Annals of Anatomy 205 (2016) 85–89

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

Research Article

The unique axon trajectory of the accessory nerve is determined by intrinsic properties of the neural tube in the avian embryo

Zhongtian Bai[a,b,c,1], Qin Pu[b,d,1], Ziaul Haque[b,e], Jianlin Wang[c], Ruijin Huang[b,f,*]

[a] The 2nd Department of General Surgery, the First Hospital of Lanzhou University, Key Laboratory of Biotherapy and Regenerative Medicine, Gansu Province, China
[b] Department of Neuroanatomy, Institute of Anatomy, University of Bonn, Nussallee 10 53115, Bonn, Germany
[c] Institute of Zoology, School of Life Science, Lanzhou University, China
[d] Institute of Anatomy, Department of Anatomy and Molecular Embryology, Ruhr-University of Bochum, Bochum, Germany
[e] Department of Anatomy and Histology, Bangladesh Agricultural University, Mymensingh, Bangladesh
[f] Department of Molecular Embryology, Institute of Anatomy and Cell Biology, University of Freiburg, Germany

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form 10 February 2016
Accepted 20 February 2016

Keywords:
Branchiomotor nerve
Accessory nerve
Somite
Neural tube
Chick embryo

ABSTRACT

The accessory nerve is a cranial nerve, composed of only motor axons, which control neck muscles. Its axons ascend many segments along the lateral surface of the cervical spinal cord and hindbrain. At the level of the first somite, they pass ventrally through the somitic mesoderm into the periphery. The factors governing the unique root trajectory are unknown. Ablation experiments at the accessory nerve outlet points have shown that somites do not regulate the trajectory of the accessory nerve fibres. Factors from the neural tube that may control the longitudinal pathfinding of the accessory nerve fibres were tested by heterotopic transplantations of an occipital neural tube to the cervical and thoracic level. These transplantations resulted in a typical accessory nerve trajectory in the cervical and thoracic spinal cord. In contrast, cervical neural tube grafts were unable to give rise to the typical accessory nerve root pattern when transplanted to occipital level. Our results show that the formation of the unique axon root pattern of the accessory nerve is an intrinsic property of the neural tube.

1. Introduction

Neurons in the brain and spinal cord project into the periphery to form connections to target tissues (Kandel et al., 2000). All nerve roots project tangentially from the brain stem (cranial nerves) and spinal cord (spinal nerves) into the periphery with one exception, the accessory nerve – a nerve consisting purely of motor axons (reviewed in Chandrasekhar, 2004; Guthrie, 2007). After exiting the neuroepithelium, its axons ascend along the neuroepithelium for many segments before turning ventrally. Hence, the accessory nerve fibres display a unique trajectory.

The accessory nerve is located within the transition from the head to the neck (head–trunk-interface) (Kuratani, 2008). Its somata are distributed within the medulla oblongata (in the head) and cervical spinal cord (in the neck), subdividing into a spinal, and a cranial accessory nerve. The caudal extent of the spinal domain varies greatly between different species. In birds, for example, the spinal accessory nerve extends to the second cervical segment of the spinal cord (Kobayashi et al., 2013). In mammals, it reaches the sixth cervical segment (Krammer et al., 1987). Axons of the spinal accessory nerve join the axon bundle coming from the cranial accessory nerve.

An essential step in the axon root development is the axon projection within the neuroepithelium. Transcription factors *Gli2, Nkx2.9, Lhx3* and *Phox2b* are involved in this pathway (Sharma et al., 1998; Pabst et al., 2003; Dillon et al., 2005, 2007; Hirsch et al., 2007). Misexpression of *Lhx, for example*, leads to ventral projection (Sharma et al., 1998), whereas ectopic expression of *Phox2b* forces a dorsal projection. Inhibition of *Phox2b* leads to an absence of the accessory nerve (Hirsch et al., 2007). *Gli2* regulates the dorsal extension of the spinal accessory axon via *Nkx 2.9* (Pabst et al., 2003; Dillon et al., 2005). *Nkx 2.9* is also required for the exit of axons of the accessory nerve from the neural epithelium (Dillon et al., 2005). Furthermore, the dorsal axon projection of the accessory nerve is controlled by the Netrin-1 pathway (Dillon et al., 2005, 2007).

Although some molecular mechanisms have been investigated, the mechanisms regulating the unique pattern of the accessory nerve root are unknown. It is noteworthy that the last two

* Corresponding author at: Institute of Anatomy, University of Bonn, Nussallee 1053115, Bonn, Germany. Tel.: +49 228 73 5618; fax: +49 228 73 3346.
E-mail address: ruijin.huang@uni-bonn.de (R. Huang).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.aanat.2016.02.003
0940-9602/

cranial nerves, the accessory (XI) and the hypoglossal nerve (XII), are located at the same axial level. However, their exit points differ; the accessory nerve is located in the dorsal neural tube, whereas the hypoglossal nerve exit is more ventrally situated. The hypoglossal axons develop according to a general neuromuscular concept of connectivity. To find their target muscle fibres, its axons enter the somites and then follow the migrating muscle progenitors to their final anatomical destination (Mackenzie et al., 1998; Huang et al., 1999; Caton et al., 2000; Amano et al., 2002). According to our previous study, the target muscle of the accessory nerve, the cucullaris muscle (homologous to the trapezius and sternocleidomastoideus muscle of mammals) is derived mainly from the lateral plate mesoderm adjacent to the first somite (Theis et al., 2010), which plays a pivotal role during the axonal pathfinding of the accessory nerve. Our previous experiments show that axons of the accessory nerve pass exclusively through the first somite (Pu et al., 2013).

It is unclear why the accessory nerve runs first along the spinal cord and hindbrain for many segments before projecting to the periphery. We first excluded a regulatory function of the somite on the pattern formation of the accessory nerve root, by performing somite ablation experiments. Then, using heterotopic transplantation of neural tube segments, we showed that the unique pattern of the accessory nerve root is determined by intrinsic properties of the neural tube.

2. Methods

2.1. Embryos

Fertilized White Leghorn chick (*Gallus gallus*) and Japanese quail (*Coturnix coturnix*) eggs were incubated at 80% relative humidity and 37.8 °C. Embryos were staged according to Hamburger and Hamilton (1951), henceforth referred to as "HH-stage".

2.2. Microsurgeries

Embryos were incubated to HH-stages 8–10. After fenestration of the eggshell, the tissue structures of interest were outlined with sharpened tungsten needle and removed using a micropipette. All the operations were performed in the right side of the embryo. The left side served as an internal control. The operated embryos were allowed to develop to HH-stages 21–24.

Somite ablations were performed in the occipital region. First, the occipital somites and their overlying surface ectoderm as well as endoderm were microsurgically separated from the neural tube and lateral plate mesoderm. The somitic tissue was then aspirated with a micropipette.

Transplantations of neural tube were performed between quail donor and chick host embryos. For heterotopic transplantation, an occipital neural tube at somites 1–4 level was isolated from a quail donor and transplanted to cervical and thoracic region of a chick host embryo. In a reciprocal experiment, a cervical quail neural tube was grafted to the somites 1–4 level of a chick embryo. As a control, an occipital neural tube of a chick host was replaced by an equivalent segment of the quail neural tube.

2.3. Immunohistochemistry

The operated embryos were fixed in Dent's fixative (Methanol/DMSO 1:4, overnight, 4 °C), and bleached (70% methanol, 20% DMSO, 10% H_2O_2) for 5 h. To block non-specific antibody binding the embryos were incubated in 10% sheep serum in PBT for 4 h. Axons were stained using an antibody to neurofilament (3A10, Developmental studies Hybridoma Bank). Quail cells were detected with a monoclonal QCPN-antibody (Developmental studies Hybridoma Bank, Iowa City, IA, USA). After extensive washes in PBS, embryos were incubated (overnight, 4 °C) with Cy3 or Cy2-labelled secondary antibodies (Dianova). A Leica (DMRB) fluorescent microscope was used for the analysis and documentation of the experiments.

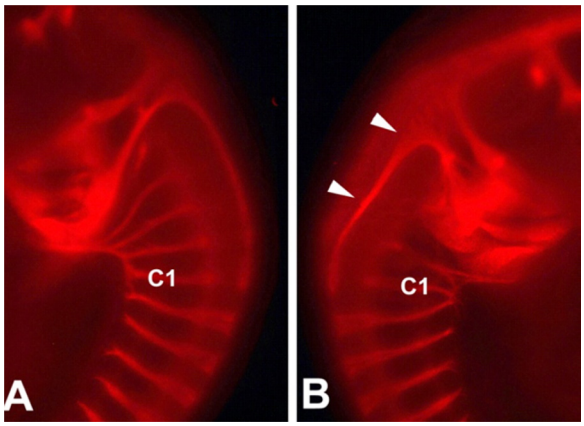

Fig. 1. The ablation of occipital somites does not alter the formation of the accessory nerve. (A) A micrograph of the control side shows the normal accessory and hypoglossal nerve outlets from the neural tube. (B) Somites 1–4 were ablated. The three cranial roots of the hypoglossal nerve (between the two white arrow heads) were not present. At the same level, the accessory nerve root developed normally. (C1) first spinal nerve, which is formed at the somite 5 level.

3. Results

3.1. Occipital somites do not influence the formation of the accessory nerve

After the accessory axons exit the lateral exit point, they extend cranially along the neural tube. Based on the observation that the accessory axons extend along the space between the neural tube and somites, we determined whether environmental cues originating from somitic tissue regulate the patterning of the accessory nerve root. Somite ablation or barrier implantation was used to test this hypothesis. After extirpation of somites 1–4 at the accessory nerve level at HH-stage 8–11, the operated embryos were allowed to develop to HH-stages 21–24, a stage when the accessory root pathway is formed. Axons were identified by staining with 3A10 antibody. The most noticeable finding in the operated embryos was the malformation of the hypoglossal nerve. At the occipital level, the hypoglossal nerve is made up of 5 segmentally organized roots, which cross the cranial half of somites 1–5 after exiting the basal plate of the hindbrain and spinal cord (Fig. 1A). In all operated embryos ($n = 4$), the segmentally organized roots of the hypoglossal nerve were no longer detectable (Fig. 1B). The abnormal development of the hypoglossal nerve roots served as an indication that the somites were no longer present during hypoglossal nerve formation and provided evidence that the somite ablation had been successful. In all successfully operated embryos ($n = 4$), we found that the accessory nerve root pattern of the operated side was indistinguishable to that of the unoperated side (Fig. 1A and B). These results imply that the absence of somitic cues did not interfere with the normal development of the accessory nerve roots. To expand on these findings, we implanted barriers between somites and the neural tube. Similar to the somite ablation, the separation of the neural tube from the somite resulted in the interruption of the segmental organization of the hypoglossal nerve. However, the accessory nerve developed normally (data not shown, $n = 8$).