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Strong sonic hedgehog signaling in the mouse ventral spinal cord is not required for oligodendrocyte precursor cell (OPC) generation but is necessary for correct timing of its generation

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ABSTRACT

In the mouse neural tube, sonic hedgehog (Shh) secreted from the floor plate (FP) and the notochord (NC) regulates ventral patterning of the neural tube, and later is essential for the generation of oligodendrocyte precursor cells (OPCs). During early development, the NC is adjacent to the neural tube and induces ventral domains in it, including the FP. In the later stage of development, during gliogenesis in the spinal cord, the pMN domain receives strong Shh signaling input. While this is considered to be essential for the generation of OPCs, the actual role of this strong input in OPC generation remains unclear. Here we studied OPC generation in *bromi* mutant mice which show abnormal ciliary structure. Shh signaling occurs within cilia and has been reported to be weak in *bromi* mutants. At E11.5, accumulation of *Patched1* mRNA, a Shh signaling reporter, is observed in the pMN domain of wild type but not *bromi* mutants, whereas expression of *Gli1* mRNA, another Shh reporter, disappeared. Thus, Shh signaling input to the pMN domain at E12.5 was reduced in *bromi* mutant mice. In these mutants, induction of the FP structure was delayed and its size was reduced compared to wild type mice. Furthermore, while the p3 and pMN domains were induced, the length of the Nkx2.2-positive region and the number of Olig2-positive cells decreased. The number of OPCs was also significantly decreased in the E12.5 and E14.5 *bromi* mutant spinal cord. In contrast, motor neuron (MN) production, detected by HB9 expression, significantly increased. It is likely that the transition from MN production to OPC generation in the pMN domain is impaired in *bromi* mutant mice. These results suggest that strong Shh input to the pMN domain is not required for OPC generation but is essential for producing a sufficient number of OPCs.

Abbreviations: CNS, central nervous system; FP, floor plate; HSPG, heparan sulfate proteoglycan; NC, notochord; MN, motor neuron; OPC, oligodendrocyte precursor cell; Shh, sonic hedgehog; Sulf1/2, heparan sulfate 6-O-endsulfatase 1/2; WT, wild type; VZ, ventricular zone.

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1. Introduction

In the developing central nervous system (CNS), several secretory factors such as Wnts, BMPs and sonic hedgehog (Shh) act as morphogens and form a concentration-dependent gradient (Yamada et al., 1993; Chiang et al., 1996; Briscoe and Ericson, 2001; Ulloa and Martí, 2010). This gradient regulates patterning and cell differentiation both temporally and spatially (Dessaud et al., 2007; Briscoe and Novitch, 2008; Kicheva et al., 2014; Cohen et al., 2015). In particular, Shh is secreted from the floor plate (FP) and notochord in the embryonic neural tube, and is involved in ventral neural tube development (Echelard et al., 1993; Roelink et al., 1995; Saha and Schaffer, 2006; Chamberlain et al., 2008). During early stages (E9.5-E10.5), it is essential to regulate the expression of transcription factors, such as Olig2 and Nkx2.2, for ventral pattern formation in regions such as the pMN and p3 domain, respectively (Goodrich et al., 1996; Jessell, 2000; Dessaud et al., 2008). During subsequent stages (E12.5 and beyond), strong Shh input initiates oligodendrocyte precursor cell (OPC) generation from the pMN domain (Orentas et al., 1999; Miller, 2002; Agius et al., 2004; Park et al., 2004; Rowitch and Kriegstein, 2010). We are interested in understanding how this strong input is generated, a mechanism which cannot be explained by simple diffusion of Shh from its source. Our group and others have shown that sulfatases, Sulf1 and 2, are involved in determining the pattern of Shh distribution in the embryonic mouse spinal cord (Danesin et al., 2006; Al Oustah et al., 2014; Ramsbottom et al., 2014; Jiang et al., 2017). Shh synthesized and released from the notochord and FP is trapped by proteoglycans, such as keratan sulfate (Weyers et al., 2013; Hashimoto et al., 2016) and heparan sulfate proteoglycan (HSPG), and is then released by sulfatase activity which removes 6-O sulfate residues from the HSPGs. This can act as the source of strong Shh input into the pMN domain and induce OPC generation. We have been analyzing sulfatase mutants and showed that both Sulf1 and 2 are necessary for normal OPC generation (Jiang et al., 2017). While OPC generation should also be affected by a decrease in Shh signaling input, it is not easy to modify Shh activity to study the effects of reduced Shh input on OPC generation because reduced Shh activity results in abnormal ventral patterning of the neural tube prior to OPC generation. Normal ventral patterning is required for expression of the Olig2 gene, which is essential for OPC generation (Miller, 2002). Thus, Shh knockout mice cannot be used for this purpose because they have severe ventral patterning abnormalities in the neural tube (Chiang et al., 1996). Even if the Shh gene is specifically inactivated within the FP, the neural tube will show a reduction in the number of Nkx2.2 (a marker of the p3 domain) and Olig2 (a pMN domain marker) positive cells (Yu et al., 2013). Normal Shh signaling requires primary cilia, and mutations in the intraflagellar transport proteins result in reduced Shh signaling (Huangfu et al., 2003; Huangfu and Anderson, 2005; Falcón-Urrutia et al., 2015; Pal and Mukhopadhyay, 2015). Motor neuron generation is abnormal in these mutants, suggesting abnormal ventral patterning. Thus, none of these mutants are suitable for exploring how strong Shh input into the E12.5 pMN domain affects OPC generation.

In this study, we focused on the *broadminded* (*bromi*) mutant mice (Ko et al., 2010). The protein encoded by *bromi* interacts with cell cycle-related kinases, through which ciliary morphology is affected. Shh signaling is reduced by this mutation and the FP is poorly formed, however, the number of MNs is unchanged, indicating that Olig2 induction and pMN domain formation are mostly normal. Here we show that the number of OPCs produced is decreased in *bromi* mutants. This suggests that the effects of decreased Shh signaling on OPC generation may be attributed to insufficient Shh input.

2. Materials and methods

2.1. Antibodies

The following primary antibodies were used in this study: mouse anti-Nkx2.2 IgG (74.5A5, Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA, USA), rabbit anti-Olig2 IgG (EMD Millipore, Billerica, MA, USA) and mouse anti-HB9 IgG (81.5C10, DSHB). For immunofluorescence, we used Alexa 568-conjugated anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) and Alexa 488-conjugated anti-mouse IgG (Life Technologies) as secondary antibodies.

2.2. Animals

Bromi mutant C57BL/6J (Ko et al., 2010) mice were used for all experiments. Mice were mated to obtain pregnant mice for the collection of embryos. Noon of the plugged date was considered to be stage E0.5. All animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of Yonsei University College of Medicine.

2.3. Tissue preparation

Pregnant mice at post-coitum day 10.5, 11.5, 12.5, and 14.5 were deeply anesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital, and their offspring were removed from the uterus. For embryos of all stages, the heads were removed and the remaining bodies were fixed with 4% paraformaldehyde (PFA) in PBS (PFA/PBS) overnight at 4 °C. The bodies were cryoprotected with 20% sucrose/PBS, embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan), and quickly frozen in liquid nitrogen. Spinal cords at the forelimb level were sectioned at 20 μm thickness with a cryostat (CM-3050; Leica Biosystems, Nussloch, Germany) and thaw-mounted onto RNase-free MAS (Matsunami-adhesive-silane)-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan). These sections were used for *in situ* hybridization and immunofluorescence.

2.4. In situ hybridization

In situ hybridization was performed as described previously (Hashimoto et al., 2016). Briefly, the sections were treated with proteinase K (1 μg/ml for 30 min at room temperature) and hybridized overnight at 60 °C with DIG-labeled antisense riboprobes in a hybridization solution consisting of 40% formamide, 20 mM Tris-HCl (pH 7.5), 600 mM NaCl, 1 mM EDTA, 10% dextran sulfate, 200 μg/ml yeast tRNA, 1x Denhardt's solution, and 0.25% SDS. The *Gli1* antisense riboprobe was hybridized for 1 h at 80 °C to overnight at 60 °C. The bound DIG-labeled probe was detected by overnight incubation of the sections with anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics), and the color was developed in the presence of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate in the dark at room temperature. After alkaline phosphatase-mediated visualization, sections were mounted with Permount (Thermo Fisher Scientific Inc., Fair Lawn, NJ, USA) after serial dehydration.

2.5. Immunofluorescence

Transverse cryosections (20 μm) of the embryonic spinal cord were immunostained as follows. Citrate antigen retrieval was performed on spinal cord sections incubated with antibodies against Nkx2.2, Olig2 or HB9. Sections were heated in a microwave oven

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