Molecular and morphological characterization of neural tube defects in embryos of diabetic Swiss Albino mice

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Summary. Background and Results: Embryos from diabetic mice exhibit several forms of neural tube defects, including non-closure of the neural tube. In the present study, embryos collected at embryonic day 11.5 from diabetic pregnancies displayed open neural tube with architectural disruption of the surrounding tissues. The percentage of proliferating cells was found to be increased in the dorsal and ventral domains of the spinal neural tube of embryos from diabetic mice, indicating a defect in the proliferation index. We have analyzed the development of various cell types, including motoneurons, interneurons, oligodendrocytes and migrating neurons, as well as radial glial cells in the open neural tube using specific molecular markers. Immunofluorescence results revealed a significantly reduced number of Pax2+ interneurons and increased number of Isl-1+ motoneurons, as well as Olig2+ oligodendrocytes in the neural tube of embryos from diabetic mice as compared to controls. In addition, these embryos exhibited a decreased number of doublecortin positive migrating neurons and Glast/Blbp positive radial glial cells with shortened processes in the neural tube. Expression levels of several developmental control genes involved in the generation of different neuronal cell types (such as Shh, Ngn, Ngn2, Ascl1) were also found to be altered in the neural tube of embryos from diabetic mice.

Conclusions: Overall, the open neural tube in embryos of diabetic mice exhibits defects in the specification of different cell types, including motoneurons and interneurons, as well as glial cells along the dorsoventral axis of the developing spinal cord. Although these defects are associated with altered expression of several development control genes, the exact mechanisms by which maternal diabetes contributes to these changes remain to be investigated.

Key words: Maternal diabetes, Developmental genes, Motoneurons, Interneurons, Oligodendrocytes, Neural tube defect

Introduction

Maternal diabetes has been widely demonstrated to induce embryopathy, which is characterized by congenital malformations in various organs in embryos of humans and in rodent models of diabetic pregnancy (Becerra et al. 1990; Phelan et al., 1997; Langer and Conway, 2000; Aberg et al., 2001; Cederberg et al., 2003; Liao et al., 2004). Neural tube defects (NTDs), including spina bifida, exencephaly and anencephaly have been frequently reported in embryos of diabetic pregnancies. NTDs are severe disabling or life-threatening congenital abnormalities which mainly exhibit incomplete fusion of the neural plate at the dorsal midline along the entire rostro-caudal length of the neural tube. Over the years, several studies attempted to unravel the molecular mechanisms by which maternal diabetes induces NTDs. Recently, maternal diabetes in vivo or high glucose in vitro has been shown to alter the expressions of several genes involved in neural tube development, subsequently contributing to NTDs (Fine et al., 1999; Liao et al., 2004; Fu et al., 2006).

During neurulation in embryos, the neural plate derived from ectoderm undergoes folding and shaping, and subsequently fuses at the midline to form the neural tube, which gives rise to the brain and spinal cord. The patterning of the neural tube along its dorsoventral axis is regulated by two different classes of proteins in an antagonistic manner. Sonic hedgehog (Shh), a secreted protein synthesized by the notochord and floor plate, functions as a ventralizing signal (Roelink et al., 1994; Marti et al., 1995b; Chiang et al., 1996). The establishment of a ventral-to-dorsal decreasing
concentration of SHH activity controls neuronal fate by either inducing or repressing the expression of several progenitor cell homeodomain transcription factors (Ericson et al., 1997). During the early stages of ventral neural tube development, three main classes of cells, such as floor plate cells, a specialized class of glial cells at the ventral midline, interneurons and motoneurons are generated at more dorsal positions to the floor plate. Motoneurons which express Is11, a homeodomain transcription factor, populate the ventral spinal cord (Pfaff et al., 1996). Interneurons that project and wire the local circuit (Brown, 1981) are crucial in integrating motor output. Earlier studies have demonstrated that Pax2, a paired-box transcription factor, is expressed in multiple interneuron cell types (Burrill et al., 1997). The ventral neurons have been divided into 5 domains, with each domain being characterized by distinct gene expression markers (Wilson and Maden, 2005).

Cells lateral to the roof plate differentiate into several classes of dorsal sensory interneurons. Experimental studies suggest that the roof plate is a source of inductive signals that control the generation of interneurons in the dorsal spinal cord (Liem, Jr. et al., 1997). There are 6 groups of dorsal interneurons, dl1-dl6, in the dorsal half of the spinal cord. These neurons are derived from progenitors that are indicated by the expression of basic helix-loop-helix (bHLH) proteins Atoh1, Neurogenin1 (Ngn1), Neurogenin2 (Ngn2) and Ascl1 (Helms and Johnson, 2003; Lee et al., 1998, 2000). These neurons connect sensory input from the periphery to spinal cord motoneurons and higher centres. Defective development of the sensory and motor neurons may impair the circuitry and functions of the nervous system.

We have previously demonstrated that maternal diabetes alters the expression of several genes involved in neural tube development (Liao et al., 2004; Fu et al., 2006; Jiang et al., 2008). The main aim of this study was to understand the specific cellular and molecular changes that occur secondary to neural tube closure defects in the offspring of diabetic mothers, as it may provide better insights into the strategy to repair neural tube defects and preserve the functional competence of the spinal cord in the offspring of diabetic mothers. In the present study, the open neural tube in embryos of diabetic mice exhibited defects in the specification of neural cells along the dorsoventral axis in the spinal cord. The defects are accompanied by alterations in the expression pattern of genes involved in generation of distinct neuronal populations during neural tube development.

Materials and methods

Experimental animals

Insulin-dependent diabetes mellitus was induced in 8-week-old Swiss Albino female mice (Laboratory Animals Centre, National University of Singapore, Singapore) by intraperitoneal injection of streptozotocin (STZ, 80 mg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) dissolved in citrate buffer (0.01M, pH 4.5) on three successive days. Blood glucose levels were monitored 1 week after STZ injection with a glucometer (Roche Diagnostics, Basel, Switzerland). Only mice with non-fasting blood glucose level exceeding 20 mmol/l were classified as diabetic and used in the experiment. Diabetic female mice were time-mated with age-matched male mice showing normal blood glucose level (4-6 mmol/l). Noon on the day when a copulation plug was observed was counted as embryonic day 0.5 (E0.5). Embryos were collected from pregnant diabetic mice anaesthetized with pentobarbitol (100 mg/kg body weight) by caesarean section on E11.5. A slight developmental delay of approximately 6 hours was observed in embryos of diabetic mice in comparison to those of control mice (Liao et al., 2004). To obtain embryos with the same number of somites, the control and diabetic mice were killed at about a 6-hour interval, i.e. at 10 a.m. and at 4 p.m. respectively. Embryos with an open neural tube phenotype from diabetic mice and normal embryos from non-diabetic mice were used as experimental and control groups respectively. All procedures involving animal handling were in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC), National University of Singapore.

Histology and immunohistochemistry

Embryos were fixed overnight in 4% paraformaldehyde (PF) in phosphate buffer solution (PBS), pH 7.4 and cryoprotected with 20% sucrose at 4°C overnight. Sections (20 µm thick) through neural tubes at comparable level of embryos from control and diabetic pregnancy were cut with a cryostat (Leica CM 3050, Leica Microsystems, Nussloch, Germany), then mounted on silane coated slides and stained with Hematoxylin and Eosin.

For immunohistochemistry, tissue sections were incubated with various primary antibodies overnight at 4°C. Primary antibodies used were rabbit polyclonal anti-Shh (1:600; R&D Systems, MN, USA) as a ventral marker against the notochord and floor plate, mouse monoclonal anti-Is11 (1:1000; DSHB, maintained by The University of Iowa) for motor neurons, rabbit polyclonal anti-Pax2 (1:200; Covance, CA, USA) for interneurons, goat polyclonal anti-Olig2 (1:800; R&D Systems, MN, USA) for oligodendrocytes, guinea pig polyclonal ant doublesclerotin (1:5000; Chemicon, USA) for migrating neuroblasts and differentiating neurons, rabbit polyclonal anti-bhlb1 (1:3000; Chemicon, USA) and guinea pig polyclonal anti-glast (1:4000; Chemicon, USA) for radial glial cells. Sections were washed with 0.1%PBS-TX and incubated with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) for 1h at room temperature, followed by incubation with avidin-conjugated FITC (1:300; Vector...
Laboratories, Burlingame, CA, USA) for 30min. Sections were washed and mounted on slides with VectaShield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Photomicrographs were taken with an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). The number of Isl-1 positive motor neurons, Pax-2 positive interneurons, Olig-2 positive cells and DAPI positive cells (to determine the total cells) in the neural tubes was scored from 8 sections (two each from 4 embryos of different litters) of each group (normal and diabetic pregnancy) separately and then the mean percentage of motor neurons and interneurons (per section) of each group was determined. For Olig-2 quantitation, the data have been presented as the mean number of cells ± S.E.M per section, since the Olig2-positive cells were spread over a larger area in the neural tubes of embryos from diabetic mice.

For analysis of cell proliferation in the neural tube, pregnant mice from both control and diabetic groups (n=4 in each group) were injected intraperitoneally with BrdU (100mg/kg body weight, Sigma-Aldrich, USA) 2h before the embryos were collected by Caesarean section. Embryos were fixed with 4% PF in PBS and cryoprotected with 20% sucrose at 4°C overnight. Transverse sections of 20 µm thickness were cut through the neural tube using a cryostat (Leica CM 3050; Leica Microsystems, Germany), mounted on silanized slides and allowed to dry at room temperature for 2 hours. Sections were washed with PBS-TX after incubation with 2N HCl at 37°C for 30 minutes, blocked with Vector Mouse On Mouse (M.O.M) blocking reagent (Vector Laboratories, CA, USA) for 1 h and incubated with mouse monoclonal anti-BrdU (1:1000; Sigma-Aldrich, USA) at 4°C overnight. Sections were washed and incubated with cy3-conjugated sheep anti-mouse secondary antibody (1:200; Sigma-Aldrich, USA) for 1 hour, washed with PBS and counter-stained with DAPI (1 µg/ml) for 5 minutes before mounting with fluorescent mounting medium. Photomicrographs were taken with a confocal microscope (Olympus, Tokyo, Japan) and the number of BrdU positive cells and DAPI positive cells (to determine the total cells) in the neural tubes was scored from 10 sections (two each from 5 embryos (n) of different litters) from each group. The dorsal and ventral domains of each section were divided symmetrically for quantitation. The proliferation index has been expressed as mean ± S.E.M. of percentage of BrdU positive cells (in relation to the total number of DAPI positive cells) per section.

**Quantitative real time reverse transcription-polymerase chain reaction (RT-PCR)**

Neural tubes were dissected out from E11.5 embryos of diabetic and non-diabetic mice (4-10 embryos from different litters) under a stereomicroscope and total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with 2 µg of total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). For real time RT-PCR, aliquots (1 µl) of the synthesized cDNA products were amplified in the reaction mixture (20 µl) containing LightCycler FastStart DNA Master SYBR Green I, 0.5 µmol/l of each primer and 3 mmol/l MgCl₂ in a LightCycler instrument (Roche Applied Sciences, Germany) according to manufacturer’s instructions. Table 1 presents the primers used in this study.

**Table 1.** Primers and thermal profile for real time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Bank Accession No.</th>
<th>Primer</th>
<th>Product size (bp)</th>
<th>Annealing temp./ Extension temp./ Time (ºC/sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>NM_007393.2</td>
<td>5’-aaccttaaggccaaacgtgaaaag-3’&lt;br&gt;5’-gcagggagtcgtgaagacgag-3’</td>
<td>199</td>
<td>60/10</td>
</tr>
<tr>
<td>Shh</td>
<td>NM_009170.2</td>
<td>5’-ccaattacaaacccgacatc-3’&lt;br&gt;5’-ccacaggtttctccttgcct-3’</td>
<td>342</td>
<td>60/10</td>
</tr>
<tr>
<td>Ngn1</td>
<td>NM_010896.2</td>
<td>5’-gcacagtgaatccttctgcttc-3’&lt;br&gt;5’-gcaggtgagacaggttccttt-3’</td>
<td>164</td>
<td>60/10</td>
</tr>
<tr>
<td>Ngn2</td>
<td>NM_009718.2</td>
<td>5’-gatgccaagctcaagagat-3’&lt;br&gt;5’-acgtggagttggaggatgac-3’</td>
<td>238</td>
<td>60/10</td>
</tr>
<tr>
<td>Isl1</td>
<td>NM_021459.3</td>
<td>5’-cgccgcaagctccaaacg-3’&lt;br&gt;5’-tcatttgcagcgctgttcttt-3’</td>
<td>217</td>
<td>60/10</td>
</tr>
<tr>
<td>Wnt1</td>
<td>NM_021279.3</td>
<td>5’-agcctcaagctccaaacgat-3’&lt;br&gt;5’-ggaatgccccattttcact-3’</td>
<td>221</td>
<td>60/10</td>
</tr>
<tr>
<td>Pax2</td>
<td>NM_011037.2</td>
<td>5’-gtgagagagggaaacgtaag-3’&lt;br&gt;5’-gtcctctgcttcgccggaag-3’</td>
<td>189</td>
<td>60/10</td>
</tr>
<tr>
<td>Atoh1</td>
<td>NM_007500.3</td>
<td>5’-gcttctctggggtatc-3’&lt;br&gt;5’-ctgaggatcgggagatg-3’</td>
<td>170</td>
<td>60/10</td>
</tr>
<tr>
<td>Ascl1</td>
<td>NM_008553.4</td>
<td>5’-gctccacgtggttccc-3’&lt;br&gt;5’-gaaaccgcataggtcaca-3’</td>
<td>225</td>
<td>60/10</td>
</tr>
</tbody>
</table>
The expression level of mRNA transcripts was analyzed by the $2^{-\Delta\Delta Ct}$ method after normalization to β-actin (Livak and Schmittgen, 2001).

**In situ hybridization**

Whole-mount in situ hybridization was carried out according to the protocol published previously (Belo et al., 1997) with modifications. *Ngn1*, *Olig2*, *Shh* and *Wnt1* antisense probes were made from plasmids containing the coding region of each gene. Briefly, Taq-polymerase-amplified PCR products of the coding regions of each gene were directly inserted into the pCR2.1-TOPO vector (Invitrogen, CA, USA). CDNAs of *Ascl1*, *Atoh1*, and *Ngn2* were kindly provided by Dr. Francois Guillemot (UK). Plasmids were linearized with specific restriction enzymes and Digoxigenin-labelled antisense mRNA probes were synthesized using a DIG RNA labelling kit (Roche, Germany) and RNA polymerases. The stained embryos were transversely sectioned with a cryostat (Leica CM 3050, Germany) and mounted on glass slides. The embryos and sections were examined and photographed with a stereomicroscope (Leica MZ APO, Germany) and light

![Control and Diabetic Embryos](image)

**Fig. 1.** Mouse embryos (E11.5) obtained from normal (A) and diabetic (B) mice. The embryo from the diabetic mouse exhibits incomplete closure of the neural tube along the rostral-caudal length of the axis (B, D arrows). Transverse sections (C, D) of the neural tube at the level of the upper limbs stained with Hematoxylin and Eosin show failure of fusion of neural plate at the dorsal midline, coupled with underdeveloped dorsal root ganglia in the embryo from the diabetic mouse. SC, spinal canal; DG, dorsal root ganglion. Scale bar: 200 µm.
Microscope (BX51, Olympus, Tokyo, Japan) respectively.

**Statistical analysis**

Statistical analysis was performed by the Student’s t-test using GraphPad Prism v5 for Windows (GraphPad Software, San Diego, CA) and expressed as mean ± S.E.M. Statistical significance was defined as p-value <0.05.

**Results**

Maternal diabetes induces neural tube closure defects in embryos

As we reported previously, about 12% of mouse embryos (E11.5) derived from 10 diabetic mice exhibited grossly malformed neural tubes, including cranial and caudal neural tube defects (Liao et al., 2004). In this study we have further characterized the
development of neural cell types in the neural tubes of E11.5 embryos from diabetic mice. In general, the normal architecture of neural tubes in embryos of diabetic mice appeared to be impaired extensively (Fig 1A-D). Moreover, the process of neurulation appeared to be perturbed, resulting in incomplete closure of the neural plate in those embryos. In a majority of the abnormal embryos, the neural tube closure defect was apparent along the rostral-caudal length of the axis throughout the thoracic and lumbar region of the neural tube (Fig 1B). Transverse histological sections stained with H&E showed that the neural plate failed to elevate and fuse at the dorsal midline in embryos of diabetic mice (Fig. 1D). Moreover, presumptive dorsal root ganglia lateral to the neural tube appear to be underdeveloped and displaced in comparison to that of embryos from normal mice (Fig. 1C,D).

**Differentiation of neuronal cell types (moto- and inter-neurons) in the neural tube of embryos from diabetic mice**

Since histological analysis clearly provided evidence of the presence of neural tube closure defects in embryos of diabetic mice, we further analyzed whether the neural patterning along the dorsal-ventral axis of the neural tube was perturbed using several appropriate molecular markers. The data obtained indicate a patterning defect along the dorsal-ventral axis of the neural tube in embryos that had neural tube closure defects.

Motoneurons and oligodendrocytes are sequentially generated in the ventral zone of the neural tube upon induction by Shh (Pringle et al., 1996; Orentas et al., 1999). Shh acts as a ventralizing signal and is expressed in the floor plate and the notochord. The whole mount in situ hybridization analysis showed that Shh expression domain was expanded in the entire neural tube of embryos from diabetic pregnancies when compared with controls (Fig. 2A-D). Quantitative real-time RT-PCR analysis further revealed the increased expression level of Shh mRNA in the defective neural tube (Fig. 2I).

Isl-1 is one of the earliest markers for motoneuron differentiation and sensory neuron differentiation in the dorsal root ganglia. It is expressed in all classes of motoneurons that innervate different groups of target muscles (Tsuchida et al., 1994; Pfaff et al., 1996) and in a distinct class of dorsal interneurons of the caudal neural tube (Liem Jr. et al., 1997). Immunohistochemical analysis showed that the percentage of Isl1+ postmitotic motoneurons appeared to be increased in the ventral zone of neural tube in embryos of diabetic mice (Fig. 2E-G). However, Isl-1 positive dorsal interneurons (dl3) were hardly detectable in the dorsal neural tube of these embryos (Fig 2E-G). In agreement with results from the immunofluorescence analysis, there was no significant difference in the overall mRNA expression of Isl-1 in the neural tubes of embryos from both groups, as revealed by the quantitative real-time RT-PCR analysis (Fig. 2I). This discrepancy could be due to the differential expression pattern of Isl-1 in dorsal and ventral domains of the neural tubes in embryos of diabetic mice.

Members of the PAX family of transcription factors control cell identity in the developing spinal cord. Among these, Pax2 is expressed in multiple spinal interneurons, including interneurons in the v0-v3 domains of the ventral horn (Burrill et al., 1997), as well as dl4 and dl6 neurons in the dorsal horn of the spinal cord (Helms and Johnson, 2003). Immunofluorescence analysis revealed that the percentage of Pax2+ interneurons in the neural tube was significantly reduced in embryos of diabetic mice as compared to controls (Fig. 2E,F,H). The reduction was found to be consistent along the dorsal and ventral axis of the neural tube. The quantitative real-time RT-PCR analysis showed that Pax2 mRNA expression level in the neural tube of embryos from diabetic mice was significantly decreased compared to that of controls (Fig. 2I).

**Cell cycle progression is altered in the neural tube of embryos from diabetic mice**

Besides the altered development of different cell types in the neural tubes of embryos from diabetic mice, the proliferation index was found to be altered. We have characterized the cell proliferation index by immunohistochemical detection of BrdU incorporation, in relation to Isl1- and Pax2-positive cells, as well as cells stained with Tuj1, which is the neuron-specific cytoskeletal component and is a widely-used marker of postmitotic neurons in the neural tube of embryos from diabetic mice. No Isl1+, Pax2+ and Tuj1+ cells were colocalized with BrdU positive cells (data not shown), suggesting that Isl1, Pax2 and Tuj1 proteins were expressed in the neurons of late stage differentiation. Along the medio-lateral axis of the neural tube, three distinct domains, such as ventricular zone adjacent to central canal containing undifferentiated cells, intermediate zone containing proliferating cells and lateral marginal zone containing differentiated cells, have been observed. These domains appeared to be perturbed in the neural tube of embryos from diabetic mice. The percentage of BrdU labeled cells (in relation to total cells as determined by DAPI staining) in the dorsal and ventral zones of the neural tube was found to be significantly higher in embryos of diabetic mice when compared to that of normal mice (Fig. 3A-G), indicating that a majority of the cells were in the proliferating zone.

**Oligodendrocyte progenitors are increased in the neural tube of embryos of diabetic mice**

Olig2 is a basic helix-loop-helix transcription factor that is specifically expressed in progenitors of oligodendrocytes and motoneurons and its expression is induced by Shh in the neural tube (Lu et al., 2000; Zhou et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001). Whole mount in situ hybridization and
immunofluorescence analyses showed that Olig2 expression domain was expanded and the number of Olig2+ cells was increased in the ventral zone of the neural tube of embryos from diabetic mice as compared to the control (Figs. 4A-E).

Development of radial glial cell lineages and migration of neurons are disrupted in the neural tube of embryos from diabetic mice

Doublecortin (Dcx), a microtubule-associated protein, is expressed in migrating neuroblasts and differentiating young neurons throughout the central and peripheral nervous system during embryonic development (des, V et al., 1998; Gleeson et al., 1998; Couillard-Despres et al., 2001). The domain containing Dcx positive cells was found to be markedly attenuated in the ventral zone of the neural tube of embryos from diabetic mice when compared to that of embryos from control mice (Fig. 5A-D).

During early development, migration of cells in the neural tube occurs along the radial glial cells, which serve as neuronal progenitors, neuronal migration guides and astrocyte progenitors (Rakic, 1972; Hatten, 1999; Hunter and Hatten, 1995; Noctor et al., 2001; Parnavelas and Nadarajah, 2001; Malatesta et al., 2003). There are three subsets of radial glial cells that are identifiable by RC1/2 (Misson et al., 1988), Glast (Shibata et al., 1997) and Blbp (Feng et al., 1994; Kurtz et al., 1994) antibodies. In the control neural tube, Blbp is expressed in cells in the ventromedial zone of the neural tube. Immunofluorescence analysis revealed that the number of Blbp positive cells appeared to be decreased in the neural tube of embryos from diabetic pregnancies (Figs. 5D). Glast was localized in the cells of the floor plate and the ventromedial zone of the neural tube with processes that were radially-oriented towards the pial surface (Fig. 5E). In the neural tube of embryos from diabetic mice, the number of Glast+ cells appeared to be markedly reduced (Fig. 5F). In addition, processes that extended towards the pial surface from the Blbp and Glast positive cells in the ventromedial zone were found to be disrupted or absent when compared to that of control (Fig. 5C-F).

Altered expression of development control genes in the neural tube of embryos from diabetic mice

We analyzed the expression pattern of several genes involved in the development of a variety of neuronal cell types, including subsets of dorsal interneurons in the neural tube. Wnt1 is expressed in the dorsal neural tube from the forebrain to the spinal cord levels at the time of neural crest generation (Parr et al., 1993; Wilkinson et al., 1987). In situ hybridization analysis showed that the Wnt1 expression was restricted to the roof plate and the adjacent area of the neural tube in control embryos. Neural progenitors that are adjacent to the roof plate expressed Atoh1, the bHLH transcription factor (Helms et al., 1997). Neural crest cells that were associated with the roof plate expressed Atoh1, the bHLH transcription factor (Helms et al., 1997).
and Johnson, 1998). These Atoh1-expressing neural progenitors give rise to the dl1 class of dorsal interneurons (Bermingham et al., 2001). There seem to be no apparent differences in the expression pattern of Wnt1 (Fig. 6A-D) and Atoh1 (Fig. 6E-H) in both the normal and open neural tubes. Ngn1 (another bHLH transcription factor) expression is located ventral to the Atoh1 expression domain in the neural tube (Lee et al., 1998; Gowan et al., 2001). Coordination with its related factor, Ngn2, is required for normal formation of the dorsal root ganglia (DRG) and the dorsal interneuron population dl2 (Ma et al., 1999; Gowan et al., 2001; Nieto et al., 2001). in situ hybridization showed Ngn1 was expressed in the domain

Fig 4. Transverse sections of the neural tube showing mRNA expression by in situ hybridization (A, B) and protein expression by immunofluorescence (C, D) of Olig2. Expression domains of Olig2 mRNA (arrows) and Olig2 protein (arrowheads) appear to have increased in the neural tube of embryos from diabetic mice. Consistently, the percentage of Olig2 positive cells (arrows) is also increased in the ventral zone of the neural tube in embryos of diabetic mice, compared to that of embryos of control mice (C-E). Data represent mean ± S.E.M (n=4) number of Olig-2 positive cells per section. *p<0.05. Scale bar: A, B, 100 µm; C-D, 50 µm.
containing dl2 and dl6 interneurons (Fig. 6I-L). Expression domain in the open neural tube appeared to be expanded dorsally (Fig. 6L). On the other hand, Ngn2 was found to be expressed in both ventral and dorsal domains of neural tubes. In the dorsal domain, expression of Ngn2 was restricted in the region where dl2-5 neurons were formed. In the open neural tube Ngn2 expression domain appeared to be expanded ventrally (Fig. 6M-P). Ascl1 was shown to be necessary for the development of most dl3 and all dl5 neurons with low level of Ascl1, giving rise to dl4 neurons (Helms et al., 2005). Ascl1 was expressed in progenitor domains containing dp3-5 cells in the control neural tube, while expression was observed to be intensified and expanded.

Fig. 5. Transverse sections through neural tube of E11.5 embryos showing double immunofluorescence labelling for Dcx (green) and Blbp (red) (A-D) and single immunofluorescence staining for Glast (green) (E, F). The sections are counterstained with DAPI (blue). Dcx expression has been observed in the cells of ventrolateral neural tubes in embryos of control mice (A, C) while the Dcx+ cells are markedly reduced in embryos of diabetic mice (B, D). Processes that extend towards the pial surface (arrows) from Blbp and Glast positive cells are found to be disrupted in neural tubes of diabetic mice (C-F). fp, floor plate. Scale bar: 100 µm.
Neural tube defects in embryos of diabetic mice

Fig. 6. Whole mount in situ hybridization for Wnt1 (A-D), Atoh1 (E-H), Ngn1 (I-L), Ngn2 (M-P) and Ascl1 (Q-T) in embryos from control and diabetic mice and the transverse sections of stained embryos through neural tube. Expression of Wnt1 and Atoh1 appears to be unaltered in embryos from control and diabetic mice. However, expression domains of Ngn1 and Ngn2 in the neural tube of embryos from diabetic mice seem to be expanded. Ascl1 expression domain has also been found to have expanded in the neural tube of embryos from diabetic mice. The real time RT-PCR analysis (U) shows the significant upregulation of Ngn1, Ngn2 and Ascl1 mRNA expression in the neural tubes of embryos from diabetic mice. The data represent the mean ± S.E.M. *p<0.05; **p<0.01. Scale bar: 200 µm.
Neural tube defects in embryos of diabetic mice

dorsoventrally in the open neural tube (Fig. 6Q-T). Moreover, the real time RT-PCR analysis also confirmed the increased expression levels of Ngn1, Ngn2 and Ascl1 in the neural tubes of embryos from diabetic mice (Fig. 6U). However, there was no change in mRNA expression levels of Wnt1 and Atoh1 in the neural tubes of embryos from diabetic mice.

Discussion

About 12% of embryos derived from diabetic mice exhibit neural tube closure defects resulting from failure of fusion of the neural plate at the dorsal midline during neurulation, which occurs at E8.5-9.5. The neural tube closure involves a coordinated suite of distinct cell shape changes, differential rates of cell proliferation and cell movements (convergent extension of neural plate and tube). It has been proposed that failure of neural tube closure is followed by in utero degeneration of exposed spinal cord tissue, resulting in neurological deficiency (Heffez et al., 1990; Stiefel et al., 2007). However, the specific cellular and molecular changes following the neural tube closure defects in embryos of diabetic mice had not been investigated in detail. An understanding of those changes would provide better insight into the development of fetal surgery to repair defects and prevent neurodegeneration, thereby preserving the functional competence of the spinal cord (Stiefel et al., 2007). The present study demonstrated the impaired development of different cell types, including neurons and glia and their patterning defects, following spinal neural tube closure defects, as was reported earlier in the forebrain in embryos of diabetic mice (Liao et al., 2004). Although the present study does not reveal the molecular mechanisms that contribute to the neural tube anomalies, it provides a clue for changes in molecular cues that guide the development of different functional regions of the spinal cord after the period when the neural tube closure occurs.

The complete neurulation and neural patterning require precisely regulated interactions among several signaling molecules and transcription factors that control cell proliferation and differentiation, as well as the position of different cell types in the developing neural tube. During pregnancy, environmental factors or teratogenicity, including maternal diabetes, seem to alter those molecules, contributing to neural tube anomalies. It has been previously shown that exposure to high glucose alters the cell-cycle progression in NSCs, microvessel endothelial cells and mesangial cells (Wolf et al., 2001; Abraham et al., 2003; Fu et al., 2006). In the present study the percentage of proliferating cells was found to be increased in the dorsal and ventral domains of the spinal neural tube of embryos from diabetic mice, indicating altered cell cycle machinery, possibly involving delay in cell-cycle exit and the differentiation process. However, the process of differentiation appeared to vary between the cell types as there were more motoneurons, as well as oligodendrocytes and less interneurons, in the open neural tube of embryos from diabetic mice.

The patterning of the central nervous system depends on the subdivision of the neural epithelium into distinct regional domains and the generation of a diverse array of neuronal cell types within each domain. The early regionalization of the neural epithelium is regulated by inductive factors secreted by signaling centers, the notochord and floor plate of the neural tube (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). Shh secreted initially from the notochord and later from floor plate cells, has been implicated in the induction of ventral neuronal cell types, including the Isl-1+ motor neurons in the spinal cord (Martì et al., 1995a; Chiang et al., 1996). In the present study, upregulation of Shh in the neural tube of embryos from diabetic mice appeared to be associated with the increased percentage of ventral Isl-1+ motoneurons in the neural tube. However, marked loss of Isl-1+ dl3 interneurons in the dorsal neural tube indicates the involvement of dorsal signals which may be negatively regulated by maternal diabetes. On the other hand, the decreased Pax-2 mRNA expression and percentage of Pax-2+ interneurons that are found in v0-v3 domains of the ventral neural tube and dl4 and dl6 domains of the dorsal neural tube in embryos of diabetic mice indicate that these embryos exhibit defects in the specification of neuronal cell types along the dorsoventral axis of the developing spinal cord. It is possible that upregulation of Shh in embryos of diabetic mice promotes motoneuron differentiation in the ventral neural tube at the expense of Pax2-expressing interneurons. Moreover, involvement of dorsal signal in the loss of Pax-2+ dl4 and dl5 interneurons in embryos of diabetic mice cannot be excluded, as dorsal midline cells have been shown to provide signals that are required for the generation of distinct classes of dorsal interneurons (Liem, Jr. et al., 1997).

Oligodendrocytes are the myelinating glial cells of the CNS and their precursors are identifiable in the same ventral region of the neuroepithelium of the developing spinal cord where motoneurons are generated (Sun et al., 1998). The generation of oligodendrocyte precursors is regulated by ventralizing signaling of Shh, which acts by inducing the expression of Olig1/2 genes, the differentiation factors of oligodendrocytes (Lu et al., 2000; Zhou et al., 2000; Albert et al., 2001). In the developing spinal cord, Olig2 is expressed in the pMN progenitor domain, and is required for the sequential generation of motoneurons and oligodendrocytes from the pMN domain (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002). It has been shown that Olig 2 regulates the expression of Ngn2, which promotes the expression of pan-neuronal markers in the pMN domain of the spinal cord (Novitch et al., 2001) and both Olig2 and Ngn2 appear to function downstream of Shh to direct the generation of motoneurons (Mizuguchi et al., 2001). In the present study, the increased expression of...
was associated with expanded Olig2 and Ngn2 expressing domains in the ventral spinal cord, suggesting that upregulation of Shh is the key factor that expands the boundaries of gene expression domains in the ventricular zone of the spinal cord. These changes could alter the landscape of the neuronal and glial progenitors domains established at earlier stages, resulting in a series of changes in specification of various cell types in the developing ventral spinal cord in embryos of diabetic mice.

The radial glial cells which express Glast/Blbp (Barry and McDermott, 2005) extend their processes from the ventricular zone to the pial surface in the neural tube and serve as a guidance scaffold for migrating neurons. These cells appear to be necessary for cell patterning and regionalization of the developing nervous system (Campbell and Gotz, 2002; Kriegstein and Gotz, 2003). The decreased number of Glast/Blbp positive radial glial cells showing short processes in the neural tube of embryos from diabetic mice indicates an impaired neuronal migration. The migration of immature neurons to their respective positions is a crucial step in the organization and development of the neural tube. The migrating neurons express the Dcx gene, a mutation of which results in abnormal neuronal migration in the human (Reiner et al., 2006). The marked reduction of Dcx positive cells in the developing ventral spinal neural tube of embryos from diabetic mice further confirms impaired neuronal migration, which could in turn result in topographic positional disarray.

The dorsal spinal cord contains a diverse array of neurons which are largely differentiated between E10-E14 in mouse. These neurons connect sensory input from the periphery to motoneurons in the spinal cord and higher centers in the brain. The specification of dorsal neural cell fates requires dorsalizing signals, including secreted proteins of Wnt families from the roof plate. Wnt1 is required for the expansion of dorsal neural progenitors (Ikeya et al., 1997). There are six dorsal neuronal populations (dI1-dI6) defined by expression of homeodomain and bHLH factors and position in the dorsoventral axis (Casparay and Anderson, 2003; Helms and Johnson, 2003). The generation of dI1-dI3 neurons are dependent on roof plate signals, whereas dI4-dI6 neurons are formed independently of these signals (Lee et al., 2000; Gross et al., 2002; Zhou and Anderson, 2002). Moreover, these neurons (dI1-dI6) have been found to be originated from Atoh1+, Ngn1+, Ascl1+ progenitors (Gowan et al., 2001; Helms et al., 2005). Expression domains of Atoh1, Ngn1 and Ascl1 within the dorsal neural tube appear sequentially along the dorsoventral axis from the roof plate (Gowan et al., 2001; Guillemot and Joyner, 1993; Helms and Johnson, 1998; Lee et al., 1998; Lo et al., 1991). Although expression domains of these genes do not overlap in an obvious manner, the expression domain of Ngn2, another bHLH factor, partially overlaps with Atoh1, Ngn1 and Ascl1 expression domains. Except Wnt1 and Atoh1, which are expressed in the roof plate and ventricular zone cells adjacent to the roof plate, expression levels and domains of the rest of the genes appear to be increased in the developing spinal neural tube of embryos from diabetic pregnancy, indicating that the generation of the correct number and types of neurons in the dorsal neural tube is perturbed. For example, Ascl1 promotes the generation of most dI3 and all dI5 neurons and antagonizes the generation of dI4 neurons (Helms et al., 2005). Upregulation of Ascl1 expression appears to be associated with decreased number of Pax2+ dI4 interneurons in the spinal neural tube of embryos from diabetic mice. However, the decreased number of Isl-1+ d13 interneurons in these embryos indicates the complexity of the defect in the differentiation process.

Overall, the open neural tube in embryos of diabetic mice exhibit impaired specification of different cell types, including motoneurons and interneurons as well as glial cells along the dorsoventral axis of the developing spinal cord. Although these defects are associated with altered expression of several development control genes, the exact mechanisms by which maternal diabetes contributes to these changes remain to be investigated systematically. However, the present study forms the basis to unravel the mechanisms that would reverse the neural tube malformations which occur in embryos of diabetic pregnancy.

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