Enhanced expression of collapsin response mediator protein 4 in spinal cords of rat fetuses with spina bifida aperta

Feng-Hua Zhou¹, Yang Fan², Xiao-Wei Wei², Li-Li Wang², Jia-Ning Miao², Hui Li¹, Li-Ping Shan³, Zheng-Wei Yuan²

Departments of ¹Physical Medicine and Rehabilitation, ²Urologic Surgery, Shengjing Hospital, China Medical University, Shenyang 110004, P. R. China; ³Key Laboratory of Health Ministry for Congenital Malformation, Shengjing Hospital, China Medical University, Shenyang 110004, P. R. China

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Abstract: Neural tube defects (NTDs) are severe congenital malformations that result from incomplete neurulation of the central nervous system. While collapsin response mediator protein 4 (CRMP-4) plays a critical role in the regulation of neurulation, information regarding the temporal expression of CRMP-4 during embryonic development of the spinal cord has yet to be reported. We therefore investigated the expression pattern of CRMP-4 in fetal spinal cords of healthy rats and of rats with spina bifida from prenatal day (E) 11 to postnatal day 1 (P0). Specifically, the mRNA and protein levels of CRMP-4 were measured in spinal cord tissues harvested from healthy and all-trans retinoic acid (atRA)-induced spina bifida rat fetuses. For these experiments, spina bifida was induced at discrete stages of neural development, including at E11, E12, E13, E15, E17, and P0. Expression of CRMP-4 mRNA and protein was detected in the spinal cords of both normal fetuses and fetuses with spina bifida as early as E11 and E12, respectively. The expression levels then gradually increased throughout embryonic development and peaked at E15 and P0, respectively. Notably, both the protein and mRNA levels of CRMP-4 were significantly enhanced in the spinal cords of fetuses with spina bifida, compared to the levels detected in healthy fetuses. Furthermore, the expression of CRMP-4 was time-dependent and occurred at a distinct stage of normal embryonic development. Lastly, CRMP-4 was overexpressed at both the mRNA and protein levels in the spinal cord tissue of fetuses with spina bifida, suggesting that CRMP-4 may contribute to the pathogenesis of this disease by regulating neurite outgrowth and neuronal apoptosis.

Keywords: Spina bifida aperta, collapsin response mediator protein 4 (CRMP-4)

Introduction

Neural tube defects (NTDs) are severe congenital malformations of the central nervous system that result from incomplete neurulation. Anencephaly, exencephaly, and spina bifida are the most common forms of NTDs, affecting approximately 0.6-6 in 1000 live births worldwide [1]. While fetal surgical repair of NTDs reduces the incidences of shunt-dependent hydrocephalus and hindbrain herniation, most patients still suffer from neurological deficits, including motor and sensory defects in the legs and urinary and fecal incontinence, and require long-term care and assistance. Our previous research indicates that these clinical presentations might result from developmental defects in motor, sensory, and ganglion neurons [2, 3], which may be linked to changes in the cellular processes of neuronal development [4]. Neuronal development is tightly regulated by intricate molecular networks. Factors that impact the proliferation and/or differentiation of neural progenitor cells are likely involved in the neuronal defects associated with NTDs [5, 6].

Animal models are essential for studying the etiologic mechanisms of birth defects. Drug-induced animal models are particularly advantageous in that they exhibit deformities similar to those observed in humans, high rates of teratogenic effects, and high levels of reproducibility. The all-trans retinoic acid (atRA)-induced rat
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model is one of the most widely used animal models for studying NTDs [7, 8]. Retinoic acid, which is the oxidized form of Vitamin A, controls anterior/posterior patterning during early developmental stages [9]; however, prenatal exposure of rat embryos to this compound induces severe malformations that result in the development of NTDs. The atRA-induced rat model therefore provides a powerful experimental system for characterizing the developmental, pathological, and molecular mechanisms of the neural tube closure processes, as well as the interactions between protein and environment that contribute to the multifactorial cause of NTDs.

Neural tube closure is a dynamic process that is mediated by a network of protein-protein and protein-DNA interactions that organize the appropriate spatial and temporal expression of various factors involved in neurulation. Meanwhile, NTDs are complex disorders that involve both genetic and environmental factors. However, both abnormal gene expression and environmental teratogenic factors are eventually manifested as abnormalities in protein synthesis or function.

To understand the factors involved in NTD development, we performed a 2-D gel electrophoresis-based proteomics assay and detected increased expression of the collapsin response mediator protein (CRMP) family member CRMP-4 in the fetal spinal cords of rats with spina bifida at prenatal day (E) 17 [10]. The CRMP family, which includes important cytosolic phosphoproteins, is comprised of 5 homogeneous isoforms: CRMP-1, CRMP-2, CRMP-3, CRMP-4, and CRMP-5. Recent studies indicate that CRMPs play critical roles in neuronal proliferation, differentiation, axonal guidance, neurite outgrowth and elongation, and signal transduction [11-13]. Furthermore, CRMP-4 is highly expressed in the dorsal root ganglion (DRG) and spinal cord during embryo development. Although the mechanisms by which CRMP-4 contributes to embryonic development are unclear, several studies indicated that CRMP-4 is essential for the regulation of neurite and dendrite elongation and branching [14, 15]. In addition, several studies have demonstrated that CRMP-4 is also involved in the degenerative or regenerative processes involved in neuritis, including the regulation of neuronal apoptosis, axonal degeneration, and axonal death [16-20]. While the role of CRMP-4 during embryo development has begun to be elucidated, the time course of CRMP-4 expression during embryonic spinal cord development has yet to be reported. In the current study, we addressed this question by examining the expression pattern of CRMP-4 in the spinal cords of healthy rat fetuses and fetuses with spina bifida between E11 and post-natal day 1 (P0).

Methods

Fetal rat spina bifida model

All animal experiments were approved by the local ethics committee. Outbred 10-12-week-old Wistar rats (250-300 g) were purchased from the animal center of China Medical University. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University. The appearance of vaginal plugs in female rats on the morning after mating was considered E0. A total of 36 pregnant rats were divided randomly into two groups: the atRA treatment group and the control group (n=18 for each). Spina bifida aperta was induced with a single intragastric injection of atRA (4% wt/vol in olive oil; 140 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) at 8:00 am on E10, as previously described [10, 21]. Animals receiving the control treatment were injected with an identical amount of atRA-free olive oil on the same day. Pregnant rats were then sacrificed at E11, E12, E13, E15, E17, and P0 (the day of birth) via administration of an overdose injection of 10% chloral hydrate into the abdominal cavity. The embryos were examined microscopically for the presence of deformities, and quickly collected in ice-cold phosphate buffered saline (PBS).

For immunoblotting and real-time quantitative PCR (qRT-PCR) analysis, spinal cords were isolated from 9 fetuses in both the spina bifida and control groups at each time point (3 from each group were used for immunoblotting and 6 for qRT-PCR analysis) and stored at -80°C. For immunohistochemistry analysis, 3 embryos with spina bifida and 3 control embryos were
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harvested at E15 and E17 and fixed in freshly prepared 4% paraformaldehyde (in PBS). Embryos were subsequently processed and embedded in paraffin, and serial transverse sections (2.5 µm) were cut through the lumbo-sacral regions with a microtome (Thermo, Walldorff, Germany). At each time point, embryos in each group were harvested from at least 3 independent dams.

Quantification of mRNA by qRT-PCR

Total RNA was harvested from the posterior spinal cord using TRIzol reagent (Invitrogen, Waltham, MA, USA), according to the manufacturer’s protocol, and RNA concentrations were determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). RNA samples with an A260 nm/A280 nm ratio less than 1.8 were discarded. For analysis of CRMP-4 (accession number: AF389425) mRNA expression levels, total RNA (3 µg) was mixed with random 6-mer oligos (50 pmol), oligo dT primer (25 pmol), and the components of the PrimeScript RT Reagent Kit (TaKaRa Bio, Inc., Shiga, Japan) in 20 µL reactions, and reverse-transcribed into cDNA by incubating at 37°C for 15 min, 85°C for 5 sec, and then at 4°C. Diluted cDNA (1:10) was then subjected to qRT-PCR analysis using a SYBR® Premix Ex Taq™ II Kit (TaKaRa) and the following primers: forward, 5'-ACGGTGATGGCACGGAACA-3'; reverse, 5'-CCCAGGAGCAGGCACGAAT-3'. The reaction mixtures contained 2 µL of cDNA templates, 0.4 µM of each primer, 10 µL of 2×SYBR Green Master Mix, and were brought to a final volume of 20 µL with RNase-free water. Reactions were performed in triplicate using a 7500 Fast Real-time PCR System (Applied Biosystems, Waltham, MA, USA) with the following program: pre-denaturation at 95°C for 30 sec followed by 45 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 20 sec. Reaction mixtures lacking reverse transcriptase were used as a negative control. The relative levels of CRMP-4 mRNA in each sample were calculated using the 2−ΔΔct method and were expressed as a fold induction relative to the levels detected in the control spinal cords at E11. The expression levels of β-actin (accession number: NM_031144; Primer sequences: forward, 5'-GGAGATTACTCCTGGCTCCA-3'; reverse, 5'-GACTCATCGTACTCCTGCTG-3') were used for normalization.

Immunoblot analysis

Protein extracts were generated by suspending fetal posterior spinal cords in lysis buffer (7 mM urea, 2 mM thiourea, dithiothreitol [DTT], and phenylmethylsulfonyl fluoride [PMSF]), and total protein concentrations were quantified using an Enhanced BCA Protein Assay Kit (Beiyotime, P0010, Haimen, China). Aliquots of protein extracts (50 µg) were mixed with sample buffer, incubated at 95°C, and separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA) using a Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA, USA) and Tris-HCl methanol buffer (20 mM Tris, 150 mM glycine, and 20% methanol). Membranes were then washed with PBS containing 0.05% Tween 20 and incubated for 2.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit (Santa Cruz Biotechnology, Dallas, TX, USA) or goat anti-actin primary antibody (1:2000; Santa Cruz Biotechnology) overnight at 4°C. Membranes were then washed with PBS containing 0.05% Tween 20 and incubated for 2.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit (Santa Cruz Biotechnology) or anti-goat IgG antibody (CWBIO, Beijing, China). Antibody complexes were visualized using enhanced chemiluminescence (ECL) reagents (GE Healthcare, Little Chalfont, UK), and protein bands were quantified with GelPro4.0 software (Media Cybernetics, LP, Rockville, MD, USA). The relative density of each protein band was calculated by dividing the optical density value of each protein by that of the loading control (β-actin).

Immunohistochemistry

Immunohistochemical staining of CRMP-4 was performed on transverse sections of the lumbo-sacral spinal cords of E15 and E17 embryos. Sections were de-waxed in xylol, rehydrated in decreasing concentrations of alcohol, and then subjected to microwave antigen retrieval (10 min in 0.1 M citrate acid buffer solution [pH 6]). Sections were blocked with 0.3% hydrogen peroxide and PBS containing 10% fetal calf serum (FCS) and 0.1% Triton X-100. Sections were then incubated with a CRMP-4-specific rabbit antibody (1:500; Santa Cruz Biotechnology) in 10% FBS overnight at 4°C. After washing, sections were incubated with peroxi-
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**Figure 1.** Spina bifida model of rat embryo at E13 and E17. Embryos from atRA-treated pregnant rat showed spina bifida aperta (arrow). SB, spina bifida; N, normal control.

**Figure 2.** Relative expression of CRMP-4 mRNA (VS. E11) and protein (Relative optical density) in spinal cords of normal fetuses with embryonic development stage.

Results

atRA treatment induces neural tube defects in a rat model of spina bifida

A total of 151 live embryos were harvested from the 18 pregnant rats that received atRA treatment; embryos were harvested at E11, E12, E13, E15, E17, and P0. Gross morphologic examination with a stereomicroscope revealed that 79 of these embryos (52.3%) exhibited spina bifida aperta in the lumbosacral region (Figure 1). In contrast, none of the 172 embryos harvested from the control rat group exhibited signs of the disease.

Expression of CRMP-4 in spinal cords of normal fetuses

To investigate the temporal expression pattern of CRMP-4 during embryonic spinal cord development, we measured the mRNA and protein levels of CRMP-4 in the spinal cords of fetuses at multiple developmental stages, including E11, E12, E13, E15, E17, and P0. CRMP-4 mRNA was detected as early as E11 in normal rat fetuses, and the levels of this protein gradually increased throughout embryonic development (from E11 to P0), peaking at E17 (Figure 2). Specifically, CRMP-4 mRNA levels increased gradually between E11 and E13 and sharply between E15 and E17, before decreasing at P0. Notably, the CRMP-4 mRNA levels were significantly higher between stages E12 and P0 than that at E11. Meanwhile, immunoblot analyses demonstrated that CRMP-4 protein levels followed a slightly different trend than the mRNA levels. The CRMP-4 protein was detected as early as E12 in normal rat fetuses.
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Table 1. Temporal expression of CRMP-4 mRNA (relative gene expression) in spinal cords of rat fetuses by qRT-PCR

<table>
<thead>
<tr>
<th>E11</th>
<th>E12</th>
<th>E13</th>
<th>E15</th>
<th>E17</th>
<th>P0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>1.003±0.100</td>
<td>1.000±0.078</td>
<td>1.215±0.338</td>
<td>1.048±0.337</td>
<td>1.022±0.250</td>
</tr>
<tr>
<td>Spina bifida Group</td>
<td>0.817±0.095</td>
<td>1.950±0.072</td>
<td>2.410±0.762</td>
<td>3.245±1.074</td>
<td>2.258±0.413</td>
</tr>
</tbody>
</table>

Control group indicates rat fetuses without aTRA treatment, and spina bifida group indicates those with spina bifida aperta by aTRA treatment. *P<0.05 vs. control group; #P<0.01 vs. E11; *P<0.01 vs. E12; †P<0.01 vs. E13; ‡P<0.05 vs. E15; §P<0.05 vs. E17.

CRMP-4 expression is elevated in the spinal cords of rats with spinal bifida

As was the case in the normal fetuses, CRMP-4 mRNA and protein was first detected in the spinal cords of rat fetuses with spina bifida at E11 and E12, respectively. However, both the protein and mRNA levels of CRMP-4 were significantly enhanced in the spinal cords of fetuses with spina bifida, compared to those of the healthy fetuses (Table 1, Figures 3, 4). Specifically, while the spinal tissues from both groups exhibited similar levels of CRMP-4 mRNA at E11, there were significantly higher mRNA levels of CRMP-4 in the spinal cords of fetuses with spina bifida than in those of the control fetuses at E12, E13, E15, E17, and P0. The temporal expression trends of CRMP-4 mRNA and protein in fetuses with spina bifida at different embryonic stages were consistent with those of the control fetuses. To detect the distribution of CRMP-4 protein in the developing spinal cord, both normal rat fetuses and fetuses with spina bifida were subjected to immunohistochemistry staining at E15 and E17. CRMP-4 protein was primarily distributed in the spinal cord cortices and in the dorsal root ganglia in both the control and spina bifida sections. Compared with normal spinal cords, however, those from rats with spina bifida exhibited significantly higher levels of CRMP-4 protein expression (Figure 5).
Nervous system development is a complicated process that involves the proliferation of neuronal precursor cells and the differentiation, migration, and extension of axons to the appropriate synaptic targets. Recent studies indicated that CRMPs contribute to axonal guidance during neural development by inhibiting growth-cone extension. Despite numerous studies of CRMP-4, the functional role of this protein has yet to be fully characterized. Recent studies, however, showed that CRMP-4 contributes to the regulation of neurite and axon outgrowth [15, 22], and to neuron development [15, 19, 20, 23].

Despite the important role played by CRMP-4 in promoting normal neural network formation, abnormally high levels of CRMP-4 expression may be the principal cause of neuronal death associated with pathological conditions. CRMP-4 is expressed primarily during embryonic development, and during this period is expressed exclusively in the nervous system. Previous studies demonstrated that CRMP-4 is expressed at distinct stages of neural development. For example, CRMP-4 is expressed in the central and peripheral nervous systems at E17. Notably, however, CRMP-4 was shown to exhibit a distinct distribution pattern, being expressed at high levels in the dorsal root ganglia and throughout the spinal cord [24]. Consistent with these findings, our results demonstrated that both CRMP-4 protein and mRNA were expressed at discrete stages of spinal cord development, and as early as E12, in both normal fetuses and fetuses with spina bifida. Furthermore, CRMP-4 protein was primarily distributed in the spinal cord cortexes and dorsal root ganglia of both normal and spina bifida-induced fetuses. However, both the protein and mRNA levels of CRMP-4 were significantly higher in the spinal cords of fetuses with spina bifida than in those of normal fetuses, suggesting that CRMP-4 may contribute to the pathogenesis of this disease.

Previously, there was a lack of comprehensive information regarding the expression pattern of CRMP-4 during embryonic development. To address this gap in knowledge, we examined CRMP-4 expression in rat fetuses with or without spina bifida at distinct stages (between E11 to birth). In this study, the expression of CRMP-4 mRNA and protein was time-dependent. CRMP-4 mRNA was detected as early as E11 in the spinal cords of both normal fetuses and fetuses with spina bifida, and the levels gradually increased throughout embryonic development. Notably, however, while peak levels of CRMP-4 mRNA were detected in normal fetuses at E17, such levels were detected in fetuses with spina bifida at E15. Meanwhile, the pattern of CRMP-4 protein expression was similar to that of mRNA expression. However, the stages at which peak protein levels were detected were notably later than those at which the peak mRNA levels were detected; in both the normal fetuses and the fetuses with spina bifida, the expression of CRMP-4 protein peaked at P0. Together, the temporal expression patterns observed in this study are consistent with the characterized role of CRMP-4 during neurogenesis, and suggest that abnormally high levels of CRMP-4 may contribute to the process of neural tube closure and to the pathogenesis of spina bifida.

While the molecular mechanisms that mediate neural tube closure are poorly understood, a recent study indicated that the planar cell
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Polarity (PCP) pathway is important in neural tube development [25]. This pathway plays a key role in convergent extension, which is required to ensure sufficiently close proximity of neural folds to allow for the initiation of tube closure during neurulation. The so-called Dishevelled (DVL) protein is the key signaling molecule of the planar cell polarity pathway. Dishevelled contributes to the regulation of downstream effectors, which act on the cytoskeleton and induce cellular polarizing events, through activation of RhoA [26]. Furthermore, CRMP-4 is a substrate of RhoA, and these two proteins were shown to colocalize within the neural growth cone and to regulate actin cytoskeletal dynamics. Recent studies [14] have also shown that CRMP-4 mediates axonal inhibition via RhoA. Meanwhile, a previous report demonstrated that siRNA-mediated knockdown of CRMP-4 expression attenuated the inhibition of neurite outgrowth, and that CRMP-4 exhibited a functional interaction with RhoA during axonal outgrowth and axonal protection [27]. These findings indicate that CRMP-4 may be a common regulatory factor that is shared by the planar cell polarity and Rho signaling pathways.

Furthermore, CRMP-4 plays a regulatory function in the cytoskeletal dynamics of neuronal cells to control axonal growth, which may contribute the pathogenesis of spina bifida. Lastly, a recent report [17] demonstrated that overexpression of CRMP-4 in lumbar motoneurons resulted in the inhibition of neurite outgrowth, followed by cell death, while inhibition of CRMP-4 expression prevented cell death and promoted synaptic regeneration.

In this study, we specifically demonstrated that CRMP-4 expression was time-dependent and occurred at a distinct stage of normal embryonic development. Furthermore, we demonstrated that both the mRNA and protein levels of CRMP-4 were enhanced in the spinal cords of fetuses with spina bifida. We therefore propose that CRMP-4 contributes to the pathogenesis of spina bifida by regulating neurite outgrowth and neuronal apoptosis.

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Figure 5. CRMP-4 immunohistochemistry in spinal cord of rat fetuses. Transverse sections of spinal cords stained for CRMP-4 in control and spina bifida fetuses at E15 and E17. CRMP-4 protein was distributed in spinal cord cortex and dorsal root ganglion. The CRMP-4 protein expression in spinal bifida fetuses were significantly higher compared with normal fetuses. *P<0.05 vs. control group.
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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zheng-Wei Yuan, Key Laboratory of Health Ministry for Congenital Malformation, Shengjing Hospital, China Medical University, No. 36 Sanhao Street Heping District, Shenyang 110004, China. Tel: +86 024 23929903; Fax: +86 024 23929903; E-mail: ZhengweiYuancn@163.com

References

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