Early neonatal loss of inhibitory synaptic input to the spinal motor neurons confers spina bifida-like leg dysfunction in a chicken model

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Summary statement
Consistent with the symptoms of human with SBA, chickens with SBA-like features exhibit lower-limb paralysis within two weeks after hatching which appeared to coincide with synaptic change-induced disruption of spinal motor networks.
Abstract

Spina bifida aperta (SBA), one of the most common congenital malformations, causes lifelong neurological complications particularly in terms of motor dysfunction. Fetuses with SBA exhibit voluntary leg movements in utero and during early neonatal life but these disappear within the first few weeks after birth. However, the pathophysiological sequence underlying such motor dysfunction remains unclear. Additionally, because important insights have yet to be obtained from human cases, an appropriate animal model is essential. Here, we investigated the neuropathological mechanisms of progression of SBA-like motor dysfunctions in a neural tube surgery-induced chicken model of SBA at different pathogenesis points ranging from embryonic to post-hatch ages. We found that chicks with SBA-like features lose voluntary leg movements and subsequently exhibit lower-limb paralysis within the first 2 weeks after hatching, coinciding with the synaptic change-induced disruption of spinal motor networks at the site of the SBA lesion in the lumbosacral region. Such synaptic changes reduced the ratio of inhibitory-to-excitatory inputs to motor neurons and were associated with a drastic loss of γ-aminobutyric acid (GABA)ergic inputs and upregulation of the cholinergic activities of motor neurons. Furthermore, most of the neurons in ventral horns which appear to be suffering by excitotoxicity during the early postnatal days, underwent apoptosis. However, the triggers of cellular abnormalization and neurodegenerative signaling were evident in the middle to late gestational stages, probably attributable to the amniotic fluid-induced in ovo milieu. In conclusion, we found that early neonatal loss of neurons in the ventral horn of exposed spinal cord affords novel insights into the pathophysiology of SBA-like leg dysfunction.
Introduction

Spina bifida aperta (SBA), also known as myelomeningocele, one of the most common congenital malformations that causes lifelong disability, develops in approximately 1/1,000 neonates worldwide; the lifetime cost is more than US$600,000 (Copp et al., 2015). SBA is characterized primarily by defective fusion of the neural tube, which causes in utero damage to the exposed spinal cord (Osaka et al., 1978; Copp et al., 1990; Heffez et al., 1990; Millicovsky and Lazar, 1995). Fetuses with SBA exhibit voluntary leg movements in utero and during early neonatal life but these disappear within the first few weeks after birth (Korenromp et al., 1986; Sival et al., 1997, 2004, 2006, 2008). In utero surgical closure of the exposed cord seeks to preserve spinal tissue and improve motor dysfunction (Meuli et al., 1995a, 1996; Sival et al., 1997; Adzick et al., 1998; Tulipan et al., 1999); the procedure reduces some SBA-related neurological complications but does not preserve motor function in humans (Tulipan et al., 1999; Sival et al., 2004; Fichter et al., 2008; Adzick, 2013). The direct causes of such dysfunction remains unclear; ethical and technical limitations render it difficult to obtain information from humans. Thus, research in analogous animal models of SBA is needed to better understand the cellular and molecular mechanisms of leg dysfunction and to develop novel therapeutic interventions.

Surgically induced exposure of the spinal cord causes human-like spina bifida lesions in different mammals (rat, pig, and sheep) triggering in utero damage to the exposed spinal cord and various neurological disorders (Heffez et al., 1993; Meuli et al., 1995a, 1995b). A criticism of such models is that laminectomy is performed from middle to advanced gestation, limiting the experimental relevance to secondary spinal cord injuries induced by amniotic fluid in early gestation. Thus, studies in these animal models have focused principally on optimizing surgical coverage, refining fetal surgery techniques, and evaluating tissue-engineering approaches (Meuli and Moehrlen, 2014; Papanna et al., 2016; Watanabe et al. 2016). Several genetic (Sim et al., 2013; Nikolopoulou et al., 2017) and drug-induced (Alles and Sulik, 1990; Danzer et al., 2005)
rodent models of neural tube-related disorders exist, but are of limited utility in SBA research on postnatal leg dysfunction because the fetuses generally die in utero (Copp et al., 1982; Selcuki et al., 2001). However, incision of the roof plate of the chicken neural tube during early gestation triggers in ovo exposure of the spinal cord ab initio (Mominoki et al., 2006; Tsujimura et al., 2011). Also, SBA-like features including motor dysfunction, quite similar to those of human neonates, are evident during neonatal life (Fig. 1; Table 1). Thus, the chicken model should yield valuable data on the pathological sequence of events in the spinal cord associated with SBA-like motor dysfunction.

Many neurological complications including motor dysfunctions at postnatal age have been associated with alterations of synaptic transmission in spinal motor networks, mainly dysregulation of the balance between excitation and inhibition (Schütz, 2005; Sunico et al., 2011; Ramírez-Jarquín et al., 2014). It is postulated that alterations in synaptic transmission, particularly inhibitory neurons, by spinal motor neurons may be associated with SBA-related motor dysfunction because of changes in the extent of γ-aminobutyric acid (GABA)ergic activities, the principal inhibitory modulator, play key roles in stepping (Sinnamon and Benaur, 1997), hind limb paralysis (Gottesfeld et al., 1976), and motor dysfunction (Sanna et al., 1993; Ramírez-Jarquín et al., 2014). Thus, we explored the excitatory and inhibitory synaptic inputs to spinal cord motor neurons at different ages ranging from gestation to post-hatching in a chicken SBA model.

Results

Motor behavior and neurological assessments

Neuropathological dysfunction was evident from the day of hatching (PD0) (Fig. 1a; Table 1). Shortly after hatching, the chicks with SBA could stand, move the leg joints, and walk, but the voluntary control of movement (sitting to walking) was poor compared to normal chicks. Over the next 2 weeks, the SBA chicks lost function in the toe, ankle, and knee joints, and subsequently developed lower-limb paralysis (Fig. 1a; Table 1), although concurrent leg flapping and/or
hitching, principally in the hip joints, was evident during wing, head, and neck movements (Fig. 1b). In addition, severe clubfoot deformities appeared in both legs within the first 2 weeks of neonatal life (Fig. 1a, arrows).

**Histopathological analysis**

To explore whether spinal cord exposure to amniotic fluid triggered progressive destruction of neural tissue, we compared cord sections at the SBA lesion to those of normal controls at times ranging from embryonic day (ED) 14 to PD10. Gross necropsy of SBA chicks revealed open wounds (SBA-like lesions) in the lumbosacral region of the back, varying degrees of spinal cord deformation in the lesional area (Fig. 1c), quite similar to human myelomeningocele (Hutchins *et al.*, 1996). The area of greatest compression (the gross lesion epicenter) was at L2–L4 (the open defect, Fig. 1c–ii). Apart from the changes in anatomical shape, cross-sectional images of the lesional epicenter revealed prominent tissue loss, particularly the dorsal horn (Fig. 1d–f). The extent of deformation of neural tissue area (gray matter) in the exposed spinal cord varied by age, being similar during the embryonic period but increasing with age after hatching and becoming severe at PD10. Furthermore, the number of motor neurons seemed to be preserved during the embryonic period and the early neonatal days (ED14 to PD4) but decreased by PD10 (Fig. 5b). Another prominent change was large vacuolation between motor neurons at PD10. These changes were not noted earlier in SBA chicks or in control chicks of any age (Fig. 1g), and led to the loss of motor neuron synaptic terminals (Sunico *et al.*, 2011).

**Excitatory and inhibitory synaptic boutons on spinal cord motor neurons**

To clarify whether actual synaptic alterations took place on the lumbar cord motor neurons in SBA chicks during early neonatal days, we used electron microscopy to analyze the ultrastructural parameters of synaptic boutons attached to motor neurons of the exposed lumbar cord (Fig. 2a). In control chicks, segments of the synaptic boutons were attached to the motor neuron cell body, but the boutons were detached in SBA chicks at severe symptomatic stage on PD10. At P-10, active zones were observed in excitatory synaptic boutons of SBA chicks but not
in inhibitory boutons, indicating loss of inhibitory inputs on motor neurons (Sunico et al., 2011).

Quantitative analyses revealed that the average number of synaptic boutons per motor neuron in SBA chicks did not differ from that of controls on PD0, PD2, or PD4 (Fig. 2b-ii). However, SBA chicks had significantly greater numbers of excitatory boutons and fewer inhibitory boutons than age-matched controls, commencing from the day of hatching (Fig. 2b-i). The proportions of inhibitory and excitatory boutons on motor neurons of normal neonatal chicks were ca. 75% and 25%, respectively; these proportions were not evident in SBA chicks at any time. In fact, the value of inhibitory-to-excitatoty boutons per motor neuron was significantly lower in SBA chicks than in age-matched normal controls from PD0, and gradually decreased with progression of leg dysfunction and this was drastic at PD10 (Fig. 2b-iii), when SBA chicks completely lost motor coordination and their voluntary leg movements and showed lower-limb paralysis (Fig. 1a, b; Table 1).

**Excitatory and inhibitory immunoreactivities of spinal cord motor neurons**

To explore whether changes in the inhibitory and excitatory inputs to motor neurons were associated with the levels of excitatory and inhibitory neurotransmitters, and whether such changes commenced during embryogenesis or after hatching, we conducted immunostaining analyses using acetylcholine synthesis, choline acetyltransferase (ChAT) and GABA synthesis, glutamic acid decarboxylase 67 (GAD67) (markers of excitatory and inhibitory transmission, respectively) at different time points from E18 to PD10 (Fig. 2c). In normal chicks, both immunoreactivities were low in advanced gestation but increased after hatching (Fig. 2c-i–iv, x–xiii). However, clear increases in immunoreactivities at the open defect spinal cord of SBA chicks were evident at E18 (Fig. 2c-v, xiv), indicating that the synaptic abnormalities commenced during embryonic development. Furthermore, in line with the electron microscopy data (Fig. 2b-i), ChAT immunoreactivity increased whereas that of GAD67 decreased at PD2 and PD4 (Fig. 2c-vi, vii, xv, xvi), but both had decreased by PD10, at which time GAD67 immunoreactivity was almost absent (Fig. 2c-viii, xvii). When we compared immunoreactivity intensity, a significantly higher
level of ChAT was observed in motor neurons of SBA chicks than age-matched normal controls during early neonatal days (Fig. 2c-xviii). On the other hand, the GAD67 expression level around motor neurons of SBA chicks was significantly decreased at post-hatch days (Fig. 2c-ix), suggesting an association between GABAergic transmission and reduction of inhibitory inputs to motor neurons.

**GABAergic transmission by spinal cord motor neurons**

To explore whether a reduction of inhibitory inputs to motor neurons was associated with the loss of GABAergic transmission, we immunohistochemically analyzed GABA and its subpopulations, calbindin-D-28K (CB), and calretinin (CR) at different time points (E14 to PD10). As for GAD67, more GABA-synaptic terminals were observed on exposed cord motor neurons at E14 and E18 than age-matched controls. These gradually declined with post-hatch age and were almost absent at PD10 (Supplementary Fig. S2i–x). In fact, when we compared immunoreactivity intensity, a significantly higher level of GABA was observed around motor neurons of SBA chicks than age-matched normal controls at E-18 (control, 11.46±1.21; SBA, 26.52±1.70; P<0.01, Student's t-test) whereas it was gradually decreased with post-hatch age which was drastic at PD10 (control, 30.59±1.18; SBA, 2.05±1.27; P<0.01, Student's t-test), suggesting that both GABA synthesis and release gradually decreased in exposed cords as motor dysfunction progressed. Similarly, the immunoreactivities of both CB (Supplementary Fig. S2xi–xx) and CR (Supplementary Fig. S2xxi–xxx) gradually fell after hatch. Furthermore, double-labeling of GABA and either CB or CR was prominent during middle and late gestation but was greatly reduced at PD4 and almost absent at PD10 (Fig. 3), at which time a drastic decrease in the number of inhibitory synaptic boutons on motor neurons was evident (Fig. 2b-i).

**Neurodegeneration in the spinal cord**

To clarify the mechanisms underlying the loss of GABAergic synaptic terminals on motor neurons in SBA chicks, the immunoreactivity of caspase 3, a marker of apoptosis, in interneurons expressing GAD67 and CB was evaluated in the ventral horn of the lumbar cord at different
pathogenesis points (Fig. 4). There were pathogenesis point-dependent changes in caspase 3 immunoreactivities in GABAergic interneurons in the SBA chicks, such that weak caspase 3 immunoreactivity was evident in GAD67-expressing interneurons at E18 (Fig. 4a-v) while moderate to high caspase 3 immunoreactivity was found on PD2 and PD4 (Fig. 4a-vi, vii). However, most of these interneurons had likely degenerated by PD10 because only a few interneuron-like structures remained at this point (Fig. 4a-viii, star marks). Similar patterns of caspase 3 expression were observed in CB-expressing Renshaw cells of SBA chicks (Fig. 4b-v–viii). In addition to the GABAergic interneurons, strong caspase 3 immunoreactivity was observed in the glial cells and tissue area of the exposed cord in SBA chicks on P-4 (Fig. 4a-vii), which suggests loss of glial cells and tissue area due to apoptosis by PD10.

We also analyzed the immunoreactivity of caspase 3 on motor neurons to determine whether apoptosis was associated with their degeneration in SBA chicks (Fig. 5). As in the GABAergic interneurons, weak caspase 3 immunoreactivity was observed in the motor neurons of SBA chicks at E18 (Fig. 5a-v), which increased with post-hatch age. In fact, from PD4, abundant immunoreactivity was evident and was very strong on PD10 (Fig. 5a-vii, viii), which is when SBA chicks had only approximately half the number of motor neurons in exposed cords as age-matched normal controls (Fig. 5b-vii). On the other hand, very little immunoreactivity was identified in either GABAergic interneurons (Fig. 4ai–iv; 4bi–iv) or motor neurons (Figs. 5a-i–iv) of normal chicks during the experimental period, suggesting that the degeneration of GABAergic interneurons and motor neurons in the lumbar cord of SBA chicks might occur via apoptosis.

Activities of GABAergic, cholinergic, and caspase 3 in the sham control chicks

To clarify whether mechanical and environmental insults played roles in the changes in GABAergic, cholinergic, and apoptosis activities, the immunoreactivities of GABA, ChAT, and caspase 3 were compared between SBA chicks and two sham-injured groups: the ES-sham group, in which embryos received the same treatment as the SBA group except that the length of the
incision of the roof plate was less than three somites; and the PS-sham group, in which an SBA-like open defect in the back of the chicks was made at PD0 via laminectomy at three vertebral segments, L2–L4 (Supplementary Figs. S1b, c). Although PS-sham chicks showed reduced ability to walk during the first 2 days after laminectomy, their voluntary leg movements on PD10 remained functional. Neither sham group showed spinal cord deformation or changes in tissue area at the lesion sites. In addition, sham injuries did not alter the expression levels of GABA, ChAT, or caspase 3 in the lumbar cord motor neurons on E14, E18, PD4, or PD10 (Fig. 6; Supplementary Fig. S4). These findings suggest that the mechanical and environmental insults had little or no influence on GABAergic, cholinergic, and caspase 3 activities in SBA chicks; rather, the changes of synaptic transmission and neurodegeneration were likely a consequence of secondary and/or tertiary effects of spinal cord exposure to amniotic fluid during in ovo development (Meuli et al., 1995a; Hutchins et al., 1996; Stiefel et al., 2007; Adzick, 2013; Kowitzke et al., 2016).

Discussion

Chicks with SBA-like features initially had normal voluntary leg movements but quickly after hatching (within 2 weeks) exhibited lower-limb paralysis. Such motor dysfunction has also been observed in human neonates with SBA (Korenromp et al., 1986; Sival et al., 2004, 2006, 2008). However, why these changes occur within this short period of time remains to be determined. Because there are no genetic or drug-induced factors involved in the exposure of the spinal cord to amniotic fluid, our results may be relevant for understanding the pathological events responsible for the progression of SBA-like leg dysfunctions.

Consistent with studies investigating human neonates with SBA, this study is the first to provide experimental evidence that the transiently present voluntary leg movements of SBA chicks disappear shortly after hatching. In fact, the degree of SBA-like motor dysfunction, particularly the loss of functionality in toe, ankle, and knee joint movements and the progression of lower-limb paralysis during early neonatal days (Fig. 1a; Table 1), were correlated with the
synaptic change-induced disruption of motor networks in exposed lumbar cords (Figs. 2 and 7). Furthermore, these changes were associated with a drastic loss of GABAergic transmission and the upregulation of cholinergic activities (Figs. 2 and 3; Supplementary Fig. S2). Such loss of GABA may lead to increase excitatory drive to the motor neurons. The decrease in the number of inhibitory synaptic boutons that resulted in a lower ratio of inhibitory-to-excitatory inputs on the motor neurons (Fig. 2) provides further evidence of disinhibition of motor neurons, as do the deteriorated motor coordination (sit to walk), and rise of hyperreflexia and tremors function observed in SBA chicks during the early days of lower limb paralysis (Fig. 1a, b; Table 1). Therefore, it is possible that this type of disinhibition may have induced overexcitation of the motor neurons, which could have led to deteriorated motor functions in SBA chicks in early neonatal life.

In addition, such disinhibition-induced overexcitation could have exacerbated existing excitotoxic events and accelerated the degeneration of synaptic boutons and motor neurons (Suníco et al., 2011; Ramírez-Jarquín et al., 2014) that lead to skeletal muscle weakness, disconnection of motor signals, and paralysis of limbs (Schütz, 2005). The possibility of disconnected motor signals between spinal cords and leg muscles is also supported by the drastic reduction in CB immunoreactivity in Renshaw cells (Supplementary Fig. S3), which are the only GABAergic interneurons that receive afferents directly from motor neurons (Sanna et al., 1993; Ramírez-Jarquín et al., 2014). In fact, on PD10, SBA chicks not only exhibited drastic decreases in GABAergic transmission (Figs. 2c-viii, ix and 3-viii, xvi; Supplementary Fig. S2x, xx, xxx) and inhibitory synaptic boutons (Fig. 2b-i) on motor neurons but had also lost approximately half of their motor neurons (Fig. 5b-vii), at which point the chicks completely lost their motor coordination and voluntary leg movements, and showed lower limb paralysis (Fig. 1a; Table 1). Taken together, these results indicate that the disinhibition-induced disruption of motor networks is a neuropathological hallmark contributing to enhanced SBA-like leg dysfunctions in the early neonatal days of SBA chicks. Our findings also strengthen the possibility of a correlation between
the loss of GABAergic tones and motor dysfunction in SBA cases, because as shown previously, intrathecal treatment with a GABA agonist relieves SBA-induced spasticity in humans (Bergenheim et al., 2003).

Qualitative deterioration of leg movements was evident from the first neonatal day in SBA chicks (Fig. 1a, Table 1), which has also been reported in human neonates with SBA (Sival et al., 2006), suggesting that the functional abnormalities in the spinal motor networks in these cases begin during the embryonic period. In our study, this was supported by the lower ratio of inhibitory-to-excitatory inputs to motor neurons in SBA chicks on PD0 (Fig. 2b-iii). Although the number of motor neurons seemed to be preserved in deformed spinal cords of SBA chicks during the gestational period (Fig. 5b), increases in GABAergic and cholinergic immunoreactivities were found in motor neurons at midgestation and these activities were very strong at advanced stages (Figs. 2c and 3; Supplementary Fig. S2). The widespread GABAergic immunoreactivity indicates that this neurotransmitter system may play a neuroprotective role against amniotic fluid-induced neurotoxicity and/or other neurotoxic insults including cholinergic-induced excitotoxicity, as has been observed in various nervous system tissues (Schütz, 2005; Nabeka et al., 2014).

Alternatively, to maintain normal neurological functions during the gestational period, the increased levels of GABAergic transmissions might be necessary for sufficient inhibition of the increased cholinergic-induced excitation of motor neurons or vice versa (Ramírez-Jarquín et al., 2014). Moreover, the possibility that excess GABA may be a source of energy for abnormal neurons in exposed cords to promote proliferation and migration cannot be excluded (Neman et al., 2014; Van Swearingen et al., 2014). These possibilities are supported by our results, as SBA chicks had preserved interneurons (Fig. 4) and motor neurons (Fig. 5) with few apoptosis signals, along with enlarged areas of abnormal tissue (Fig. 1e) in advanced gestational stages. Although further studies are needed to clarify the actual roles that increased GABAergic and cholinergic activities play, our results indicate that the functionality of motor neurons in SBA chicks is compromised during the embryonic period starting from at least midgestation.
The loss of neurons due to apoptosis is an important pathophysiological component of many neurological diseases (Tatton et al., 1997; Lawson and Lowrie, 1998; Keane et al., 2001; Scholz et al., 2005). The loss of spinal neurons at lesion sites may be associated with SBA-related neurological dysfunction (Adzick, 2012), however, little is known about the mechanism underlying neurodegeneration or the type of degenerated neurons that are associated with SBA-related motor dysfunction. In a previous study, we proposed that the loss of small neurons, which are most likely interneurons, in exposed spinal cords of SBA chicks at an early neonatal age is likely related to motor dysfunction (Mominoki et al., 2006). The present study provides evidence that apoptosis-induced degeneration of GABAergic interneurons and motor neurons in lumbar cords at lesion sites were associated with SBA-like motor dysfunctions (Figs. 4 and 5). Kowitzk et al. (2016) also suggested that neurons in neonatal human myelomeningocele cases may be degenerated via apoptosis, although Sival et al. (2008) was unable to detect apoptosis markers in motor neurons of autopsied spinal segments of human fetuses with SBA. This discrepancy may be due to differences in the pathological stage of neurodegeneration because activation of caspase 3 is the final step required for the execution phase of apoptosis (Holtzman and Deshmukh, 1997). In this study, the immunoreactivity of caspase 3 in motor neurons of SBA chicks was evident in an advance gestational stage (E18) but not at midgestation (E14), which is when the cellular abnormalities were observed (Supplementary Fig. S4), supporting the possibility of pathogenesis stage-dependent activation of caspase 3 in exposed spinal cords during embryonic development. In fact, the immunoreactivity of caspase 3 gradually increased with the progression of pathogenesis in early neonatal days and was very strong on PD10, which is when SBA chicks exhibited only a few damaged interneuron-like structures and approximately half the normal number of motor neurons (Figs. 4 and 5).

Taken together, the present results indicate that apoptosis-induced degeneration of spinal neurons in lumbar cords are associated with SBA-like motor dysfunctions and that these pathological changes occur within the first 2 weeks after hatching. However, the trigger of cellular
abnormalization and propagation of neurodegenerative signals were evident from mid to advanced gestational stage, and those are likely a consequence of amniotic fluid-induced in ovo milieu because mechanical and environmental insults in sham operated chicks had no, or very little, effect on neurodegeneration processes (Fig. 6; Supplementary Figs. S1 and S4). These results provide experimental evidence of the well-accepted “two-hit” (Meuli et al., 1995a; Hutchins et al., 1996; Stiefel et al., 2007; Adzick, 2013) or recently proposed “three-hit” hypothesis (Kowitzke et al., 2016) of neurodegeneration in SBA. Indeed, cellular abnormalization and changes in synaptic transmissions in motor neurons on E14 (Fig. 3; Supplementary Fig. S4) provide clues as to why the in utero closure of SBA lesions after midgestation cannot completely preserve motor functions in human with SBA (Tulipan et al., 1999; Sival et al., 2007). Collectively, the present findings demonstrate that the SBA-like leg dysfunctions, reductions of inhibitory inputs to motor neurons, and increases in apoptotic activity have a causal relationship with the pathogenesis of motor disorders in SBA chicks.

In conclusion, the present findings indicate that early postnatal loss of inhibitory transmission in the lumbar cord motor neurons may contribute to the pathophysiology of SBA-like leg dysfunctions. Our results shed light on the mechanisms underlying the timing and causes of cellular abnormalization, disinhibition of motor neurons, and neurodegeneration, thus opening up the possibility of new approaches to prevent neuronal function and degeneration in the progression of SBA-related motor complications. If our results are not unique to SBA chickens, thus also relevant in humans with SBA, our work should have a major impact on the development of novel therapeutic interventions for this congenital disease.
Materials and Methods

Animals

The fertilized eggs of chickens (Gallus gallus; Mori Hatchery, Kagawa, Japan) were incubated in a commercial incubator (Showa Furanki, Saitama, Japan) at 37.8 ± 0.2°C with 60% relative humidity to obtain embryos at developmental stages 17–21 inclusive on gestational day 2.5–3.5. The developmental stage of each embryo was determined using the developmental table of Hamburger and Hamilton (Hamburger and Hamilton, 1951). The embryos were then divided into two groups: the SBA group, in which the roof plate of the neural tube was incised, and the normal control group, in which the neural tube was left intact. The hatched chicks were raised in a room kept at 30°C with continuous lighting and fed a commercial diet (crude protein: 24%, metabolizable energy: 3,050 kcal/kg; Toyohashi Feed Mills Co. Ltd., Aichi, Japan) with water available ad libitum. Since the SBA chicks were unable to eat food by themselves during early neonatal days, they were gavaged a feed slurry (tube feeding) at a mass of 4.0% body weight into the crop (4 times in a day). The feed slurry was made by mixing 40% powdered diet with 60% distilled water on a weight basis. The animal experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the Ehime University Graduate School of Medicine, Japan (No. 05A27-10).

Surgical manipulation for generating SBA chicks

To produce SBA chicks, surgical manipulation of the neural plate was carried out according to a previously described procedure (Mominoki et al., 2006). Briefly, after removal of approximately 1 mL of albumin, the egg shell and amnion were opened and placed under a stereomicroscope to determine the developmental stage of the embryo. Then, the roof plate of the neural tube was incised longitudinally, starting at the level of the cranial margin of the 26th somite, which forms the sixth and the seventh thoracic segments, using a custom-made microknife (Kinutani et al., 1985, 1986). The incision extended caudally for a distance equivalent to the length of seven somites and was made by inserting the microknife into the neural tube to
approximately half the depth of the tube. The roof plate was incised with care taken not to damage other parts of the neural tube. After the incision was made and the surgical manipulation was complete, the shell window was closed using transparent adhesive tape and the eggs were re-incubated at 37.8 ± 0.2°C with 60% relative humidity.

**Motor behavior and neurological assessments**

The induction of SBA in the chicks was confirmed by observing open defects on the backs of the chicks with leg dysfunction (Supplementary Fig. S1d). For motor behavior and neurological assessments, the chicks (both SBA and control) which was used at P0 was also used at P2, P4 and P10. To characterize the leg movements of the chicks, the spontaneous locomotor activities of the normal and SBA chicks were videotaped at 08:00 and 20:00 for 10 min at each evaluation age (PD0, PD2, PD4 and PD10) and scored offline by two independent observers. The bilateral leg movements were categorized following assessments of movement at each joint (hip, knee, ankle, and toes) for each leg as follows: no movement (0 points), slight movement (1–2 points; defined as movement of the joint that was less than half of full range), reduced movement (3–4 points, defined as movement of the joint that was greater than or equal to half of full range), and normal movement (5 points). Motor dysfunction was evaluated by assessing the ability to sit-stand-walk as follows: unable (0 points), minimal (1–2 points), reduced (3–4 points), and normal (5 points). Additionally, the total number of leg movements (stepping and/or leg flapping) per 10 min were assessed in the SBA and normal chicks. There were six chicks in each group at each age point for behavioral analyses.

**Sample collection and tissue preparation**

Spinal cord sections from the lumbosacral region were collected from the normal and SBA chicks on ED14, ED18, PD2, PD4, and PD10. The incisions of the roof plate of the neural tube in the SBA embryos were confirmed by observing open defects on the backs of the chicks (data not shown). Six chicks from each group at each age point were transcardially perfused with a fixative solution containing 4% paraformaldehyde with 0.5% glutaraldehyde in 0.1 M phosphate-
buffered saline (PBS). After the perfusion fixation, the spinal cord was removed from the location of open defect (exposed area, lesion location) (Fig. 1c-ii). Spinal tissue samples from the normal control chicks were collected from location that was similar to those of the SBA chicks. Following the sample collection, the tissues were immersed in 4% paraformaldehyde overnight at 4°C. Next, the samples were dehydrated and embedded in paraffin.

For the electron microscopy analysis, three chicks from each group on PD0, PD2, PD4, and PD10 were transcardially perfused using the procedures described above, except that 3% glutaraldehyde was used instead of 0.5% glutaraldehyde. Following the perfusion, spinal cord samples were collected from the location of the open defect in the SBA chicks and from a similar location in the normal control chicks. After a rinse in 0.2 M PB and post-osmification, the samples were stained en bloc with a saturated aqueous uranyl acetate solution for 2 h, dehydrated in a graded ethanol series, and then embedded in Epon epoxy resin (Hexion, Columbus, OH, USA).

Surgical manipulation for generating sham control chicks

To clarify whether the mechanical and environmental insults altered the spinal motor networks, two types of sham injury control groups were created. In the first group, the embryos were treated in the same manner as the SBA group, except that the length of incision in the roof plate was less than three somites (ES-sham; Supplementary Fig. S1c). The incidence of open neural tube defect and leg dysfunctions in survivors of ES-sham group was 0% in this study (Supplementary Fig. S1c) and previous study (Mominoki et al., 2006). In the second group, a laminectomy was performed at three vertebral segments, L2–L4, to make an SBA-like open defect (about 1 cm) in the backs of the chicks (PS-sham; Supplementary Fig. S1b). Briefly, the day-old (6–10 h after hatching) normal chicks were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (0.5 mg/kg) and anesthesia was verified by the absence of both the leg withdrawal reflex to a toe-pincho and the corneal blink reflex. After shaving and cleansing the surgical field, the skin of the back was incised, the underlying muscles were retracted to expose the lumbar vertebrae, and a total laminectomy was performed under a microscope; special care
was taken not to injure the spinal cord and to keep the dura intact. Following the surgical procedure, the chicks were placed in a heating chamber and their body temperatures were maintained at approximately 37°C until fully awake, when they were returned to their cages. Using the methods described earlier, spinal cord samples were collected from similar location of lumbar cord of both sham groups on ED14, ED18, PD4 and PD10; there were four chicks in each group at each age point.

**Histopathological analysis**

Serial coronal sections (7 µm) from the location of the open defect (exposed area) of the lumbar cord in the SBA chicks, and from a similar location in the normal chicks (Fig. 1c), were stained with hematoxylin-eosin. The tissues were observed under a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan), images were acquired using a digital camera attached to the microscope (Nikon Digital Sight DS-L2), and the total cross-sectional and gray matter areas were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The tissue sections were treated with a Nissl stain (cresyl violet) and the total number of motor neurons was quantified by a blind rater. The ventral horn was defined as gray matter ventral to the central canal and only motor neurons with a clearly identifiable nucleus were counted. The total tissue area, gray matter surface area, and number of motor neurons were calculated and averaged across six cross sections per chick at each age point; there were six chicks in each group at each age point.

To assess the presence of neuropathological alterations in motor neuron area, the tissue embedded in resin for the electron microscopy analyses was used for light microscopy analyses. Semi-thin sections (0.5 µm) were cut using an ultramicrotome (Leica VIM-535; Reichert-Nissei Ultracuts, Vienna, Austria), collected on a slide, and then lightly stained with a solution containing 1% toluidine blue and 1% borax in distilled water. The tissue was covered with a toluidine blue solution, incubated for 2–5 min on a heater at 60°C, rinsed with distilled water, and then air-dried. Finally, it was observed under a light microscope (Nikon).
**Electron microscopy**

For electron microscopy, a rectangular area of the lateral ventral horn was removed; ultrathin sections (80 nm) were made with a diamond knife and mounted on single-slot grids coated with Formvar film. The double-stained specimens stained with uranyl acetate and lead citrate were examined with an electron microscope (JEM-1230, 80 kV; JEOL USA, Peabody, MA, USA). Electron microscopic profiles were identified as synaptic boutons only if the profile had all of the characteristics of synaptic structures with respect to synaptic vesicles, synaptic density, and typical membrane apposition. Classification and quantification of the boutons were conducted according to the type of synaptic vesicle, as previously described: synaptic boutons were classified as S-boutons if more than 80% of the vesicles were spherical, while those that contained more than 20% flat vesicles were classified as F-boutons because the S-type (Fig. 2a, star marks) or F-type (Fig. 2a, hash marks) vesicles are presumably excitatory and inhibitory synapses, respectively (Uchizono, 1965; Matsuda *et al.*, 2004; Sunico *et al.*, 2011). Approximately 2% boutons were omitted from further morphometric or statistical analyses because they contained only a few vesicles or were irregularly shaped. To be considered for the analysis of synaptic boutons, each motor neuron was required to have 18–22 electron micrographs, obtained at a magnification of 20k. The numbers of each type of synaptic boutons on the motor neurons were calculated and averaged across 10 motor neurons in each chicks at each age point. Furthermore, the total number of synaptic boutons (both inhibitory and excitatory) and the ratio of inhibitory-to-excitatory boutons per motor neuron was calculated and averaged in each chicks at each age point. There were three chicks in each group at each age point.

**Immunofluorescence staining**

Immunofluorescence staining was performed as described previously (Nabeka *et al.*, 2014). The sections were incubated with one of the following primary antibodies for 60 h at 4°C: rabbit polyclonal anti-GABA (1:3,000; Sigma, St. Louis, MO, USA), mouse monoclonal anti-GAD67 (1:500; Millipore, Temecula, CA, USA), mouse monoclonal anti-CB (1:500; SWANT, Bellinzona,
Switzerland), mouse monoclonal anti-CR (1:500; SWANT, Bellinzona, Switzerland), rabbit polyclonal anti-ChAT (1:500; Abbiotec, San Diego, CA, USA), or rabbit polyclonal anti-caspase 3 (1:500; Bioss, Woburn, MA, USA). After a wash in PBS, the sections were treated for 2 h at room temperature with either Alexa Fluor 546 goat anti-rabbit IgG (H+L) (1:1,000; Invitrogen, Carlsbad, CA, USA) or Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:1,000; Invitrogen) and DAPI. The sections were then washed with PBS, mounted in Vectashield (Vector Laboratories; Burlingame, CA, USA), and examined in high-resolution confocal images obtained using a Nikon A1 confocal microscope equipped with a 100× objective lens (Nikon).

For double immunofluorescence staining, the sections were incubated for 60 h in a solution containing rabbit polyclonal anti-GABA (1:3,000; Sigma) plus mouse monoclonal anti-CB or mouse monoclonal anti-CR (1:500; SWANT); and rabbit polyclonal anti-caspase 3 (1:500; Bioss) plus mouse monoclonal anti-GAD 67 (1:500, Millipore) or mouse monoclonal anti-CB (1:500; SWANT). After washing in PBS, the sections were treated for 2 h at room temperature with either Alexa Fluor 546 goat anti-rabbit IgG (H+L) (1:1,000; Invitrogen) or Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:1,000; Invitrogen) and DAPI, then washed with PBS, mounted in Vectashield (Vector Laboratories), and examined in high-resolution confocal images obtained using a Nikon A1 confocal microscope equipped with a 100× objective lens (Nikon).

The specificities of the antibody staining were tested using a negative staining procedure with normal rabbit IgG instead of the primary antibodies; the samples were processed as described above. There was a lack of non-specific staining (data not shown).

**Staining intensity measurement**

The staining intensities of GABA, GAD67 and ChAT in the ventral horn (motor neuron area) of the open defect lumbar cord in SBA chicks and similar location in normal chicks at different age points were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). To correct for background in images, three background intensity readings were taken per image. These readings were subsequently averaged and subtracted from the signal intensity...
around motor neurons (for GABA and GAD67) or within motor neurons (for ChAT) to give an accurate reading of the candidate protein staining intensity. The intensity data were presented as means ± SEM. Six random sections per chicks at each age point in each group were analysed for quantification; there were six chicks in each group at each age point.

**Statistical analysis**

All quantitative parameters (stepping/leg flapping, tissue area, number of synaptic boutons, immunoreactive intensities and number of motor neurons) were quantified and compared between the normal control and SBA groups, except mentioned elsewhere. Statistics were performed using the average values and all data are reported as means ± SEM. The data were analyzed with two-way analyses of variance (ANOVA) and Tukey–Kramer post hoc comparisons, and $P$ values $< 0.05$ were considered to indicate statistical significance.
Acknowledgements

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Author contributions

M.S.I.K. and S.M. planned, designed and performed experiments, analyzed results and compiled the manuscript; M.S.I.K. generated surgery-induced SBA chicks; H.N., F.I., T.S. and T.T. performed experiments and analyzed data; X.L provided support with high-content image acquisition and analysis; S.S., S.K. and F.H. involved in data interpretation and manuscript editing; All authors discussed the study and approved the final manuscript.

Additional Information

The authors declare no competing financial interests.
References


Table 1: Assessment of neurophysiological dysfunctions in SBA chicks

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<th>Age</th>
<th>Scoring of leg joint movement and motor dysfunction</th>
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<td>(a) Leg joint movement</td>
<td>(b) Motor coordination</td>
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<td></td>
<td>Hip</td>
<td>Knee</td>
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A 5-point scoring system was used to categorize movement of hip, knee, ankle and toe joint; motor coordination ability in the normal and SBA chicks on the neonatal days. (a) Bilateral leg movements were assessed based on the movement in each leg for each joint of the hips, knees, ankles, and toes. Leg joint movements were characterized as no movement (0 points), slight movement (1–2 points; defined as movement of the joint that was less than half of full range), reduced movement (3–4 points, defined as movement of the joint that was greater than, or equal to half of, the full range), and normal movement (5 points). (b) Dysfunctional motor coordination was evaluated as the ability to sit-stand-walk and was graded as unable (0 points), minimal (1–2 points), reduced (3–4 points), and normal (5 points). #1 of 6 and ##2 of 6 SBA chicks with representative joint movements in only one leg (left or right).
Figure 1. Assessments of motor behavior and spinal cord gross histology at the lesion sites in spina bifida aperta (SBA) chicks. (a) Representative photographs showing the phenotypes of normal (i–iv) and SBA (v–viii) chicks. The normal control chicks were able to stand firmly shortly after hatch (i). The SBA chicks were able to put their toes on a surface and had the ability to sit-to-walk at PD0 (v) but this gradually deteriorated with age (days) until the chicks completely lost the functional ability to sit-to-walk from PD4 (vii and viii). Severe clubfoot deformities (arrows) were appeared in both legs of SBA chicks within 10 days of hatching. (b) Total numbers of leg movements (stepping and/or leg flapping/hitching) per 10 min in normal and SBA chicks. (c) Gross necropsy revealed open wounds (SBA lesion) on the lumbosacral region of the back of the SBA chicks as well as varying degrees of spinal cord deformation in the lesion area (ii, location of open defect). (d) Cross-sectional images of exposed cord from SBA chicks stained with
hematoxylin showing varying degrees of alterations in anatomical shape (iv–vi); this was not observed in the normal chicks (i–iii). (e and f) Total tissue area and gray matter area in the exposed lumbar cord (location of open defect) of SBA chicks and in a similar location in normal chicks on the embryonic and neonatal days. (g) Pathological alterations in the lumbar cord motor neuron area of SBA chicks. Representative photographs of semi-thin toluidine-blue stained sections from the exposed lumbar cord of SBA chicks (iv–vi) and in a similar location in normal control chicks (i–iii) on neonatal days. Vacuolated and swollen axons and feasible dendrites (#) were evident in SBA chicks on post-hatch days. The data in Figs. 1b, 1e and 1f are presented as means ± SEM; n=6 in each group at each age point. *Significantly different from the SBA group at each age point ($P < 0.01$), two-way ANOVA and post hoc Tukey's test. Arrows indicated clubfoot deformities.
Figure 2. Postnatal loss of inhibitory synaptic transmission in the spinal cord motor neurons of SBA chicks. (a) Illustrative examples of synaptic boutons attached to the plasma membrane of motor neurons with either spherical (*, excitatory) or flat/pleomorphic (#, inhibitory) vesicles. Dotted lines indicate the segments of the bouton that were detached from the motor neuron cell body; arrows indicate the active zones; triangle indicate absence of active zones. (b) Characterized by the type of vesicles (i, excitatory or inhibitory boutons), average number of synaptic boutons (ii) and ratio of inhibitory-to-excitatory synaptic boutons per motor neuron (iii) from chicks with the indicated genotypes at the indicated conditions. The data in Fig. 2b are presented as means ± SEM; ten motor neurons from each chick at each age point; there were three chicks at each age point in each group. (c) GABA synthesis, glutamic acid decarboxylase 67 (GAD67; i–viii, arrows), and acetylcholine synthesis, choline acetyltransferase (ChAT; x–xvii, arrowheads) immunoreactivities in the lumbar cords of normal and SBA chicks at different developmental stages. Representative confocal images from the ventral horn of the open defect in the lumbar cord of SBA chicks and in a similar location in normal controls at ED18, PD2, PD4, and PD10.
Each panel represents the immunoreactivities of GAD67 (i–viii, green) and DAPI (blue) or ChAT (x–xvii, red) and DAPI (blue). The staining intensities of GAD67 and ChAT in the ventral horn (motor neuron area) of the open defect lumbar cord in SBA chicks and similar location in normal chicks at different age points were measured using ImageJ software. The intensity data in Fig. 2c are presented as means ± SEM. Six random sections per chicks at each age point in each group were analysed for quantification; there were six chicks in each group at each age point. *Significantly different from the normal control group at each age point (2b-ii and iii; 2c-ix and xviii). Groups with different letters are significantly different at each age point (2b-i); ($P < 0.05$), two-way ANOVA, post hoc Tukey’s test.
Figure 3. Postnatal loss of GABAergic terminals around motor neurons in the exposed lumbar cord of SBA chicks. (a) Double-labeled immunofluorescent staining performed with antibodies directed against GABA and either calbindin-D-28K (CB) or calretinin (CR). Representative confocal images showing the localization patterns of GABA and CB (i–viii, arrows) or GABA and CR (ix–xvi, arrows) in the normal and SBA chicks on ED14, ED18, PD4, and PD10. Images of the ventral horn in the open defect of the lumbar cord in SBA chicks and in a similar location in normal chicks. Each panel represents the immunoreactivities of GABA (red) or CB or CR (green), with sites of co-localization (yellow) and DAPI (blue).
Figure 4. Caspase 3-mediated apoptosis of spinal cord GABAergic interneurons in SBA chicks. (a) Representative confocal images showing immunofluorescence staining for caspase 3, which is a marker of apoptosis, and glutamic acid decarboxylase 67 (GAD67), which is a GABA-synthesizing enzyme, in the ventral horn interneurons (arrowheads) from the location of open defect in the lumbar cord of SBA chicks (v–viii) and in a similar location in normal chicks (i–iv). (b) Representative confocal images showing immunofluorescence staining for caspase 3 and CB-expressing Renshaw cells (arrows) in normal (i–iv) and SBA (v–viii) chicks. Images of the ventral horn in the open defect of the lumbar cord in SBA chicks and in a similar location in normal
chicks. Each panel represents the immunoreactivities of caspase 3 (red) and GAD67 or CB (green) with sites of co-localization (yellow) and DAPI (blue). *Interneuron-like damaged structures observed in SBA chicks at PD10.
Figure 5. Caspase 3-mediated apoptosis of spinal cord motor neurons in SBA chicks. (a) Representative confocal images showing immunofluorescence staining for caspase 3, which is a marker of apoptosis, and glutamic acid decarboxylase 67 (GAD67), which is a GABA-synthesizing enzyme. Images of the ventral horn in the open defect of the lumbar cord in SBA chicks (v–viii) and in a similar location in normal chicks (i–iv). Each panel represents the immunoreactivities of caspase 3 (red), GAD67 (green), and DAPI (blue). (b) Loss of motor neurons in the lumbar cord of SBA chicks. Nissl-stained images obtained from the ventral horn of the location of open defect in the lumbar cord of SBA chicks (iv–vi) and in a similar location in normal chicks (i–iii) at different age points. Quantitation of the numbers of motor neurons in...
the ventral horn of the lumbar spinal cord sections of SBA and normal control chicks (vii). The numbers of motor neurons were calculated and averaged across six cross sections per chick at each age point; there were six chicks at each age point in each group. Data are expressed as means ± SEM. *Significantly different from the normal control group ($P < 0.01$), two-way ANOVA, post hoc Tukey’s test.
Figure 6. Mechanical injuries at the embryonic and post-hatch stages did not influence GABAergic, cholinergic, or caspase 3 reactivity in the sham control chicks. Representative confocal images from the ventral horn at the location of open defect in the lumbar cord of SBA chicks and similar locations in the normal controls, ES-sham, and PS-sham chicks at PD4 and PD10. Each panel represents the immunoreactivities of GABA (a–d and m–p; red, arrows) and DAPI (blue) or choline acetyltransferase (ChAT; e–h, and q–t; red, large arrowheads) and DAPI (blue) or caspase 3 (i–l and u–x; red, small arrowheads) and DAPI (blue).
Figure 7. Schematic summary of the pathological events in the spinal cord motor neurons during progression of SBA-like leg. The roof plate of the neural tube was incised on embryonic day 3 (ED3) to expose the spinal cord to amniotic fluid. The trigger of cellular abnormalization, (over expression of inhibitory and excitatory transmissions) and propagation of neurodegenerative signals (activation of caspase 3), were evident from mid to advanced gestational stage (ED14–ED18). At early neonatal life (PD0–PD4), the degree of SBA-like leg dysfunctions were correlated with the synaptic change-induced disinhibition of motor neurons in exposed lumbar cords which were associated with a drastic loss of GABAergic transmission and the upregulation of cholinergic activities. At PD10, the SBA chicks not only exhibited drastic decreases in inhibitory tones on motor neurons but had also lost approximately half of their motor neurons via apoptosis which is when the SBA chicks showed lower-limb paralysis.
**Supplementary Figure S1. Schematic depicting the process used to generate SBA and sham-control chicks.** (a) Normal control group: chicks from intact eggs were used as the normal controls. (b) PS-sham group: an SBA-like open defect was made in the lumbosacral area via a laminectomy at three vertebral segments (L2–L4) in normal chicks at P-0. (c) ES-sham group: embryos underwent the same surgical manipulation as the SBA chicks, except that the length of incision in the roof plate was shorter than three somites. (d) SBA group: the length of incision in the roof plate was longer than five somites. Although PS-sham chicks showed reduced ability to walk during the first 2 days after laminectomy, their voluntary leg movements on PD10 remained functional. Neither sham group showed spinal cord deformation or changes in tissue area at the lesion sites.
Supplementary Figure S2. Analysis of GABAergic immunoreactivities on motor neurons in spinal cord of normal and SBA chicks. Immunofluorescence staining was performed with antibodies directed against GABA (i–x) or calbindin-D-28K (CB; xi–xx) or calretinin (CR; xxi–xxx). Representative confocal images from the ventral horn of the open defect in the lumbar cord of SBA chicks and in a similar location in normal controls at ED14, ED18, PD2, PD4, and PD10. GABA (i–v; arrows), CB (xi–xv; arrows), or CR (xxi–xxv; arrows) immunoreactivity around the lumbar cord motor neurons increased in normal chicks as the developmental stage increased.
Comparing with control chicks, very strong GABA (vi and vii; arrows), CB (xvi and xvii; arrows), or CR (xxvi and xxvii; arrows) labeling was observed around the motor neurons of the SBA chicks on ED14 and ED18 but decreased by the post-hatch ages; in particular, there was a drastic decrease in, or almost complete absence of, GABA (x; arrows), CB (xx; arrows), or CR (xxx; arrows) labeling around the motor neurons in the exposed cord area in SBA chicks on PD10.
Supplementary Figure S3. Analysis of CB immunoreactivity in Renshaw cells in spinal cord of normal and SBA chicks. Representative confocal images showing calbindin-D-28K (CB) immunoreactivity in the Renshaw cells of normal (a–d) and SBA (e and f) chicks at PD4. The immunoreactivities for CB and DAPI are shown in green and blue, respectively. The intensity of CB immunoreactivity in Renshaw cells (arrowheads) was weaker in SBA chicks relative to normal chicks.
Supplementary Figure S4. Mechanical injuries at the embryonic stage did not influence GABAergic, cholinergic, or caspase 3 reactivity in the sham control chicks. Representative confocal images from the ventral horn at the location of open defect in the lumbar cord of SBA chicks and similar locations in the normal controls and ES-sham chicks at ED14 and ED18. Each panel represents the immunoreactivities of GABA (a–c and j–l; red, arrows) and DAPI (blue) or choline acetyltransferase (ChAT; d–f, and m–o; red, large arrowheads) and DAPI (blue) or caspase 3 (g–i and p–r; red, small arrowheads) and DAPI (blue). The sham injury, induced by early embryonic neural tube incision, had little or no influence on GABAergic, cholinergic, and caspase 3 activities in the SBA chicks.