (*5*, *6*).

Because the differentiation of motor neuron progenitors is dependent on retinoid signaling, we conducted a differential subtraction screen with cDNAs derived from ventral spinal cord explants grown in the presence or absence of retinol to identify genes involved in this process (Fig. 1B) (*7*). Probing reverse Northern blots with cDNAs from both sets of explants demonstrated that expression of clone 45.1 was increased about 50-fold in explants exposed to retinol compared with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 1C). Furthermore, in situ hybridization analysis revealed that clone 45.1 was expressed within or directly adjacent to developing tissues that synthesize RA, such as the spinal cord, paraxial mesoderm, mesonephros, heart, lung, and eye (fig. S1) (*8*). Sequence analysis revealed that clone 45.1 is a chick gene (AY910750) encoding a predicted protein of 599 amino acids with 67% identity to the human predicted protein PP1665 and 66% identity to mouse glycerophosphodiester phosphodiesterase 2 (GDE2) (*9*, *10*) (fig. S2), suggesting clone 45.1 is the chick homolog of GDE2. These proteins all contain a glycerophosphodiester phosphodiesterase (GDPD) domain, known to be involved in glycerophosphodiester metabolism (*11*). Analysis of the Conserved Domain Database revealed that GDE2 is a member of a large, heterogeneous family of GDPD-containing proteins for which in vivo functions are largely unknown (*9*). GDE2 is a transmembrane protein, and epitope tagging studies demonstrated that the GDPD domain is extracellular with intracellular localization of the N- and C-termini (fig. S3).

GDE2 is highly expressed by all somatic spinal motor neurons, irrespective of their rostrocaudal position, from the time they are generated (Fig. 2, A to F) until at least Hamburger-Hamilton (HH) stage 29 (*8*). These data are consistent with the induction of GDE2 expression by paraxial mesoderm-derived RA signaling. In order to determine when GDE2 might act in motor neuron development, the onset of GDE2 expression was examined. The differentiation of motor neuron progenitors can be monitored accurately by the

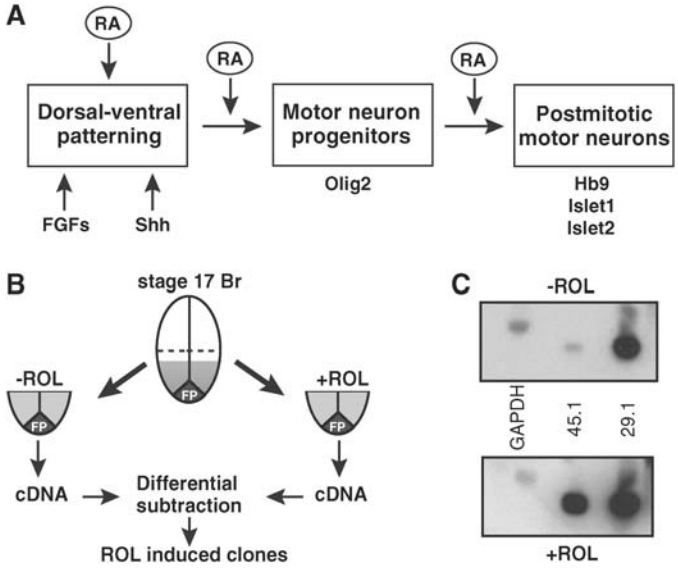

Fig. 1. GDE2 isolation and characterization. (**A**) Schematic depicting requirement for RA signaling at three distinct steps in motor neuron generation. Shh, Sonic hedgehog; FGFs, fibroblast growth factors. (**B**) Subtractive screen to isolate retinoid-responsive genes in motor neurons. Br, brachial neural tube; FP, floor plate; ROL, retinol. (**C**) Reverse Northern blots showing RA responsiveness of clone 45.1 when probed with cDNA from explants grown in the presence or absence of ROL compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and a non-RA-responsive clone, 29.1.

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sequential expression of molecular markers as well as the position of their cell bodies along the medial-lateral axis of the spinal cord. Actively cycling motor neuron progenitors located in the VZ of the spinal cord express large amounts of the transcription factor NK-homeobox 6.1 (Nkx6.1) and Olig2 (*4*, *6*) (Fig. 2G). These progenitor markers are extinguished as the cells exit the cell-cycle, migrate laterally, and begin to express motor neuron-specific transcription factors such as homeobox factor 9 (HB9), islet1, and islet2 (*4*) (Fig. 2G). GDE2 was localized in postmitotic, laterally located neurons that also expressed HB9, islet1, and islet2 (Fig. 2H) (*8*) but was not detected in medially located progenitor cells that expressed Nkx6.1 and Olig2 (Fig. 2, I and J). However, an intermediate population of cells weakly stained for Nkx6.1 and Olig2 also contained GDE2, suggesting that GDE2 expression may be initiated as cells transition to a postmitotic state (Fig. 2, I and J, arrows).

Once ventral neuronal progenitors undergo their terminal mitosis at the medial margin of the VZ, resulting daughter cells migrate laterally into the intermediate zone (IZ) (*12*). In the IZ, they increase expression of the cyclin-dependent kinase inhibitor p27 (*13*), undergo cell-cycle arrest, and respond to signals that trigger terminal differentiation (Fig. 2G). In embryos incubated with bromodeoxyuridine (BrdU), GDE2 was not detected in any cells that incorporated BrdU or were stained by the antibody MPM-2 (*14*), indicating that GDE2 is not expressed by progenitors undergoing S or M phase in the VZ (Fig. 2, K and L). The border between the VZ and the IZ is defined by S-phase nuclei labeled by BrdU (*12*). Lateral to this border, there was a subset of BrdU-labeled cells that expressed Olig2 as well as GDE2 (Fig. 2, K and M). Consistent with their location in the IZ, these cells contained small amounts of the cell-cycle inhibitor p27 (Fig. 2N). In summary, GDE2 was primarily expressed by mature motor neurons; however, its expression was initiated within cells in the IZ as they differentiated into postmitotic motor neurons.

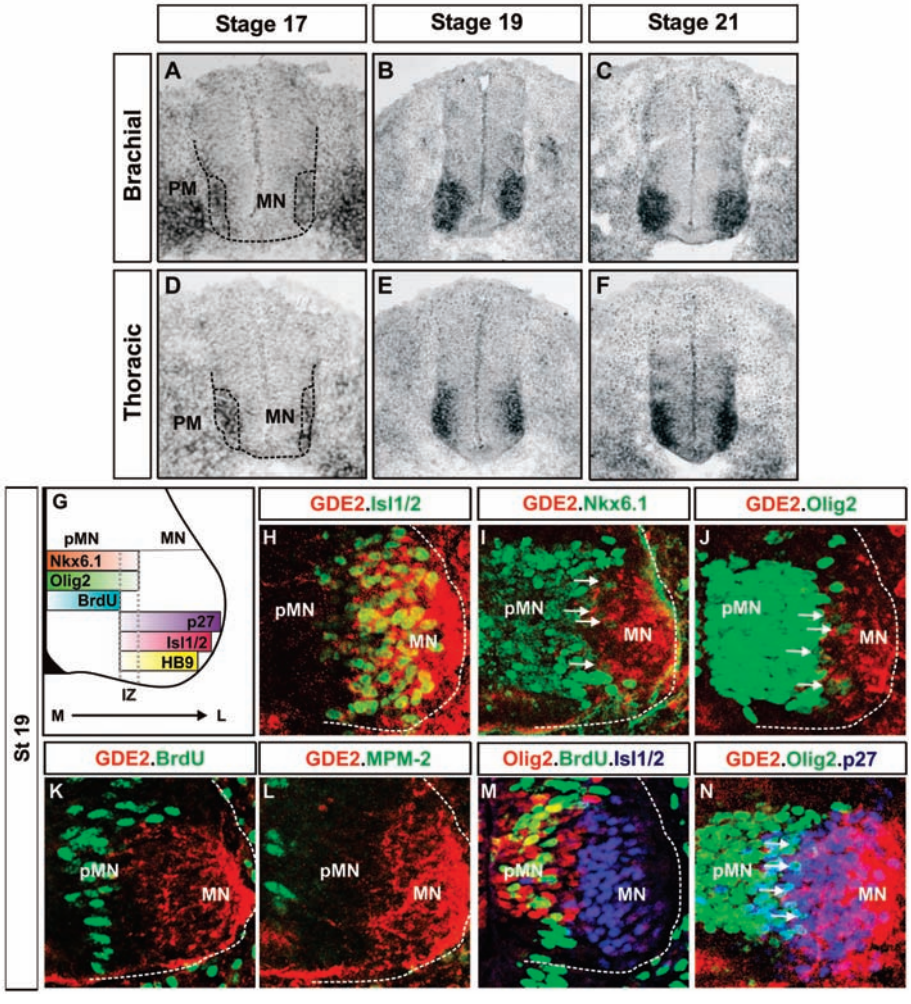

Fig. 2. GDE2 expression in spinal motor neurons. (**A** to **F**) In situ hybridization of *GDE2* mRNA in sections of chick spinal cord from limb (brachial) and nonlimb (thoracic) levels. Dotted lines mark the margins of the spinal cord and the motor neuron domain (MN). PM, paraxial mesoderm. (**G**) Schematic of molecular marker expression in ventral spinal cord. Arrow indicates medial (M) to lateral (L) axis. Dotted lines outline the intermediate zone (IZ). pMN, motor neuron progenitor domain. (**H** to **N**) Expression of GDE2 and molecular markers in HH stage 19 chick spinal cords. Ventral right quadrants are shown; medial is to the left and lateral to the right. Arrows in (I), (J), and (N), respectively, highlight cells that weakly stain for Nkx6.1, Olig2, or p27 and express GDE2 (BrDU, 30 min incubation). Dashed lines outline the margins of the spinal cord. Isl1/2, islet 1 and islet 2.

To test whether GDE2 might mediate the retinoid-dependent differentiation of Olig2 progenitors, we ablated GDE2 expression in the spinal cord by in ovo electroporation of small interfering RNAs (siRNAs) (*15*). All experiments used a green fluorescent protein (GFP) reporter plasmid to identify the electroporated side of the spinal cord. Electroporation of GDE2 siRNA typically resulted in a 70% loss of GDE2 mRNA and protein in spinal motor neurons (Fig. 3, A to C). Loss of GDE2 expression depended on the amount of siRNA administered, and siRNAs directed against different parts of the GDE2 open reading frame and 3′ untranslated region resulted in a similar loss of GDE2 mRNA and protein (fig. S4) (*8*). GDE2 silencing was not triggered by unrelated siRNAs, and GDE2 siRNAs did not induce global changes in gene expression (figs. S4 and S5). No toxicity was detected by terminal deoxynucleotidyl transferase biotin–deoxyuracil triphosphate (dUTP) nick end labeling (TUNEL) (*15*).

Embryos lacking GDE2 were analyzed for expression of the postmitotic motor neuron markers HB9, islet1, and islet2 by immunohistochemistry on the same or serial sections. In all cases a marked decrease in the number of neurons expressing each of these markers was evident on the electroporated side of the spinal cord, with about 70% loss of HB9-expressing neurons and 30 to 40% loss of more mature motor neurons expressing islet2 (Fig. 3, D and E, and fig. S5). Mice lacking HB9 show a progressive loss of islet1-expressing cells while they maintain normal numbers of islet2-expressing motor neurons, suggesting that separate pathways of motor neuron differentiation may exist (*16*, *17*). Our observation that GDE2 silencing affects HB9 expression more severely than islet2 indicates a differential requirement for GDE2 activity in these two pathways. We found no expansion in the number of neighboring interneurons, but an increase in TUNEL together with a reduction in the width of the electroporated ventral spinal cord was observed (Fig. 3, A to C) (*8*). These results provide evidence that GDE2 silencing results in the loss of postmitotic motor neurons and that cells destined to become motor neurons likely do not convert to a different fate but instead undergo cell death.

To confirm that the loss of motor neurons upon GDE2 silencing did not result from defects in progenitor generation or proliferation, we analyzed expression of the progenitor marker Olig2 and that of the ventral patterning genes paired box 6 (Pax6), Nkx2.2, and Nkx6.1 in embryos electroporated with GDE2 siRNA. There was no

change in the dorsal-ventral boundaries of the motor neuron progenitor domain or in the number of cells expressing Olig2 (fig. S5). Consistent with this, there was also no change in the number of cells expressing motor neuron restricted 2 (MNR2), a transcription factor turned on by committed progenitors in the S phase of the final cell cycle and maintained throughout their differentiation (*8*, *14*). Thus, GDE2 silencing appears not to affect progenitor cell generation or number.

To test whether GDE2 is sufficient to drive motor neuron differentiation, we misexpressed GDE2 throughout the spinal cord including within cycling Olig2-expressing progenitors in the VZ. We engineered a bicistronic construct with GDE2 linked to an internal ribosomal entry site (IRES) upstream of a nuclear form of β-galactosidase (GDE2NLZ) under the control of the chick β-actin promoter. Electroporation of GDE2NLZ into chick spinal cords resulted in high coincident expression of GDE2 and NLZ along the entire mediolateral axis in both progenitors and postmitotic neurons (Fig. 4, A and B). In contrast to the unelectroporated side, many medial cells in the electroporated VZ expressed the motor neuron marker HB9 (Fig. 4, C and D). Furthermore all of these medial HB9-containing cells also expressed markers of terminal motor neuron differentiation, such as islet2 (Fig. 4E) and choline acetyltransferase, the enzyme required for biosynthesis of the motor neuron neurotransmitter acetylcholine (*8*, *18*). To quantify this effect, we divided the ventrolateral spinal cord into three bins that roughly corresponded to domains of motor neuron progenitors, differentiating motor neurons, and postmitotic motor neurons (Fig. 4F). More islet2-expressing neurons were detected in bin 1 and bin 2 of embryos electroporated with GDE2NLZ than in embryos electroporated with NLZ alone (Fig. 4G). However, similar numbers of NLZ-expressing cells were detected in each case (Fig. 4).

Cells differentiating in response to GDE2 within the VZ expressed large amounts of the cell-cycle inhibitor p27 and failed to incorporate BrdU (Fig. 4, H to K). Moreover, these islet2-expressing cells in the VZ had decreased expression of Sry-related HMG box 1 (Sox1) and Sox2, transcription factors required for maintenance of neural progenitor status (*8*, *19*, *20*). Lastly, GDE2NLZ-electroporated embryos showed a corresponding loss of Olig2 within the VZ, and no cells expressing both Olig2 and islet2 were detected (Fig. 4E). However, the motor neurons generated in response to GDE2 misexpression were confined to the dorsal-ventral limits of the domain containing Olig2-expressing progenitors, suggesting a prior requirement for Olig2 expression in these cells (Fig. 4E). Promoting cell-cycle exit in the developing spinal cord is not sufficient to elicit terminal differentiation of motor neurons (*19*, *21*). Our results demonstrate that GDE2 is not only capable of driving cell-cycle exit but can coordinately down-regulate progenitor determinants and promote the differentiation of motor neuron progenitors into mature motor neurons.

The presence of the GDPD domain in GDE2 supports the possibility that its catalytic activity may be required for its function. The related two-transmembrane protein GDE1 can hydrolyze glycerophosphoinositol (GPI); GPI-4, 5-bisphosphate; and glycerophosphoserine; and this activity is dependent on the integrity of the GDPD domain (*9*). The GDPD domain of GDE1 is 51% similar to the catalytic X domain of phosphoinositide phospholipase C (PI-PLC) (*22*) (fig. S6), and three amino acids essential for PI-PLC catalytic activity are conserved (*23*, *24*). One of these three amino acids, a histidine, is also crucial for GDE1-mediated hydrolysis of GPI (*9*). Because the location of this histidine residue is conserved in the GDPD domain of GDE2 (fig. S6), we altered it to alanine (GDE2H.A) and determined whether the mutated protein could still promote ectopic motor neuron differentiation. Electroporation of GDE2H.ANLZ resulted in many electroporated cells within the VZ that expressed both NLZ and GDE2 (Fig. 4, L and M). However, no motor neurons expressing islet2 were detected (Fig. 4N). Transfection of GDE2H.ANLZ into human embryonic kidney–293 cells revealed no difference in amount of expression or membrane localization compared to transfection of GDE2NLZ (fig. S7). Thus, a single amino acid change within the putative catalytic site of the GDPD domain in GDE2 is sufficient to abolish the ability of GDE2 to promote motor neuron differentiation, providing strong evidence that GDPD activity is required for GDE2 function.

The extracellular orientation of the GDPD domain raises the possibility that it may act non-cell-autonomously. To test this idea, we electroporated GDE2NLZ into chick spinal cords and analyzed the number of ectopic motor neurons expressing NLZ. If GDE2 can function non-cell-autonomously, a fraction of the HB9-expressing neurons in bin 1 (Fig. 4F) should be untransfected and lack both NLZ and GDE2 expression. Although 85% of the HB9-containing cells in bin 1 did express NLZ, 15% did not but were in direct contact with GDE2-expressing cells (fig. S8). Thus, GDE2 function appears to be primarily cell-autonomous but may also be non cell-autonomous locally, at high con-

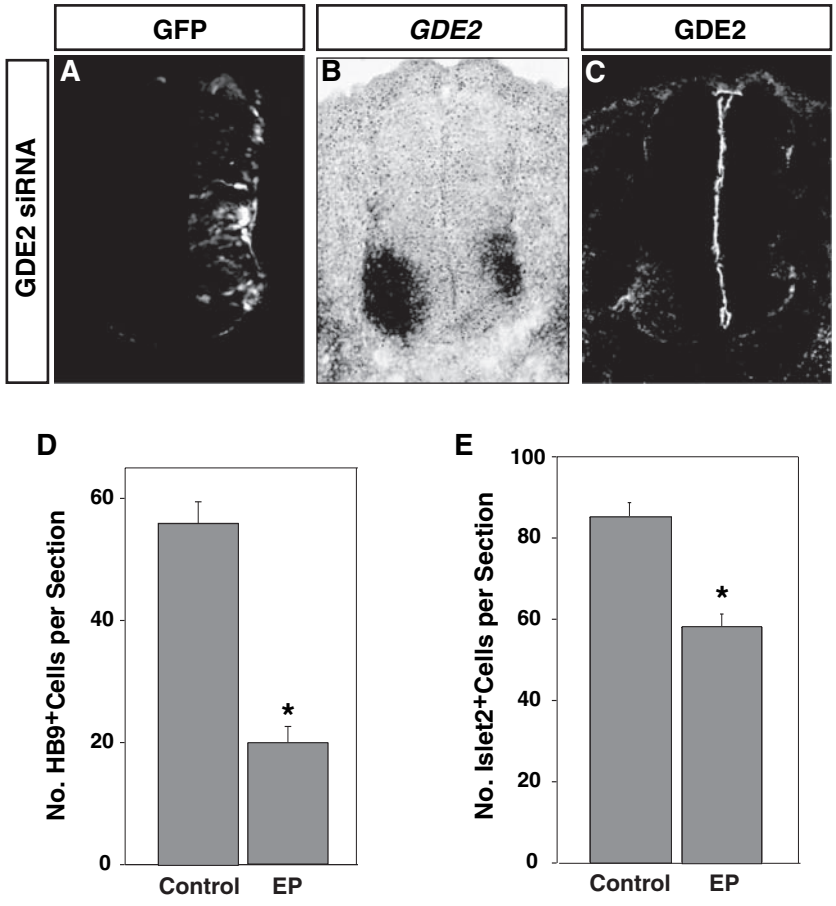

Fig. 3. Requirement for GDE2 in motor neuron differentiation. Right side of the spinal cord is electroporated. (**A** to **C**) *GDE2* mRNA (B) and protein (C) expression after electroporation of GDE2 siRNA. (**D**) Quantitation of HB9-expressing cells in electroporated (EP) and non-electroporated (control) sides of the spinal cord (n = 5, mean ± SEM). Asterisk indicates $P < 0.00000001$ (Student's t test). (**E**) Quantitation of islet2-expressing cells in electroporated (EP) and non-electroporated (control) sides of the spinal cord (n = 6, mean ± SEM). Asterisk indicates $P < 0.00000006$ (Student's t test).

centrations. Consistent with this, spinal cord explants grown in media conditioned by GDE2-expressing cells do not exhibit premature motor neuron differentiation (*8*).

We propose that paraxial mesoderm-derived RA induces expression of GDE2 in cells poised to differentiate into postmitotic motor neurons. The GDPD activity of GDE2 is required for its ability to promote cell-cycle exit and motor neuron differentiation, and this may result directly from reducing amounts of Olig2 (*5*). The extracellular location of the GDPD domain distinguishes it from other known proteins involved in lipid signaling (*22*), but the downstream pathways are unknown. One possibility is that GDE2 could act in concert with G-protein signaling pathways by analogy to GDE1, which interacts with members of the RGS (regulators of G-protein signaling) family of proteins (*25*). A related protein GDE3 induces the differentiation of osteoblast-like cell lines in vitro (*26*), raising the possibility that six-transmembrane GDPD-containing proteins may constitute a family of critical cell differentiation factors.

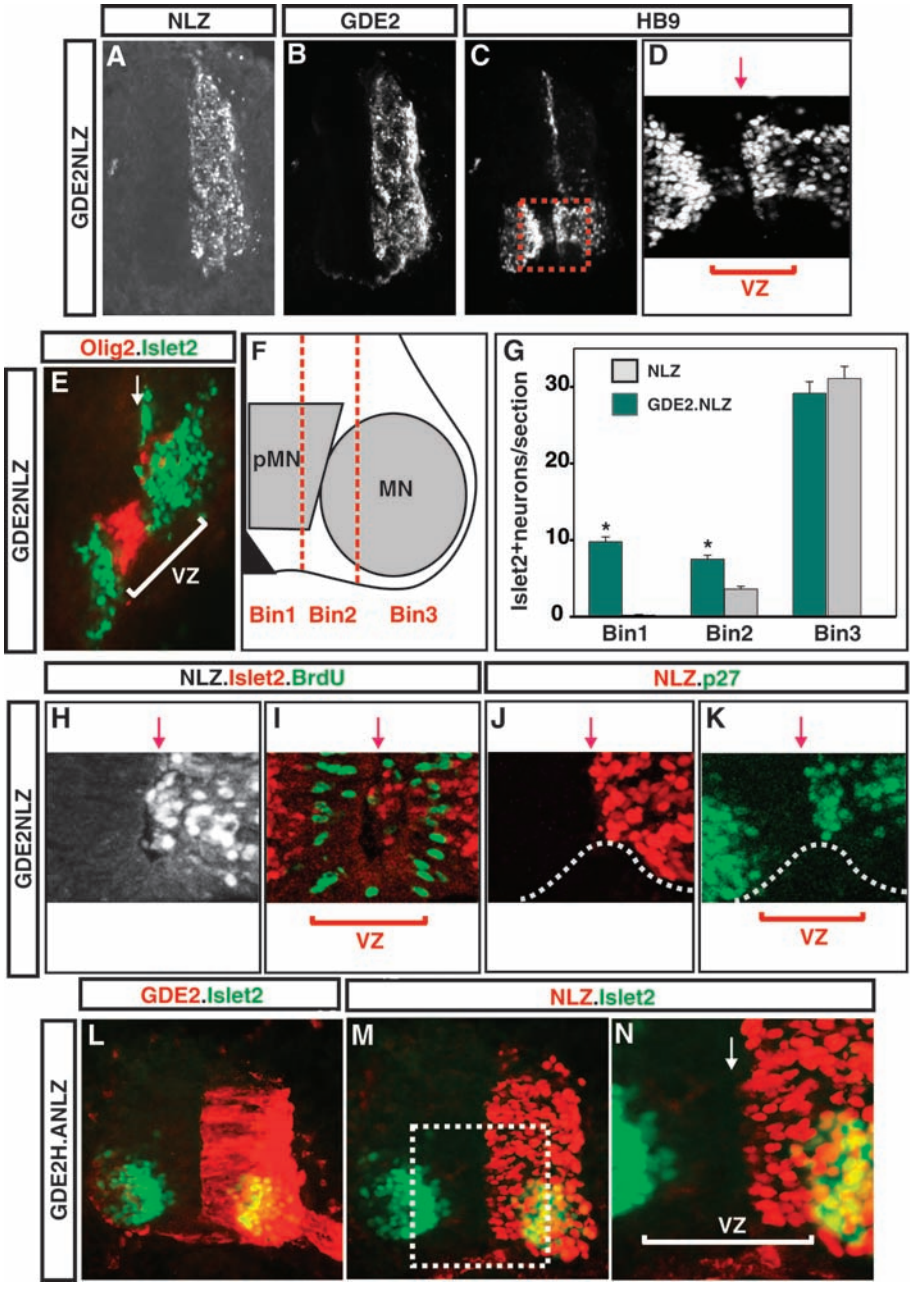

Fig. 4. Premature motor neuron differentiation induced by misexpression of GDE2. Arrows mark midline of spinal cord. Right side of the spinal cord is electroporated. (**A** to **D**) HB9 expression within the ventricular zone (VZ) after electroporation of GDE2NLZ. Boxed area in (C) is enlarged in (D). (**E**) Islet2 and Olig2 expression within the VZ after electroporation of GDE2NLZ. (**F**) Diagram of the ventral spinal cord divided into three bins: Bin 1 and bin 2 are about 20 μm wide and encompass Olig2$^+$ and Olig2$^+$/MNR2$^+$/HB9$^+$ domains, respectively. Bin 3 consists predominantly of HB9$^+$ and islet2$^+$ neurons. pMN, motor neuron progenitor; MN, motor neuron. (**G**) Number of islet2-expressing neurons located in bins 1 to 3 of embryos electroporated with GDE2NLZ versus NLZ alone (mean ± SEM, $n = 6$). With use of a Student's t test to evaluate each pair, differences between GDE2NLZ and NLZ in bins 1 (asterisk, $P < 0.00000001$) and 2 (asterisk, $P = 0.0000004$) are significant but not in bin 3 ($P = 0.396$). The total number of NLZ-staining cells is the same in both cases [bin 1: GDE2NLZ, 23 ± 1 (SEM);NLZ, 25 ± 1; asterisk, $P < 0.5$, $n = 6$] (**H** and **I**) Lack of BrdU incorporation by ectopic islet2-expressing neurons generated upon GDE2NLZ electroporation. (**J** and **K**) p27 expression within the VZ after electroporation of GDE2NLZ. Dotted lines outline the spinal cord. (**L** to **N**) Islet2 expression within the VZ after electroporation of mutant GDE2H.ANLZ. Boxed area in (M) is enlarged in (N).

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