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Short Contribution

Contralateral efferent neurons can be detected in the hindbrain outside of rhombomere 4

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ABSTRACT A group of efferent neurons whose bodies are located contralaterally and extend projections across the ventral midline of the hindbrain is considered as a rhombomere 4-specific characteristic. These neurons contribute to the vestibulo-acoustic nerve. At the level of rhombomere 2, a similar kind of efferents have only been described as a result of several experimental manipulations and have been interpreted as being due to rhombomere 2 acquiring rhombomere 4 identity. Here is shown that contralateral efferents can also be detected in rhombomere 2 of normal mouse and chicken hindbrains. These findings indicate that neural processes crossing the midline should not be considered as a rhombomere 4-specific characteristic. They also imply that the formation of the contralateral efferents at different rostro-caudal levels might be under different genetic controls, because *Hoxb-1*, which is not expressed in rhombomere 2, seems to be essential for their proper formation in rhombomere 4.

KEY WORDS: *contralateral efferents, rhombomeres, retrograde tracing*

For many years, intensive efforts have been made to elucidate how patterns are established along the anterior-posterior axis of the vertebrate neural tube (Lumsden and Krumlauf, 1996). Much of this work has been performed on the hindbrain because it is the most accessible region of the developing central nervous system to experimental analysis. During early development, the hindbrain is divided into segments called rhombomeres (r) (Lumsden and Keynes, 1989). This morphological segmentation correlates with the expression domains of a variety of genes and with patterns of neuronal organization within the hindbrain (Lumsden and Krumlauf, 1996). Cranial nerves arise from particular rhombomeres, whereas their motor neurons derive from specific rhombomere pairs (Lumsden and Keynes, 1989). For instance, motor neurons of the Vth cranial nerve derive from r2 and r3, and those of the VIIth and VIIIth cranial nerves derive from r4 and r5. The neuronal organization in the different rhombomeres is so characteristic that it provides very useful anatomical criteria to identify particular rhombomeres after experimental manipulations of the hindbrain.

A typical example for a rhombomere-specific characteristic is a group of efferent neurons in r4 that migrate contralaterally and extend projections across the ventral midline of the hindbrain to contribute to the vestibulo-acoustic nerve (Simon and Lumsden, 1993). This type of contralateral efferent neurons has been identified in r4 by retrograde neural tracings from the VIIth/VIIIth nerve exit point but such neurons have not been reported for r2 in normal embryos by similar tracings from the Vth cranial nerve (Marshall *et al.*, 1992; Simon and Lumsden, 1993; Simon *et al.*, 1995). Hence, the presence of neural processes crossing the midline has been considered as a typical phenotypic criterion to identify r4 in a number of studies concerning the differentiation of the hindbrain along its rostro-caudal axis (Marshall *et al.*, 1992; Zhang *et al.*, 1994; Simon *et al.*, 1995; Gale *et al.*, 1996).

When, in the course of some control experiments, I performed retrograde tracings from the Vth nerve exit point of mouse embryos, I found the typical r2/3 neuronal organization previously reported (Marshall *et al.*, 1992) (Fig. 1). Unexpectedly, however, when those specimens were observed at higher magnification, an additional group of axons was observed crossing the floor plate at r2 (Fig.1). I have detected those axons in 11 of 14 mouse embryos (E 10.5), although their number was quite variable. Also, as previously described for the contralateral efferents at r4 (Simon and Lumsden, 1993), contralateral cell bodies from which those axons originate were easily seen in many cases (Fig. 1). Since projections from these neurons are only seen in one direction (the contralateral), it is highly unlikely that they are dendrites from ipsilateral cell bodies rather than efferent axons. Additionally, some neural processes could also be detected crossing the midline at the r3 level (Fig. 1b).

Chicken and mouse hindbrains share many molecular and neuronal organization characteristics, but they also display some clear differences. For instance, in mouse, but not in chicken, a population of neurons contributing to the facial nerve project

Abbreviations used in this paper: r, rhombomere; DiO, 3,3'-dioctadecyloxacarbocyanine; E, mouse embryonic age in days

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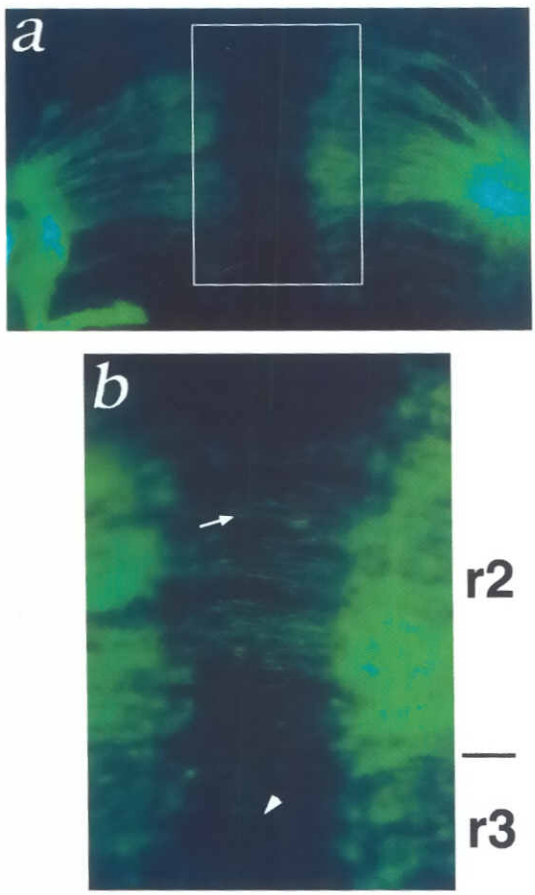

Fig. 1. Retrograde neural tracing from the vth cranial nerve of mouse embryos. *The vth cranial nerves of E10.5 mouse embryos were injected with DiO at their exit point from the hindbrain. After 24 h in the dark the hindbrains were dissected out, flat mounted and photographed.* ***(b)*** *shows a magnification of the boxed area in* ***(a)****. Many neural processes are evident crossing the midline at r2 level. The arrow points to a contralateral cell body from which an axon that crosses the midline originates. Some processes are also seen at r3 level (arrowhead).*

rostrally along the midline before turning laterally toward the root of the cranial nerve (Guthrie and Lumsden, 1992; Studer *et al.*, 1996). To find out whether the presence of contralateral efferents at the r2 level was specific to mouse embryos I also performed retrograde tracings from the vth cranial nerve exit point in chicken embryos [stage 26 (Hamburger and Hamilton, 1951)]. As in wite mouse, I found neural processes crossing the midline at r2 (5 of 10 cases) (Fig 2). These results indicate that this characteristic of the neuronal organization at the r2/3 level is not just a feature of the mouse.

The above findings are in clear contradiction with previous reports and indicate that contralateral efferent neurons, previously described as r4-specific (Marshall *et al.*, 1992; Simon and Lumsden, 1993; Zhang *et al.*, 1994; Simon *et al.*, 1995), can also be detected in r2. Since the injection of the dye was performed exclusively in the vth cranial nerve it is clear that the neural processes detected across the floor plate must belong to neurons contributing to this cranial nerve. It is not clear why those neurons were not detected previously in r2. Particularities of one technical procedure versus another cannot be claimed because I have applied here the same technique used in previous experiments. The reason might rather be related to the intensity of the labeling and the detection procedure. In my experiments, midline processes were apparent at low magnification only when a good labeling of the axons contributing to the vth nerve was accomplished. In specimens showing poor overall labeling of the neural projections the midline processes were only evident when the floor plate was observed at high magnification. In addition, all the embryos in which I could not find contralateral efferents showed particularly poor overall labeling in r1, r2 and r3.

If contralateral efferents were also present in r2, it would imply that their development at different rostro-caudal levels of the hindbrain might involve different control genes. It has been reported that *Hoxb-1* is essential for the proper formation of these neurons in r4 (Studer *et al.*, 1996). As this gene shows r4-restricted expression (Lumsden and Krumlauf, 1996), it is not likely that it participates in the formation of contralateral efferent neurons at levels other than r4. Such neurons outside r4 might therefore be formed by *Hoxb-1* independent mechanisms.

Finally, from an experimental point of view, it is important to point out that, since contralateral efferent neurons can also be detected at levels other than r4 in normal embryos, they should not be used as a phenotypic criterion to define this rhombomere.

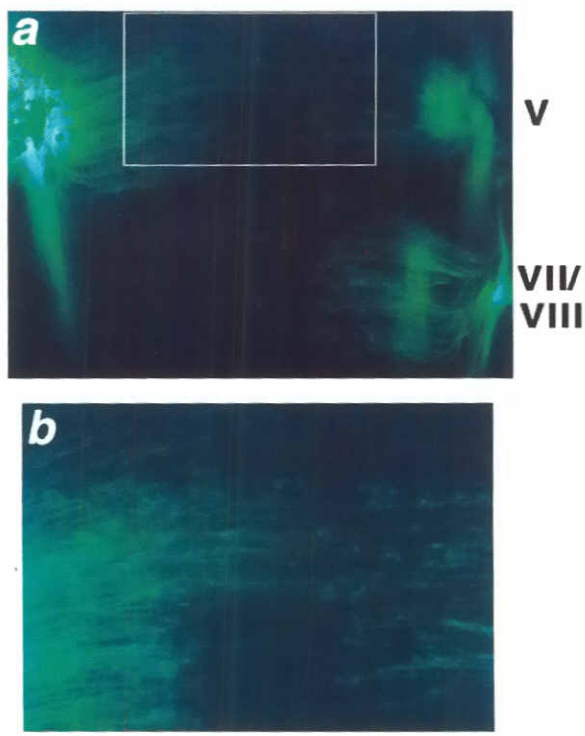

Fig. 2. Retrograde neural tracing from the vth cranial nerve of chicken embryos. *The vth cranial nerves of stage 26 (Hamburger and Hamilton) embryos were injected with DiO at their exit point from the hindbrain. In this specimen the vii/viiith cranial nerve was also injected in one side. After 24 h in the dark the hindbrains were dissected out, flat mounted and photographed.* ***(b)*** *shows a magnification of the boxed area in* ***(a)****. Many neural processes are evident crossing the midline at r2 level. v and VII/VIII indicate the positions of the vth and VII/VIIIth cranial nerves respectively.*

Experimental procedures

For this study, E 10.5 mouse embryos and stage 26 (Hamburger and Hamilton, 1951) chicken embryos were used. They were fixed in 4% paraformaldehyde for 2 h at 4°C and rinsed in PBS. The embryos were pinned ventral side up on an agarose plate, and the mesenchymal tissues of the branchial arches carefully removed to expose cranial nerves and ganglia. Small amounts of 3,3'-dioctadecyloxacarbocyanine (DiO) (5 mg/ml in dimethylformamide) were then injected at the place where the vth cranial ganglia contacts the root of the cranial nerve, contacts the root of the cranial nerve. The injection of the dye was restricted to that area and never affected the hindbrain or other neighboring areas. The embryos were left and the embryos were left in PBS at room temperature for 20 h in the dark. The hindbrains were then dissected out, flat mounted and observed under epifluorescence using a filter set for fluorescein.

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