Protein Kinase A Deficiency Causes Axially Localized Neural Tube Defects in Mice*

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We have studied the function of protein kinase A (PKA) during embryonic development using a PKA-deficient mouse that retains only one functional catalytic subunit allele, either Cα or Cβ, of PKA. The reduced PKA activity results in neural tube defects that are specifically localized posterior to the forelimb buds and lead to spina bifida. The affected neural tube has closed appropriately but exhibits an enlarged lumen and abnormal neuroepithelium. Decreased PKA activity causes dorsal expansion of Sonic hedgehog signal response in the thoracic to sacral regions correlating with the regions of morphological abnormalities. Other regions of the neural tube appear normal. The regional sensitivity to changes in PKA activity indicates that downstream signaling pathways differ along the anterior-posterior axis and suggests a functional role for PKA activation in neural tube development.

Many studies have demonstrated roles for protein kinase A (PKA) in the regulation of postnatal physiology, but limited knowledge has been gained on the function of PKA in mammalian development. Earlier studies have suggested potential requirements for PKA activity in regulating oocyte maturation in vertebrates (1, 2) and in activating the zygotic genome in pre-implantation mouse embryos (3).

Probably the most striking role of PKA in embryonic development is its negative regulation of the Hedgehog (Hh) signaling pathway. Lack of PKA activity leads to ectopic expression of Hh target genes in Drosophila imaginal discs (4). Manipulation of PKA activity in vertebrates has also suggested that the negative regulation of the Sonic hedgehog (Shh) signaling pathway is conserved (5–8). Shh signaling has been implicated in diverse processes in vertebrate development including cartilage differentiation, myotome and sclerotome specification, limb morphogenesis, and the specification of different neuronal cell types along the dorsoventral axis of the neural tube (9, 10). Whether PKA is involved in all of these processes and how PKA functions as a negative regulator (directly in the Hedgehog pathway or in a parallel pathway) are unclear.

There are two catalytic (Cα and Cβ) and four regulatory (RIα, RIβ, RIIα, and RIIβ) subunit genes of PKA identified in mice (11). These regulatory and catalytic subunits assemble into a heterotrimer composed of two C and two R subunits, and this PKA holoenzyme dissociates to release active C subunit when cAMP binds to the R subunits. Although each of the regulatory subunit genes encodes a single protein isoform, the catalytic subunit genes encode multiple variants. The Cα gene encodes two variants, Cα1 and Cα2, from two distinct promoters. In adult mice, Cα1 is expressed ubiquitously, whereas Cα2 is testis-specific (12). The Cβ gene produces three splice variants, Cβ1, Cβ2, and Cβ3. Although Cβ1 is found in all tissues examined, Cβ2 and Cβ3 are brain-specific (13). The expression pattern of PKA isoforms have also been examined by in situ hybridization in mouse embryos at late organogenesis stage, showing an expression pattern similar to that in adult mice (14).

All studies on the roles of PKA in vertebrate development have utilized transgenic or pharmacological manipulations, which have limitations in their ability to mimic the spatial and temporal patterns of endogenous activity of PKA. One way to solve this problem is to use PKA knockout mice, and we have created null mutations in each of the four regulatory and two catalytic subunits of PKA expressed in the mouse. The only single knockout to show developmental abnormalities is the RII regulatory subunit null mutation. The RII knockout mouse dies during embryonic development because of a severe defect in mesoderm formation resulting from an increase in basal PKA activity.²

The present study reports developmental consequences of decreased PKA activity in mice. A PKA-deficient mouse was generated with only one functional catalytic subunit allele, either Cα or Cβ, of PKA. The mutant mice with reduced PKA activity developed localized neural tube defects (NTDs). The spinal neural tube defect occurred at the thoracic to sacral regions of the neural tube, was 100% penetrant, and could lead to spina bifida in newborn mice, whereas exencephaly (open cranial neural tube) was partially penetrant and only present in mice with a single Cβ allele remaining. Histological examination of the abnormal spinal neural tube revealed a closed neural tube with an enlarged lumen and abnormal neuroepithelium. Marker analysis showed dorsal expansion of Shh-dependent cell types, resulting in a ventralized neuronal identity in the affected neural tube. Decreasing PKA activity also resulted in an increase in apoptotic cell death in the abnormal neuroepithelium and dorsal root ganglia, suggesting that PKA activity plays an anti-apoptotic role in the developing neural tube. All of the defects were observed in the posterior neural tube from the thoracic to sacral regions, whereas the cervical neural tube appeared normal, suggesting differential dependence on PKA activity along the anterior-posterior axis.

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The abbreviations used are: PKA, protein kinase A; C, catalytic; R, regulatory; NTD, neural tube defect; E, embryonic day; PBS, phosphate-buffered saline; DRG, dorsal root ganglia.

² P. S. Amieux, D. Howe, H. Knickerbocker, D. C. Lee, T. Su, R. L. Idzerda, and G. S. McKnight, submitted for publication.
**Neural Tube Defects in PKA-deficient Mice**

**TABLE I**

| Nos. of functional alleles of C | Genotypes | Phenotypes                  |
|--------------------------------|-----------|-----------------------------|
| 4                              | Ca⁺/⁺;CB₁⁺/⁺ | Wild-type                   |
| 3                              | Ca⁺/⁻;CB₁⁺/⁺ | Normal in appearance        |
| 2                              | Ca⁺/⁻;CB₁⁻/⁻ | Normal in appearance        |
| 2                              | Ca⁻/⁻;CB₁⁺/⁺ | Normal in appearance        |
| 1                              | Ca⁻/⁻;CB₁⁻/⁻ | Growth retardation, male sterility |
| 1                              | Ca⁻/⁻;CB₁⁻/⁻ | Normal in appearance        |
| 1                              | Ca⁻/⁻;CB₁⁻/⁻ | 100% spinal neural tube defects; 75% spinal neural tube defects; 25% spinal neural tube defects and exencephaly |
| 0                              | Ca⁻/⁻;CB₁⁻/⁻ | Early embryonic lethality   |

**Western Blots and Protein Kinase Activity Assay**—Wild type C57BL/6J embryos at E9.5 were used for dissection of the three portions of the neural tube: posterior neural tube (thoracic to sacral), anterior neural tube (cervical/brachial), and cranial neural tube. The dissection was performed in PBS, and tissues including the body wall, limb buds, heart, and the brachial arches were removed. Samples from a total of 65 embryos were pooled together and then homogenized in buffer (250 mM sucrose, 20 mM Tris, pH 7.6, 5 mM MgAc, 0.1 mM EDTA, 0.5 mM EGTA, 10 mM dithiothreitol, 1.0% Triton X-100, 10% deoxycholate sodium salt, 2 µg/ml leupeptin, 3 µg/ml aprotinin, 0.2 µg/ml soybean trypsin inhibitor, 1 mM 4(2-aminoethyl) benzenesulfonyl fluoride), sonicated, and centrifuged for 10 min at 12,000 × g at 4 °C. Supernatants were collected, and the protein concentration was measured by Bradford method (Bio-Rad). Forty micrograms of protein were loaded onto individual lanes of a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were stained with 0.2% Ponceau S before blocking overnight in blocking buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5% bovine serum albumin, 0.05% Tween 20) and probed with anti-Ca or anti-CB polyclonal antibodies. The blots were then incubated with horseradish peroxidase secondary antibody and visualized using the Amersham ECL system.

**RESULTS**

**Reduced PKA Activity Causes Neural Tube Defects Leading to Spina Bifida**—To investigate PKA function during embryonic development, we generated a PKA-deficient mouse with only one functional catalytic subunit allele by crossing the Ca and CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins. The CB1 knockout used in these studies is disrupted in exon1 preventing expression of CB1 but allowing synthesis of CB2 and CB3 in brain (13, 16). There was no significant change of PKA activity because of a compensatory increase of CB3 proteins.

**MATERIALS AND METHODS**

**Animals**—The Ca and CB1 knockout mice were generated as previously described (15, 16). Ca⁻/⁻;CB1⁻/⁻ heterozygous mice were maintained on a 129/C57BL6 mixed background. Genotyping of both Ca and CB alleles was performed on tail DNA by PCR analysis. The wild type Ca allele was detected by a pair of primers (CTGACTTTCAGGTACTGCAC and GTCGCCACACAAGTCCCAAGTA), which amplify a 250-nucleotide fragment of the intron between exons 6 and 7. The Ca knockout allele was detected by another pair of primers (CTGCATGCCTGCTTCATCGTA), which amplify a 270-nucleotide fragment of the region between the 3‘ end of the neomycin resistance gene and a portion of the intron just 3‘ to exon 8. Genotyping of the CB wild type allele was performed using the primers CB PCR-IB (GCTGATCCAGGTACTGCAC) and CB PCR 2 (CTTCAATCGCCAGCTTCCTT), which amplify a 69-nucleotide fragment in the first exon. The knockout CB1 allele was detected using the primers CB PCR-IB and CB neo-2 (ATTCCTCATCGCTCTCTCTTG), which amplify a 480-nucleotide fragment including the 5‘ portion of exon 1 and the 5‘ end of the neomycin resistance gene. PKA-deficient mice with different mutant combinations of Ca and CB alleles were generated by intercrossing Ca⁻/⁻;CB1⁻/⁻ heterozygous mice. When the vaginal plug was detected in the morning, noon of the same day was considered as E9.5 for the timing of embryos. All dissection was performed in M2 medium (Sigma). Genotyping of embryos was performed by PCR analysis using yolk sac DNA.

**Skeleton Preparation from Newborn Pups**—After removing skin and viscera, carcasses were fixed overnight in 95% ethanol and stained overnight using 0.015% alcin blue in a solution of 4 parts of 95% ethanol and 1 part of acetic acid. Samples were put back in 95% ethanol for 2–5 h and then incubated in 0.5% KOH for 4–5 days. Skeletons were stained in 0.015% alizarin red, 0.5% KOH and cleared in 0.5% KOH, 20% glycerol for about 2 days. Skeletons were stored and photographed in a 1:1 mixture of glycerol and 95% ethanol.

**Histology**—Embryos were fixed overnight in Methacarn (6 parts of methanol, 3 parts of chloroform, and 1 part of acetic acid) at room temperature and embedded in paraffin. Samples were sectioned at 8 µm and stained with hematoxylin and eosin. Sections were viewed under a Nikon microscope and photographed.

**Immunohistochemistry and Whole-mount in Situ Hybridization**—Embryos were fixed in 4% paraformaldehyde at 4 °C for 2–4 h, washed in phosphate-buffered saline (PBS), and submersed in 30% sucrose plus PBS overnight at 4 °C. Samples were embedded in O.C.T. compound (Sakura Finetek) and cryosectioned at 20 µm. Sections were washed with PBS and blocked using 10% goat serum (Zymed Laboratories Inc., San Francisco, CA) in PBS with 0.1% Triton X-100. Incubation of primary antibodies was performed at 4 °C in 1% goat serum plus PBS with 0.1% Triton X-100 overnight. Secondary antibody was fluorescein-conjugated goat IgG fraction to mouse IgG (ICN, Aurora, OH). Sections were visualized using a Bio-Rad MRC 600 confocal scanning microscope, and images were captured using COSMOS software. The antibodies used in this study are described and referenced in the Developmental Studies Hybridoma Bank data base at the University of Iowa.

**Immunohistochemistry for cleaved caspase-3** was performed according to manufacturer’s protocol (Cell Signaling Technology, Beverly, MA). The secondary antibody was biotinylated anti-rabbit IgG followed by fluorescein avidin D (Vector Laboratories, Inc., Burlingame, CA). The sections were visualized using a Leica spectral confocal microscope.

**RNA in situ hybridization** was carried out as described (17) using digoxigenin-labeled probe. Immunological detection was performed using preabsorbed anti-digoxigenin-AP Fab fragments (Roche Molecular Biochemicals) and colored with BM Purple AP substrate (Roche Molecular Biochemicals).
and only seen in embryos with a single functional Cα allele.

Embryos were examined from E8.5 to E12.5 to ascertain when the spinal column defect in PKA-deficient mice occurred during embryogenesis. The genotypes of embryos were determined by PCR analysis of DNA isolated from visceral yolk sac. Mutant embryos can be first distinguished from their wild type littermates at E9.5 by an expansion of the dorsal neural canal (data not shown). The defect was only observed in the neural tube posterior to the forelimb buds. The anterior spinal cord appeared normal. At E10.5, the neural tube defect became more obvious with varying severity in individual embryos, probably because of embryo to embryo variations in developmental stage. The most severe phenotype was observed as a blister-like bulging of the neural tube along the dorsal midline. The bulging neural tube appeared to be covered by the abnormal neuroepithelium in the PKA-deficient neural tube (Fig. 1D). The bulging neural tube appeared to be covered by epithelial tissue, which was consistent with the observation of the affected spinal column in the newborn pups. In addition, some of the Cα1−/−Cβ1−/− mutants also exhibited exencephaly (open cranial neural tube) (Fig. 1E). As summarized in Table I, all Cα1−/−Cβ1−/− embryos had only spinal neural tube defect, whereas approximately one fourth of Cα−/−Cβ1−/− embryos developed exencephaly in addition to the spinal neural tube defect. Exencephaly alone was not observed. As described below, the morphology and the neuronal patterning in the defective spinal neural tube were indistinguishable in embryos of either the Cα−/−Cβ1−/− or Cα−/−Cβ1−/− genotype.

Neural Tube Expansion and Dorsal Root Ganglion Regression in PKA-deficient Embryos—Histological examination of the developing neural tube was performed in embryos from E9.5 to E12.5. Transverse sections through the thoracic to sacral neural tube of mutant embryos at E9.5 and also some embryos at E10.5 showed a closed neural tube with an expanded alar plate and enlarged lumen (Fig. 2, A and B). The neural tube at the cervical region appeared normal (data not shown). In the longitudinal sections through the dorsal half of the neural tube, the neuroepithelium appeared expanded (Fig. 2, C and D), indicating a possible overproliferation within the dorsal neural tube at this developmental stage. A similar phenotype in the neural tube has been observed in transgenic embryos expressing a dominant negative form of PKA in dorsal aspects of the mouse central nervous system (6).

We examined bromodeoxyuridine incorporation in the spinal cord of mutant embryos to identify cells that have divided. The average number of labeled cells in sections of the affected neural tube of E9.5 and E10.5 embryos at hindlimb level was compared with that in corresponding sections of wild type controls. The result revealed no difference between the mutants and their controls in the number of proliferating cells in the whole transverse sections of neural tube (data not shown), suggesting that the aberrant morphology is not caused by overproliferation.

In embryos older than E10.5, the expansion of the neural canal increased dramatically. Transverse sections showed that the lumen was significantly enlarged and the neuroepithelium contained a higher cell density compared with wild type control (Fig. 2, E and F). In addition to the change in neural tube morphology, dorsal root ganglia (DRG) were also affected. DRG were formed in the mutant E10.5 embryos, even though they appeared disorganized (Fig. 2D), but they regressed at E12.5, suggesting that PKA activity might be required for DRG maintenance. The loss of DRG cells could indicate a possible abnormal cell death in neural crest cell derivatives, which might also occur in the neural tube.

To test the possibility of increasing apoptosis in the affected neuroepithelium and neural crest, immunohistochemistry was performed to detect activated caspase-3 in the developing neu-
PKA-deficient embryos. D, in wild type sections, apoptotic cells were rare, but they could be detected occasionally in the neural tube (yellow arrow) and in DRG (not in the section shown here). One interesting phenomenon in our study is that structures served in mutant embryos. Death may account for the regression of these structures observed in the neural tube of E10.5 embryos. Caspase-3 is one of the key executioner caspases of cell death and the activated form of caspase-3 is usually only found in cells undergoing apoptosis. In the neural tube of PKA-deficient embryos, apoptotic cells were frequently observed compared with the rare occurrence of apoptotic cells in wild type embryos (Fig. 3). Most of the apoptotic cells in the mutant were located in dorsal and/or lateral regions of the neural tube. In addition to the neural tube, a more significant increase in cell death was also detected in DRG adjacent to the affected neural tube, but not in DRG at other axial levels of PKA-deficient embryos (Fig. 3C). Despite the observed increase in apoptosis, the total number of apoptotic cells observed in the defective neuroepithelium was still very small and could easily be overcome by the large number of dividing cells in the neural tube. Therefore, the increased cell death is unlikely to be a major determinant of the neuroepithelial abnormality. The more significant increase in DRG cell death may account for the regression of these structures observed in mutant embryos.

**Differential Levels of the Catalytic Subunits of PKA along Axial Structures**—One interesting phenomenon in our study is that the neural tube defects were only detected posterior to the forelimb buds of PKA-deficient embryos. What makes the posterior part of the spinal neural tube more sensitive to the decrease in PKA activity compared with other regions? One possible reason could be that the expression of the catalytic subunits of PKA are differentially distributed along the anterior-posterior regions of the neural tube. Therefore, we examined the manifestations of Shh signaling and altered neuronal identity in the neural tube of PKA-deficient embryos. The PKA pathway has been implicated as a negative regulator of Sonic hedgehog signaling in development, and Shh signaling is a major organizer for dorsal/ventral patterning in the neural tube. Therefore, we examined the manifestations of Shh signaling and the specification of neuronal identity in the neural tube of PKA-deficient embryos at E10.5. Shh is sufficient for the induction of floor plate cells (19), which express Hnf3β and Shh itself. In PKA-deficient embryos, the expression of both Shh and Hnf3β was expanded dorsally (Fig. 5, B and F). Shh expression was detected in the ventral half of the neural tube at levels similar to a normal floor plate. The expression domain of Hnf3β was also greatly expanded in the ventral neural tube, albeit at levels lower than those found at the ventral midline (Fig. 5F). Cells with lower Hnf3β expression did not display typical floor plate morphology, i.e. a single layer with basal nuclei. Interestingly, the dorsal expansion of analyzed to quantitate the expression of C subunits. The analysis demonstrated that there was slightly less C subunit expression in the posterior neural tube compared with the anterior neural tube (Fig. 4A), and this correlated with a 20% decrease in PKA activity in the posterior compared with anterior regions (Fig. 4B). Both the results from Western blot and kinase assay indicate a modest difference in overall expression of catalytic subunits of PKA, but we believe that this is unlikely to explain the dramatic differences we see in morphology and gene expression (see below) in the affected posterior region.

The Western analysis also demonstrated that both Cα and Cβ genes were expressed as a single protein isoform, Cα1 and Cβ1, respectively, in the axial tissues of mouse embryos at E9.5. Cα2, the testis-specific isoform in adult mice, was not detectable at this developmental stage, and the brain-specific isoforms, Cβ2 and Cβ3, were also absent.

**Ectopic Expression of Shh Signaling and Altered Neuronal Identity in the Neural Tube of PKA-deficient Embryos**—The PKA pathway has been implicated as a negative regulator of Sonic hedgehog signaling in development, and Shh signaling is a major organizer for dorsal/ventral patterning in the neural tube. Therefore, we examined the manifestations of Shh signaling and the specification of neuronal identity in the neural tube of PKA-deficient embryos at E10.5.
FIG. 5. Dorsalized expression of Shh and Hnf3β in the affected neural tube at the posterior region of PKA-deficient mice, but not in the anterior neural tube. A–F, immunohistochemistry on neural tube sections of E10.5 wild type (A, C, and E) and PKA-deficient (B, D, and F) embryos. A and B, expression of Shh at the lumbar (posterior) region. C and D, expression of Shh at the cervical (anterior) region. E and F, expression of Hnf3β at the lumbar (posterior) region. G, whole-mount in situ hybridization of E9.5 embryos showing the expression of Hnf3β. Notice the dorsalized expression in the PKA-deficient embryo (left) is localized in the neural tube posterior to the forelimb buds. H, a drawing of a mouse embryo showing the regions of anterior and posterior neural tube.

Shh and Hnf3β expression was correlated only with the abnormal morphology in the affected neural tube. In the cervical neural tube, Shh and Hnf3β were expressed normally (Fig. 5, C and D; data not shown). The same localized effect was also observed with the whole mount in situ hybridization of PKA-deficient embryos at E9.5, which demonstrated that the ectopic expression of Hnf3β was localized to the region posterior to the forelimb buds (Fig. 5G).

Shh is required for the induction of motor neurons and adjacent interneuron progenitors in the ventral neural tube, which is mediated by regulating the expression of several homeodomain proteins (20–25). The dorsalized expression of Shh in PKA-deficient embryos may lead to an altered patterning of neuronal identity in the neural tube. We analyzed the pattern of ventral neuronal progenitors in the PKA-deficient neural tube by focusing on the expression of two homeodomain proteins, Pax6 and Nkx2.2. Shh signaling represses the expression of Pax6, which, in turn, represses the expression of Nkx2.2 in V3 interneuron progenitor cells at the region dorsolateral to the floor plate (24, 25) (Fig. 6, A and C). In the affected neural tube of PKA-deficient embryos, the expression of Pax6 was repressed in most of the neural tube, with some dorsal Pax6 expressing cells remaining (Fig. 6B). Consistent with this, the expression of Nkx2.2 was dorsally expanded with the dorsal boundary extending into the medial/dorsal ventricular zone of the neural tube (Fig. 6D). The dorsalized expression of Pax6 could be a consequence of ectopic activation of the Shh response in the affected neural tube. These results indicate a change of neuronal identity in the affected neural tube that leads to a ventralized neural tube, which we confirmed by examining the induction of motor neurons. Motor neuron progenitor cells, which are located immediately dorsal to V3 interneuron progenitors, express low levels of Pax6 but not Nkx2.2. The postmitotic motor neurons were detected by the expression of the homeodomain proteins Islet1/2, which mark DRG cells and differentiated motor neurons (24). In wild type mice, Islet1/2-expressing motor neurons are located immediately dorsal to V3 interneurons in ventrolateral regions adjacent to the ventricular zone of the neural tube (Fig. 6G). In PKA-deficient mice, Islet1/2-labeled motor neurons were dorsally expanded into the intermediate zone, consistent with the Nkx2.2 expansion and Pax6 repression (Fig. 6H).

Because of the appearance of ventral neuronal progenitors in the dorsal neural tube, we next examined the fate of dorsal cell types in the affected neural tube. Pax7, one of the class I homeodomain proteins (26), is normally expressed in the dorsal half of the neural tube and repressed by Shh in the ventral neural tube (Fig. 6E) (23). The ventral limit of Pax7 expression defines the ventral boundary of the dorsal neuron progenitors (26). In the affected neural tube of PKA-deficient embryos, there was no detectable Pax7 expression (Fig. 6F), indicating a significant loss of dorsal cell types.

Consistent with the observed ectopic expression of Shh and Hnf3β, the altered distribution of the neuronal cell types only occurred in the neural tube posterior to the forelimb buds in PKA-deficient embryos. In the cervical region of the neural tube, Shh and Hnf3β expression was correlated only with the abnormal morphology in the affected neural tube. In the cervical neural tube, Shh and Hnf3β were expressed normally (Fig. 5, C and D; data not shown). The same localized effect was also observed with the whole mount in situ hybridization of PKA-deficient embryos at E9.5, which demonstrated that the ectopic expression of Hnf3β was localized to the region posterior to the forelimb buds (Fig. 5G).

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Consistent with the observed ectopic expression of Shh and Hnf3β, the altered distribution of the neuronal cell types only occurred in the neural tube posterior to the forelimb buds in PKA-deficient embryos. In the cervical region of the neural tube, Shh and Hnf3β expression was correlated only with the abnormal morphology in the affected neural tube. In the cervical neural tube, Shh and Hnf3β were expressed normally (Fig. 5, C and D; data not shown). The same localized effect was also observed with the whole mount in situ hybridization of PKA-deficient embryos at E9.5, which demonstrated that the ectopic expression of Hnf3β was localized to the region posterior to the forelimb buds (Fig. 5G).

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Neural Tube Defects in PKA-deficient Mice

tube, the results of marker analysis appeared normal (data not shown). These observations indicate that the spinal neural tube is not a homogeneous structure and suggest differences in signal transduction systems along the anterior-posterior axis of the neural tube.

**Complete C-deficient Embryos Survive to Gastrulation Stage**—In our study of PKA-deficient mice, we also produced complete C-deficient embryos in which all four C alleles were mutated. From the mating between Ca+/−Cβ1+/− mice, by ratio, 1 of 16 embryos will be completely C-deficient. We examined embryos at stages E8.5 to E10.5 from timed mating of Ca+/−Cβ1+/− mice, and discovered 20 complete C-deficient embryos (Ca+/−Cβ1+/−) from 329 implantations (1/16). The expected ratio in production of complete C-deficient embryos indicated that defects in these embryos did not prevent implantation, although the complete C-deficient embryos were all in various stages of resorption. It is unlikely that maternal PKA activity is sufficient to sustain development up to the gastrulation stage. Either PKA activity is not absolutely required for very early development or other related kinases are able to partially compensate. For instance, PrRXX, an X chromosome-linked kinase related to catalytic subunits of PKA, has been shown to interact with RI subunits of PKA, suggesting that PKA is also important in neural tube, the results of marker analysis appeared normal (data not shown). These observations indicate that the spinal neural tube is not a homogeneous structure and suggest differences in signal transduction systems along the anterior-posterior axis of the neural tube.

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**Discussion**

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**Neural Tube Defects in PKA-deficient Mice**—NTDs are common congenital abnormalities in humans with an occurrence of 1 in ~1000 births. The etiology of neural tube defects is multifactorial, and both genetic and environmental factors are involved. Mouse models have been used to study the embryonic mechanisms and genetic basis of neural tube defects (28, 29), and these studies have uncovered a heterogeneous collection of genes that may be involved.

More than 60 mouse mutants have been reported to exhibit neural tube defects (29, 30). However, most of them also have syndromes involving other tissues that cause early embryonic lethality. In these mutants, NTDs are likely to be secondary to primary defects in other developmental systems. In a few mutants that survive to later stages of embryogenesis, mutations are likely to be more specific to neurulation and cause defects in the neural tube directly. For example, Splotch (Sp) mice resulting from mutation of the Pax3 gene develop neural tube defects with varying penetrance during neurulation and die by E13 because of cardiac defects (31). Targeted disruption of p53 (coding for a tumor suppressor) (32, 33), MARCKS (a substrate of protein kinase C) (34), or ApoB (a transporter protein) (35) genes all lead to exencephaly in 20–30% of embryos. These mutants can survive to birth and are not severely defective in other developmental systems except for the neural tube defects.

The PKA-deficient mice examined in this study exhibit neural tube defects without other apparent malformation, suggesting that PKA can play a specific role in neural tube formation. In contrast to the low penetrance of NTDs in other mutants, PKA-deficient mice develop spinal neural tube defects with 100% penetrance. Some mice that retain one functional Ca allele are born alive without other apparent malformations except spina bifida at their thoracic and lumbar regions. Mice that retain one functional Cβ allele display a more profound developmental abnormality that includes exencephaly in ~25% of embryos as well as the characteristic NTD described above for the mice with a single Ca allele.

In many mouse mutations, spina bifida presents as a severe form in which both vertebral arches and the neural tube fail to close. Other less severe forms of spina bifida are characterized by normal closure and development of the neural tube but open vertebral arches (30). In general, the more severe form is a result of malformation caused primarily by a neural defect, whereas the other forms of spina bifida may be caused by mesodermal defects. The anatomy of a closed but defective neural tube in PKA-deficient mice appears to represent a novel category. The abnormal neuroepithelium and the altered differentiation of neuronal precursors in the neural tube indicate that the primary defect in PKA-deficient embryos is affecting the developing neuroepithelium. We propose that the dramatic expansion of the closed neural tube causes failure of the vertebral arches to fuse in PKA-deficient mice. These special features make the PKA-deficient mice an interesting model for the study of neural tube defects.

**Increased Apoptotic Cell Death in the Affected Neural Tube of PKA-deficient Mice**—Apoptosis is a highly regulated cellular process that is essential for embryonic development. The PKA signaling pathway has been implicated in the regulation of apoptosis by modulating the levels and/or activities of Bcl-2 and related proteins, and this may depend on the isoforms of PKA expressed, their subcellular localization, and availability of specific substrates (36). Studies have suggested that PKA action is at a site upstream of caspase-3 (37, 38). Therefore, we used activated caspase-3 as a marker for apoptotic cells and observed an increased incidence of cell death in the neural tube and DRG in PKA-deficient mice. This suggests that PKA activity serves as an anti-apoptotic signal in the developing neural tube. Although apoptosis increases in the PKA-deficient neural tube, this is unlikely to be the primary mechanism leading to the neural tube defect and is more likely the result of mis specification of neuronal cell types caused by induction of Shh signaling earlier in development.

**Dorsalized Shh Signaling in PKA-deficient Neural Tube**—Shh is a notochord and floor plate-derived signal required to pattern the ventral neural tube and induce the formation of motor neurons and ventral interneurons (21–23, 39). The induction of different cell types in the ventral neural tube is controlled in a concentration-dependent manner. Cells positioned in progressively more ventral regions are exposed to higher Shh concentrations, and this triggers differentiation into distinct cell types (21, 24, 25).

Shh signal response can be negatively regulated by PKA. Either too much or too little PKA activity can result in changes in gene expression and tissue patterning by modulating the Shh signaling pathway (5–8). In the developing neural tube, expression of a dominant-negative regulatory subunit of PKA in dorsal aspects of the central nervous system activated Shh signaling in the region of the dorsal midbrain and mid/hindbrain junction. This resulted in the dorsal activation of Hnfβ/
and Shh, aberrant motor neuron induction, and ectopic expression of Patched (Ptc) and Gli1 (6). In our PKA-deficient mice, we have observed a similar effect resulting in dorsal expansion of Shh-dependent cell types and ventralization of the neural tube. This dorsal expansion of Shh-dependent response includes Shh itself, which can be found in a significantly expanded domain. Therefore, we cannot distinguish between a cell autonomous or nonautonomous mechanism explaining the expansion of the Shh-dependent cell types. The increased Shh response in ventral plate cells induces them to adopt a more ventral fate, including V3 interneurons and motor neurons. Subsequently, cells positioned in the dorsal neural tube are induced to differentiate into ventral cell types. Because the expression of the dorsal marker Pax7 is undetectable, it is likely that the dorsal cell progenitors are never induced. Despite the marked ventralization of neuronal cell types, the relative position of different gene expression domains remained, suggesting that the mutually repressive interaction between class I and class II homeoproteins (26) is maintained in the PKA-deficient neural tube. Moreover, not all of the cells that express Hnf3β in the ventral neural tube display the floor plate morphology. A possible explanation is that the floor plate cells are induced early in development and cells in the ventral neural tube of PKA-deficient mice are induced to express Hnf3β at later stages, when they are no longer capable of adopting the floor plate cell fate. This is in contrast to what has been observed in Patched mutants, in which all cells expressing Shh and Hnf3β are floor plate cell-like (40).

The dorsalized Shh signaling was only observed in the affected neural tube at the thoracic to sacral regions. In the head and cervical regions that showed wild type morphology, the distribution of Shh signaling and pattern of the neuronal cell types remained normal. The strong correlation between the dorsalized Shh signaling and the neural tube defect suggested that the ectopic expression of Shh itself might be responsible for the neural tube defect. Alternatively, the PKA deficiency may elicit downstream signaling events that lead to changes in gene expression in a completely Shh-independent manner. This could be explored further by producing PKA-deficient embryos on a Shh null background.

Studies have suggested several downstream components in the Shh pathway as potential targets for PKA phosphorylation. For instance, Smoothered (Smo) contains a cluster of putative PKA sites in its C-terminal cytoplasmic domain and PKA could target Smo for degradation by direct phosphorylation (41). Studies have also shown that Gli2 and Gli3, two major mediators of Shh signals, can be phosphorylated upon PKA stimulation (42). Phosphorylation of Gli3 has been shown to promote its proteolytic processing to generate a repressor form of the transcription factor (42). A recent study of Shh and Gli3 double mutants showed that a null mutation in Gli3 can partially rescue the mutant phenotypes of a Shh knockout embryo (43). However, it is interesting to note that this rescue of ventral cell fates was dependent on position along the rostral-caudal axis. Motor neurons were substantially rescued only in the lumbar region, and the rescue of V0 and V1 interneurons was also much greater in this region. Because PKA activity regulates the formation of Gli3 repressor, it seems likely that PKA deficiency is mimicking the Gli3 knockout by preventing proteolytic processing to the repressor form. Normally, Gli3 might function as a weak activator of Shh signaling in the ventral neural tube, and studies have shown that Gli3 is expressed throughout the neural plate (44, 45). It is possible that PKA phosphorylates Gli3, thus promoting the conversion of Gli3 from a weak activator to a repressor in the dorsal neural tube, whereas this conversion is blocked by Shh signaling in the ventral neural tube. Therefore, in PKA-deficient mice, the level of the repressor form of Gli3 could be reduced in the dorsal neural tube, resulting in changes in neuronal specification that mimics Shh signaling. However, reducing the level of Gli3 repressor alone is unlikely to be sufficient to cause a defect in the neural tube because mice lacking Gli3 show no ectopic activation of Shh signaling and no discernible phenotype in the spinal cord (46, 47). Perhaps, other factors that interact with Shh signaling, such as BMP and Wnt (48), are also modulated by PKA activity. Although endogenous processing of Gli2 has not been demonstrated, it has been shown that an N terminus-deleted form of Gli2, but not full-length Gli2, can induce Shh-dependent cell types in the dorsal neural tube when ectopically expressed (49). It is possible that PKA deficiency may lead to an increase in Gli2 activator levels.

The increased Shh signal response seen in the PKA-deficient mice is consistent with previous work in Drosophila, zebrafish, and mice. However, the localized effect in a discrete region of the neural tube suggests that PKA-dependent modulation of Shh signaling differs qualitatively along the posterior to anterior axis. Our dissection of the neural tube does not show significant changes in the expression of PKA catalytic subunits from posterior to anterior regions, suggesting that the sensitivity of the thoracic to sacral region is not the result of differential levels of PKA activity. Although the large changes in PKA activity produced in PKA-deficient mice lead to developmental defects, it is likely that smaller changes in PKA activity are normally regulating neural precursor differentiation during development. This suggests that as yet unknown factors may be interacting with receptors to regulate intracellular cAMP and modulate developmental signals such as Shh.

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