Article

Early emergence of axons and formation of accessory nerve (XI) in avian embryos

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Abstract: Development of the nervous system involves specification of distinct classes of neurons at defined locations within the central nervous system (CNS). In the head–trunk-transitory region accessory nerve (XI) display a unique axonal trajectory in that they ascend along the lateral margin of the spinal cord and the medulla oblongata. At present, we lack a detailed description of early emergence and development of the accessory nerve (XI) in avian embryos. To know the early projection of accessory axons from the central nervous system (CNS) and formation of accessory nerve (XI), whole-mount immunostaining of chick embryos was performed. Our results showed that axons start to project from the CNS as early as Hamilton and Hamburger (HH) stage 18 at the level of occipital somites (1 to 5). In the succeeding developmental stages, the axons developed, united with each other and ran dorsolaterally along the longitudinal axis of the embryo. Finally, it formed a bent near the first occipital somite and passed along with vagus nerve. This study will give us an idea on the topographic anatomy of accessory nerve (XI) during development of chick embryos.

Keywords: medulla oblongata; spinal cord; axonal trajectory; spinal accessory nerve; chick embryos

1. Introduction

The head of the embryonic vertebrate has a mesodermal component that consists of pharyngeal arch (visceral) mesoderm ventrally and paraxial (somatic) head mesoderm dorsally, giving rise to the branchial muscles and somatic muscles, respectively (reviewed by Sambasivan *et al.,* 2011). The ventral part of the head mesoderm, in contrast, gives rise to branchial muscles innervated by branchial cranial motor nerves (cranial nerves V, VII, IX, X and XI). The accessory (XI) nerve roots are located at the level of occipital somites because they contribute to the occipital bone (Couly *et al.,* 1993; Christ and Ordahl, 1995; Wilting *et al.,* 1995; Huang *et al.,* 2000). The accessory nerve, also called spinal accessory nerve (SAN), is the eleventh cranial nerve (XI). The accessory nerve composed of two parts: a cranial part (Ramus internus, accessory portion) and a spinal part (Ramus externus, spinal portion) (Standring *et al*., 2008). The spinal portion innervates the neck and back muscles, the trapezius and sternocleidomastoid muscles (Theis *et al.,* 2010), while the accessory portion innervates laryngeal and pharyngeal muscles. Fibers of both parts have a common trajectory and exit the hindbrain through the jugular foramen, similar to the vagus nerve (Ryan *et al.,* 2007). The cranial part (accessory portion) is the smaller of the two. Its fibers arise from the cells of the nucleus ambiguus and emerge as four or five delicate rootlets from the side of the medulla oblongata, below the roots of the vagus nerve. It runs laterally to the jugular foramen, where it interchanges fibers with the spinal portion or becomes united to it for a short distance; here it is also connected by one or two filaments with the jugular ganglion of the vagus. It then passes through the jugular foramen, separates from the spinal portion and is continued over the surface of

the ganglion nodosum of the vagus, to the surface of which it is adherent, and is distributed principally to the pharyngeal and superior laryngeal branches of the vagus. The spinal part (spinal portion) is firm in texture, and its fibers arise from the ventral horn cells in the cord between C1 and C5 of the cervical plexus. The fibres emerge from the cord laterally between the anterior and posterior spinal nerve roots to form a single trunk, which ascends into the skull through the foramen magnum. It then exits the skull through the jugular foramen, through which it passes, lying in the same sheath of dura mater as the vagus, but separated from it by a fold of the arachnoid.

2. Materials and Methods
2.1. Chick embryos
Fertile White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the farm maintained by the Institute of Animal Science, University of Bonn, Germany. Eggs were incubated in a humidified (80%) atmosphere at 37.8°C for the desired length of time and staged according to Hamburger and Hamilton, 1951. The embryos were fixed by 4% PFA in PBS or Dent's fixative for four hours to overnight at 4°C.

2.2. Immunohistochemistry
Immunostaining of the whole-mount embryos were performed as described by Tuschida *et al.,* 1994. All embryos were fixed in Dent's fixative (1 h to overnight) and then bleached in Dent's bleach overnight before immunohistochemical staining. Anti-Desmin (1:100) and 3A10 (1:50) antibodies were used to identify myotomes and axons, respectively. The antibodies were purchased from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA. Goat-anti-mouse-Cy2 and -Cy3 conjugated secondary antibodies (1:200) were used to detect the primary antibody.

2.3. Photographic documentation and data analysis
Stained embryos were photographed by using Nikon digital camera DXM1200C connected to a Nikon SM21500 fluorescence microscope. In all cases, images were assembled and annotated using Adobe Photoshop CS3.

3. Results and Discussion
During morphogenesis, axons of the accessory nerve join the vagal nerve and collectively ascend along the rostro-caudal axis and turning ventrally before reaching the otic vesicle. At this stage axons of these nerves are hardly distinguishable from each other. Using histological sections of embryos in which the first somite was marked, the vagal nerve was seen to pass through the territory of the developing somite 1 (Huang *et al.,* 1997). In this study, we investigated the topography of the accessory nerve (XI) using whole-mount immunohistochemistry of chick embryos (HH stage 12-23) with 3A10 and anti-Desmin antibodies. The 3A10 marked the axons and the Desmin marked the myotomes. We found that accessory nerve rootlets first appeared in the occipital region at HH stage 18 (Figure 1). This result varied from the report of Rogers, 1965; who reported early appearance at HH 19. The rootlets from the occipital region united to each other at HH stage 19 and developed in the succeeding stages (HH 20-21) and formed accessory nerve (XI). The accessory nerve (XI) formed a bent at the level of first somite and ran along with the vagus nerve (X) (Figure 2). This result supports the report of Pu *et al.,* 2013. Compared to the myotome of the second somite, the myotome of the first one was considerably smaller. The nervus glossopharyngeus (IX), vagus (X) and accessorius (XI) passed through the space between the second somite and the otic vesicle (Figure 3).

The head and trunk are not simply juxtaposed onto each other anterio-posteriorly, but the pharyngeal arches and rostral somites co-exist in the postotic region; the boundary of these two components forms an S-shaped head/trunk interface that is conserved in all vertebrate species (Kuratani, 1997). This interface develops into the future 'neck' region of some vertebrates (Matsuoka *et al.,* 2005), corresponding to a region that develops a set of intermediate structures, partly reflecting head-specific features and partly trunk, like the neck muscles that are derived from somites and associated with crest-derived connective tissues (reviewed by Kuratani, 1997). The cucullaris, tongue, and infrahyoid muscle complexes, as well as the nerves that innervate them (cranial nerves XI and XII), can be counted among these structures. The morphological and evolutionary evaluation of the cucullaris muscle (which became the trapezius and sternocleidomastoid muscles in mammals and SAN that innervates it have long been controversial, primarily because these structures arise in the interface between the head and trunk domains (Kuratani, 2008). Recently, anatomical, embryological, and molecular studies propose

that the SAN is a transitional nerve that does not belong either to general somatic efferent (GSE) nor special visceral efferent (SVE) (Benninger and McNeil, 2010).
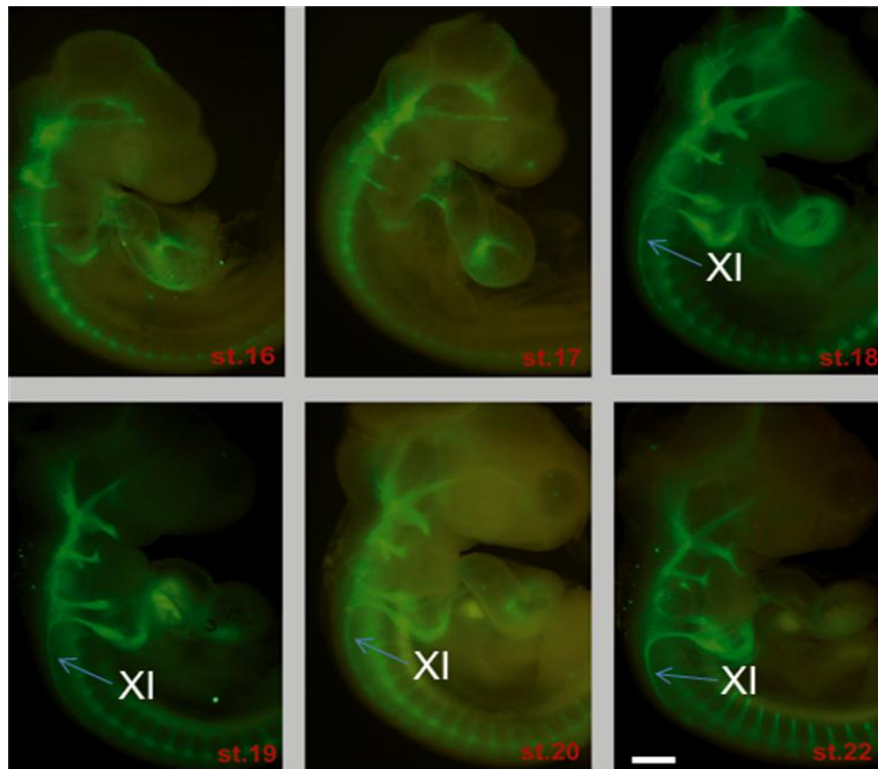

Figure 1. Whole-mount immunostaining of chick embryos with neurofilament (3A10) antibody. Accessory axons first appear at HH 18, unite to form accessory nerve (XI) at HH 19. Scale bar in all images 200 μm.
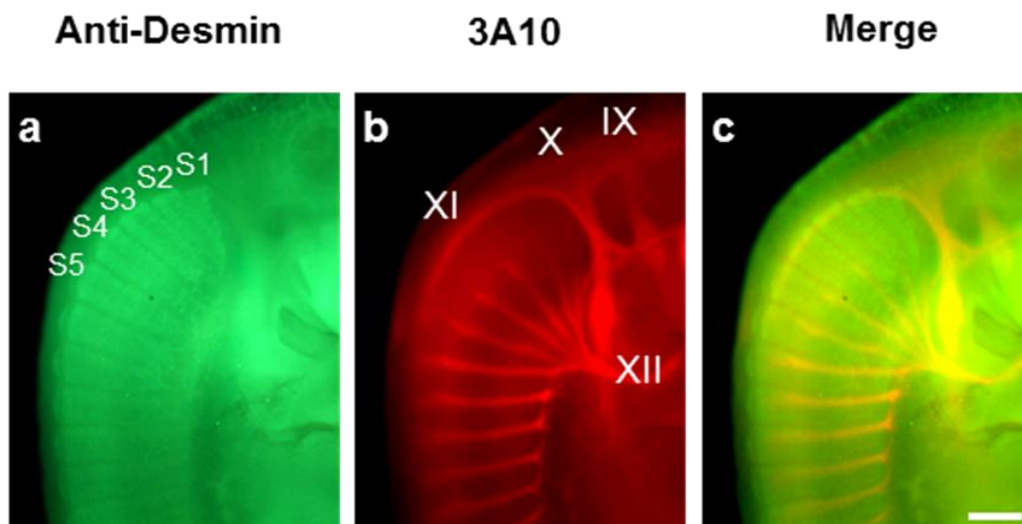

Figure 2. Whole-mount immunostaining of chick embryos (HH 23) with anti-Desmin and neurofilament (3A10) antibodies. Anti-Desmin stains myotomes of somites (a) and 3A10 stains axons (b). Roman numerical indicate cranial nerves. Accessory nerve (XI) emerges around the level of first somite, S1 (c). Scale bar in all images 250 μm.
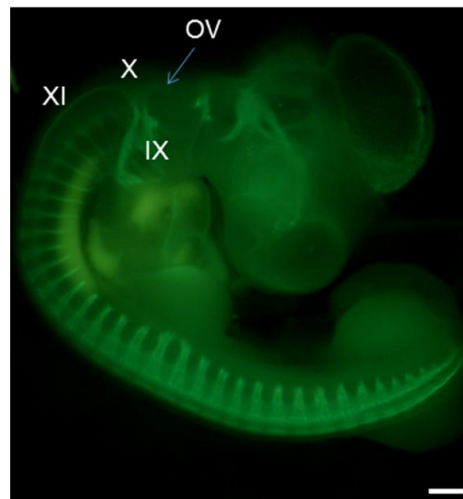
Figure 3. Whole-mount immunostaining of chick embryo (HH 22) with neurofilament (3A10) antibody. Roman numerical indicate cranial nerves and OV= otic vesicle. Scale bar 250 μm.

Mammalian SAN nuclei have been localized in adults of many animal species, based on retrograde labeling with a variety of tracers (HRP, DiI, fluorescent tracers or cobalt placed in the cucullaris muscle or directly into the SAN (McKenzie, 1962; Krammer *et al.,* 1987). The results of some of the reports differ—in a few cases, even for the same animal species by using similar retrograde labeling methods. For example, SAN nuclei have been identified in the caudal part of the medulla and the rostral part of the spinal cord in sheep (Flieger, 1967), monkey (Ueyama *et al.,* 1990), and rabbit (Ullah and Salman, 1986), whereas other researchers located them in the rostral part of the spinal cord in cat (Satomi *et al.,* 1985), monkey (Jenny *et al.,* 1988), and sheep (Clavenzani *et al.,* 1994). In our study, similar results were found in chick. Moreover, disagreement also exists as to the number of cell columns of the SAN longitudinal nuclei. The above discrepancy may be ascribed, at least in part, to the difference in accuracy of observation derived from different labeling methods. Recently, Ullah *et al.,* 2007 reported that there are three longitudinal columns that innervate the sternocleidomastoid muscle in addition to one trapezius column in the adult rat (Ullah *et al*., 2007), whereas Flieger, 1964 and Clavenzani *et al.,* 1994 found two groups of longitudinal nuclei for the sheep SAN. Hayakawa *et al.,* 2002 reported that there are two columns, one each for the sternocleidomastoid and trapezius muscles. Finally, a single longitudinal column was identified in the rat by retrograde labeling of the SAN branches with DiI (Yan *et al*., 2007), and similarly in the baboon (Augustine and White, 1986) only one column of somata in the spinal cord has been reported.

There are two SAN cell populations in the adult rat as found by retrograde labeling of SAN axons (Yan *et al.,* 2007). These authors clarified the localization of motor neurons, which extended axons either directly through the SAN or through the ventral rami of the C2–C6 cervical nerves to innervate the trapezius. The somata of trapezius-innervating neurons whose axons grow through the spinal accessory nerve are larger than those of neurons that pass through the cervical nerves. The medial column, which innervates the sternomastoid and cleidomastoid muscles, is located at the dorsomedial edge of the ventral horn at the level of C1 to C3, and the lateral column, which innervates the trapezius and cleido-occipital muscles, is located in the dorsolateral part of the ventral horn at the level of C2 to C6 (Matesz and Szekely 1983; Krammer *et al.,* 1987; Hayakawa *et al.,* 2002). In avians, on the other hand, the spinal motor axons that innervate the cucullaris sprout from dorsal roots, and these neurons exhibit branchiomotor properties by expression of *Phox2b* and *Isl1* (Kobayashi *et al.,* 2013; Hirsch *et al.,* 2007).

4. Conclusions
The current study highlights the details topographic anatomy of accessory nerve (XI) during chick embryo development. It would be the basis for understanding the developmental pathobiology of this nerve. However, further research can be conducted to explore the molecular mechanisms involved in the unique pathway of accessory nerve (XI).

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