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Research Report

# Chiasmatic neurons in the ventral diencephalon of mouse embryos—Changes in arrangement and heterogeneity in surface antigen expression

Ling Lin, Anny W.S. Cheung, Sun-On Chan*

*Department of Anatomy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, PR China*

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**Abstract**

We have investigated the changes in arrangement of the SSEA-1 immunoreactive chiasmatic neurons in the mouse ventral diencephalon from embryonic day (E) 9 to the end of gestation. A regionally specific staining of SSEA-1 was first detected in the ventricular layer of the caudal diencephalon at E10 and later at E11 on the cells in the subventricular layer. At E12, these cells formed the characteristic V-shaped configuration caudal to the optic axons in the chiasm. At E13–E15, this neuronal array changes gradually to a configuration that facilitates contact with the optic axons only at the midline and the initial segment of the optic tract. Colocalization studies showed that CD44 was localized strongly on the neurons in the central but not lateral domains of the array, suggesting existence of heterogeneity in these neurons in terms of surface antigen presentation. This difference between the central and lateral domains raises the possibility that the chiasmatic neurons may regulate the patterning of axon orders at the midline and the optic tract through presentation of distinct combination of guidance cues at these strategic positions in the optic pathway. Furthermore, exogenous Lewis-x/SSEA-1 inhibited neurite outgrowth from the E14 retinal explants; this inhibition was observed in neurites from both ventral temporal and dorsal nasal retina. These findings suggest an action of this surface carbohydrate on the control of axon growth and guidance in the mouse optic pathway.


*Theme:* Development and regeneration
*Topic:* Axon guidance mechanism and pathways

*Keywords:* Chiasm; Axon guidance; Midline; Optic tract; SSEA-1

## 1. Introduction

In the mouse embryos, the optic chiasm and the proximal segment of the optic tract are two critical regions where significant sorting and reordering of axons occur in the retinofugal pathway [18,28]. A number of studies have indicated the contribution of a population of early-generated neurons in the ventral diencephalon to these axon sorting processes. These cells express surface molecules including CD44 and stage-specific embryonic antigen-1 (SSEA-1) [26,31]. The functional significance of these chiasmatic neurons is first demonstrated in the study which shows a failure of axon entry into the chiasm after elimination of the CD44 immunoreactive neurons [32]. A more recent report has demonstrated that CD44 is the molecule on these neurons that serves the permissive role for axon crossing at the midline [24]. Furthermore, chondroitin sulfate proteoglycans, which are localized on and around these neurons, have been shown to affect the development of axon divergence at the midline and formation of chronotopic axon arrangement in the optic tract [7,8,21]. These findings indicate a multiple role of the chiasmatic neurons on axon growth and patterning in the mouse optic pathway.

SSEA-1 is another surface molecule that is expressed prominently on the chiasmatic neurons. This molecule is

* Corresponding author. Fax: +852 26096898.
*E-mail address:* sunonchan@cuhk.edu.hk (S.-O. Chan).

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originally defined by a monoclonal antibody against murine teratocarcinoma cell line F9 [30]. It is a carbohydrate moiety carried by glycoproteins or glycolipids and has a structure as Gal-β-(1-4)-[Fuc-α-(1-3)]-GlcNAc (lacto-*N*-fucopentaose, LNFIII or FAL) [17]. It is also known as CD15 (leukocyte cluster of differentiation 15) and Lewis-x, a member of the Lewis blood group antigens in human [2]. Expression of SSEA-1 has been shown in mouse embryos at late eight-cell stage [30] and is expressed abundantly on the surface of trophectoderm, primitive ectoderm and endoderm and primordial germ cells during migration [13,16]. In the mouse diencephalon, SSEA-1 immunoreactivity is observed on the chiasmatic neurons, which exist in patterns closely related to the growth of the earliest generated axons [26] and the turning of the uncrossed axons in the chiasm [27]. Although SSEA-1 is one of the first identified molecules on the chiasmatic neurons [26], whether it is involved in the regulation of retinal axon growth and patterning in the chiasm and the optic tract is undetermined. In this study, we investigated the changes in arrangement of these neurons in the mouse ventral diencephalon and related these changes to fiber order changes at the chiasmatic midline and the optic tract. Furthermore, we determined the possible contribution of the SSEA-1 to axon growth in the chiasm by examining effects of exogenous SSEA-1/Lewis-x carbohydrate on neurite outgrowth from mouse retinal explants.

## 2. Materials and methods

### 2.1. Animals

Experimental procedures in the present study were approved by the University Animal Ethic Committee. Time-mated pregnant pigmented C57 mice were obtained from the Laboratory Animals Services Center of the Chinese University of Hong Kong. The day on which the vaginal plug was found was considered as embryonic day 0 (E0).

### 2.2. Immunohistochemistry

Pregnant mice were killed by cervical dislocation, and embryos at the ages of E9–E18 were removed by Cesarean section and killed by decapitation. The heads of the embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) overnight at 4 °C. The heads were embedded in a gelatin–albumen mixture and sectioned on a vibratome at the thickness of 100 μm. Frontal or horizontal sections containing the retinofugal pathway from the eyes to the proximal parts of the optic tract were collected in 0.1 M phosphate buffer saline (PBS, pH 7.4). Localization of SSEA-1 in these brain sections was examined using immunocytochemistry, which followed the procedures described in our early studies [7,9]. Sections were incubated with a mouse monoclonal antibody against SSEA-1 (IgM, from Developmental Studies Hybridoma Bank, Iowa, USA, under the contract N01-HD-6-2915 from NICHD) [30] at dilution of 1:5 overnight at 4 °C. Sections were then incubated in FITC-conjugated goat anti-mouse IgM (1:100, Jackson Laboratories, Maine, USA) for 90 min at room temperature. Control sections were processed with the same procedures but in the absence of the primary antibody. For double-label studies, sections were probed with antibodies against SSEA-1 (1:5) and CD44 (IM7; 1:50, rat IgG, PharMingen, USA) simultaneously overnight followed by incubation with goat anti-mouse IgM conjugated to FITC and donkey anti-rat IgG labeled with Cy3 (both at 1:100, Jackson Laboratories, Maine, USA). This IM7 antibody recognizes an extracellular epitope outside the hyaluronic acid binding site of the CD44 molecule [35]. It was used in this study because it gives a more distinct label of the chiasmatic neurons than the other CD44 antibody, Hermes-1 [24]. Sections were coverslipped with 50% glycerol in PBS and examined by using a confocal imaging system. In all control preparations, there was no obvious staining in the brain sections. In this part of the study, at least 4 litters of embryos were examined in each age group.

### 2.3. Preparation of retinal explants and treatment with Lewis-x

Retinal explants were prepared from E14 mouse embryos according to the procedure described in an earlier study [5]. The eyes were collected in cold DMEM/F-12 medium, and the retinas were dissected free of the lens, vitreous and pigmented epithelium. Retinal explants were isolated from peripheral regions of either ventral temporal or dorsal nasal quadrant, which give rise to mostly uncrossed and crossed axons, respectively, in the chiasm at this developmental stage [4,10,15]. Explants were placed evenly on polylysine–laminin coated coverslips and cultured in DMEM/F-12 supplemented with 1% bovine serum albumin, 0.4% methylcellulose, 0.5% insulin, 0.5% transferrin and 0.1% sodium selenite (all purchased from Sigma, USA). The trisaccharide Lewis-x [Galβ1,4(Fucα1,3)GlcNAc, Cat. No. 434630, from CalBiochem, USA] at various concentrations was added into the medium at the start of the culture. After 18 h in culture, explants were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h. Control for this part of the study consisted of preparations without addition of the Lewis-x carbohydrate, with added lactose, or with addition of another carbohydrate Lewis-y [Fucα1,2Galβ1,4(Fucα1,3)GlcNAc; Cat. No. 434634; CalBiochem, USA]. Neurite outgrowth from the whole explant was imaged sequentially using the Neurolucida Image Analysis System (MicroBrightField, Inc., USA) under phase contrast optics (20×, Plan-Neofluar, NA 0.5, from Zeiss, Germany). The images were then reassembled into a montage with clear visualization of the whole explant and its neurites. Using the