Generation of newborn rat brainstem-spinal cord preparations with defined rostral boundary

Supplemental material for

Dependence on extracellular Ca^{2+}/K^{+} antagonism of inspiratory centre rhythms in slices and en bloc preparations from newborn rat brainstem

A. Ruangkittisakul[+], L. Secchia[+], T. D. Bornes, D. M. Palathinkal & K. Ballanyi
Department of Physiology & Perinatal Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

(For details of references, see main paper)

The isolated brainstem-spinal cord preparation from newborn rats is an established model to study the neural control of breathing (Ballanyi *et al.* 1999). In this preparation, the activity of various respiratory networks is retained. Within the ventral respiratory column, these networks are the pre-Bötzinger Complex (preBötC) and the more rostrally located Bötzinger Complex (BötC) plus parafacial respiratory group (pFRG) that is presumably closely associated with the retrotrapezoid nucleus (RTN) (Feldman & Del Negro, 2006). The rostrocaudal extensions of respiratory marker brainstem nuclei are surprisingly constant in newborn rats of postnatal days (P) 0-4 (Ruangkittisakul *et al.* 2006). In this report, a representative brainstem atlas consisting of 50 μm fixed and thionin-stained transverse brainstem sections was introduced. In this "reference" atlas, the locations of marker structures were referred to their distance in mm from the caudal end of the facial motonucleus (VII_c), with a negative sign assigning a position caudal to VII_c. According to this terminology, the centre of the preBötC is presumably located close to -0.50 and extends ~100 μm each in rostral and caudal direction, thus it is located between -0.40 to -0.60 (compare Figs. S1, S2 with Fig. 1 in Smith *et al.* 1991, Fig. 2 in Ruangkittisakul *et al.* 2006 and Fig. 1 of the present study). The rostrocaudal extension of the RTN/pFRG greatly overlaps with that of the VII nucleus, but appears to extend ~200 μm caudal to VII_c (Onimaru & Homma, 2003, 2006). It should be noted that pre/post-inspiratory active neurons are also found within and caudal to the preBötC (Arata *et al.* 1990; Smith *et al.* 1990; Onimaru *et al.* 2003).

Here, we describe a method for generation of newborn rat brainstem-spinal cord preparations with defined rostrocaudal boundaries that have either the preBötC more or less exposed to the rostral boundary or contain more rostral tissue with (portions of the) VII and thus, the BötC and RTN/pFRG. For their generation, we used structural landmarks on the ventral surface of the brainstem for positioning of a razor blade for manual transection. As shown in Fig. S1, sectioning of brainstem-spinal cords at such landmarks revealed that the centre of the most rostral hypoglossal (XII) rootlet was located at -0.47 ± 0.04 (n= 6) while cutting just caudal to the vagal (X) nerve revealed a mean boundary of -0.06 ± 0.05 (n= 5). Transecting at the caudal end of the caudal cerebellar artery (CCA) (De Araujo & Campos, 2005) revealed a boundary of +0.54 ± 0.02 (n= 5), whereas cutting just rostral to the CCA showed caudal structures of the medial nucleus of the trapezoid body (NTB) at +0.79 ± 0.06 (n= 4). The CCA was located at +0.74 ± 0.16 (n= 14) (Fig. 1). A very caudal cutting position, where the vertebral arteries join to form the basilar artery (BA), revealed a boundary of -1.21 ± 0.08 (n= 5) (Figs. 1, S1).

The sectioning level of fixed and post-experiment thionin-stained brainstem-spinal cords with the proposed rostral boundary located between -0.20 and -0.50 was histologically identified by comparing structures of the inferior olive (IO) on their cut rostral surface with the complementary structures in the fixed and stained transected rostral aspect of the en bloc preparation (Figs. 1, S1, S2). In ~40 % of 21 preparations with the rostral boundary presumably located between -0.15 and -0.30, IO structures were not revelead in the stained preparations. In those cases, sagittal sections of the transected rostral aspect of the preparations were inspected to determine the rostral boundary via the distance of the caudal end of the block from VII_c (Fig. S3). In >70 % of cases, the histologically-identified boundary was close to that expected according to the surface landmarks.

The notable number of preparations without visible IO structures on the cut surfaces indicates that either the staining was sometimes poor or that the distance between VII_c and the rostral end of the IO may be more variable than indicated by our recent study based on transverse sections (Ruangkittisakul *et al.* 2006). To clarify this, we generated series of 200-250 μm thick sagittal sections of P2 rat brainstems (Fig. S2). In the sagittal plane, VII_c is located >1 mm laterally, whereas the most rostral extension of the IO is located close to the midline (Ruangkittisakul *et al.* 2006). To determine the mean rostrocaudal distance between these regions in sagittal slices, we

have used as reference the caudal end of the medial subregion of the NTB, which is located within the same mediolateral plane as the IO (Fig. S2). This marker region is colocalized on the one hand with the caudal end of the superior olive and on the other hand with the rostral end of VII nucleus (Paxinos G, Watson C [1982] The rat brain in stereotactice coordinates. Academic Press, Sydney, New York, London, San Diego, San Francisco, Sao Paolo, Tokyo, Toronto). In 5 sagittal sections, the mean distance between the rostral end of the IO and VII_c was 0.16 ± 0.03 mm, similar to that determined in transverse serial brainstem sections from P0-4 rats used to generate the atlas (0.13 ± 0.029 mm, n= 26; Ruangkittisakul *et al.* 2006). According to data from that report, the mean distance between the caudal end of the NTB and the rostral end of the VII nucleus was 0 ± 0.05 mm (n= 10). The analysis of transected slices revealed further that VII nucleus extended to +0.76 ± 0.07 (n= 14).

Based on this, brainstem-spinal cord preparations transected at the rostral preBötC boundary (i.e., at the rostral proximal end the most rostral XII root) show pronounced IO structures at their rostral boundary. Conversely, preparations transected between the VI nerve and the CCA would contain the entire VII and thus the RTN/pFRG. Finally, preparations cut at the X nerve would have VII_c at the cut surface and thus contain the BötC. The brainstem-spinal cord (BSC) preparations can be labelled via the rostral boundary, i.e., in the above 3 cases BSC[-0.40], BSC[0], BSC[+0.74]. Our study revealed that preparations with the boundary caudal to -0.45 did not generate inspiratory-related rhythm (Fig. S4).

Legends to supplemental figures

Fig. S1. Ventral brainstem surface markers for the generation of brainstem-spinal cords (BSC) with identified boundaries.

A, a fixed and thionin-stained BSC with pons attached was subjected to 5 consecutive manual transections using a razor blade. The 4 resulting transverse slices and the rostral brainstem block were exposed to thionin again for staining the cut surfaces (caudal slice boundaries are shown in the upper panels). Numbers indicate the distance of the caudal surface from VII_c as reference (compare Fig. 1). *B*, schema of ventral brainstem surface marker structures. The centre of the most rostral XII rootlet was located 0.47 mm caudal to VII_c, thus at "-0.47" according to the terminology of Ruangkittisakul *et al.* (2006), whereas cutting just caudal to the vagal (X) nerve revealed a mean boundary at -0.06. Transecting just caudal to the caudal cerebellar artery (CCA, De Araujo & Campos, 2005) revealed a mean boundary at +0.54, whereas cutting caudal to the abducens (VI) nerve and rostral to the latter artery showed caudal structures of the nucleus of trapezoid body (NTB) at +0.79. A very caudal cutting position, specifically where the vertebral arteries joined to form the basilar artery, revealed a mean boundary of -1.21. The ovals labelled preBötC, BötC and pFRG indicate the positions of respiratory structures (compare Fig. 1). Scale in *B* (in mm) also applies for *A*.

Fig. S2. Brainstem marker nuclei for localizing respiratory structures in newborn rat brainstem-spinal cord preparations.

Images show sagittal fixed and thionin-stained 200 μm sections from a postnatal day (P) 2 Wistar (W) rat brainstem, the uppermost photograph showing the most lateral section. The pair of dotted lines on the left indicates the rostral end of the inferior olive (IO) and the caudal end of the VII motonucleus (VII_c). The line on the right indicates the caudal end of the medial subregion of the NTB, which is colocalized in midline sections with the caudal end of the superior olive (Paxinos & Watson, 1982). The rostral end of the VII nucleus is colocalized with the caudal end of the medial NTB, though in a more lateral sagittal plane. This allows determination of the rostrocaudal extension of the VII nucleus, which proposedly greatly overlaps with the pFRG (see also Figs. 1, S1). The centre of the ~200 μm spanning preBötC is presumably located at ~0.50 mm caudal to VII_c (Smith *et al.* 1991; Ruangkittisakul *et al.* 2006). Note that the inspiratory active dorsally-located hypoglossal (XII) motonucleus has a rostrocaudal extension similar to that of the IO.

Fig. S3. Determination of the rostral brainstem-spinal cord boundary for transection levels lacking histological respiratory marker regions.

A, fixed and thionin-stained caudal and rostral brainstem tissue blocks from a P2 W rat after manual transection aiming to obtain a BSC[-0.20]. The rostral boundary could not be determined due to lack of presence (or lack of staining) of IO structures (*B*). *C*, serial sagittal sectioning (250 μm) of the rostral brainstem block revealed that the closest distance of VII_c from the caudal boundary of the tissue block was 0.2 mm. Accordingly, the preparation could, indeed, be labeled BSC[-0.20]W-P2.

Fig. S4. Absence of inspiratory rhythm in a brainstem-spinal cord preparation with the rostral boundary at the proposed centre of the preBötC.

A, photographs of a transected live (left panel) and fixed plus thionin-stained (right panel) BSC[-0.50]W-P1 preparation. *B*, the uppermost trace shows non-respiratory cervical nerve (C_4) bursts occurring at irregular intervals and variable amplitude within the first 12 min of recording in 3 mM [K^+] before "rhythm" stopped (not shown). In 6.2 mM [K^+] solution, irregular small amplitude cervical bursts were intermingled with large amplitude, long duration (0.5-4 min) activity. The massive long duration discharge was reversibly suppressed in 6.2 mM [K^+] by "high Ca^{2+}/Mg^{2+}" superfusate in which [Ca^{2+}] was raised from 1.2 to 2.4 mM and [Mg^{2+}] from 1.0 to 1.3 mM. In that saline, large amplitude bursting occurred at variable strength and single burst duration of 1-10 s. Return to solution with physiological cation content abolished all activities. Similar to that example, 2 further BSC preparations with the boundary at -0.50 and one with a boundary at -0.55 did not show respiratory rhythm.

Fig. S1

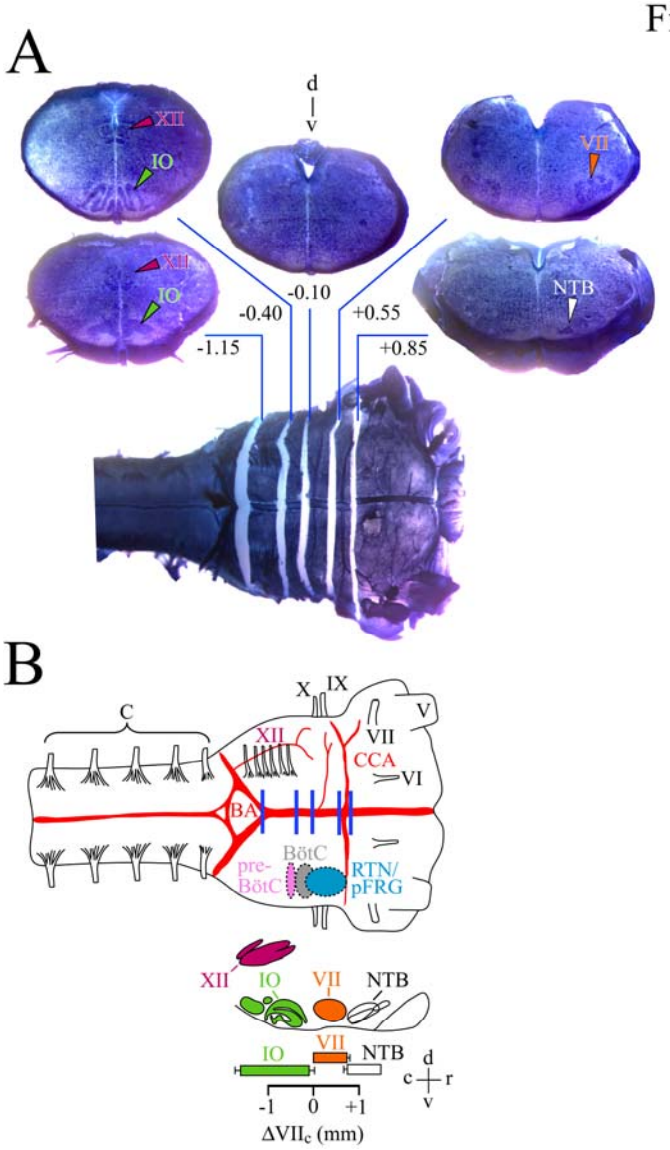

A
d
v
XII
IO
VII
XII
IO
NTB
-0.10
-0.40
+0.55
-1.15
+0.85
B
X
IX
C
V
XII
VII
CCA
VI
BA
BötC
pre-
BötC
RTN/
pFRG
XII
IO
VII
NTB
IO
VII
NTB
d
c
r
v
-1
0
+1
ΔVIIc (mm)

Fig. S2

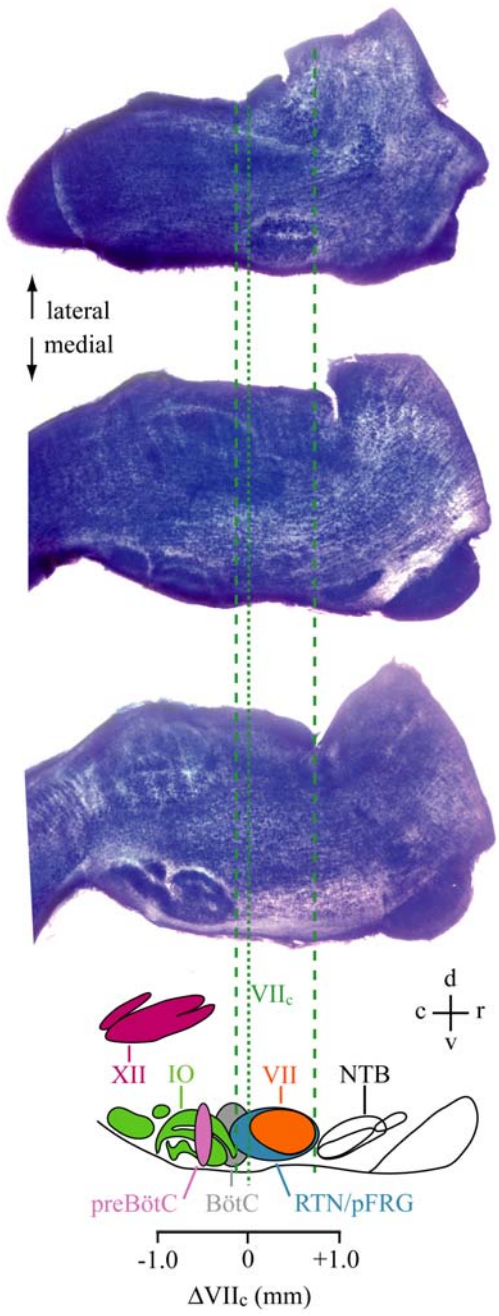

lateral
medial
VIIc
d
c
r
v
XII
IO
VII
NTB
preBötC
BötC
RTN/pFRG
-1.0
0
+1.0
ΔVIIc (mm)

Fig. S3

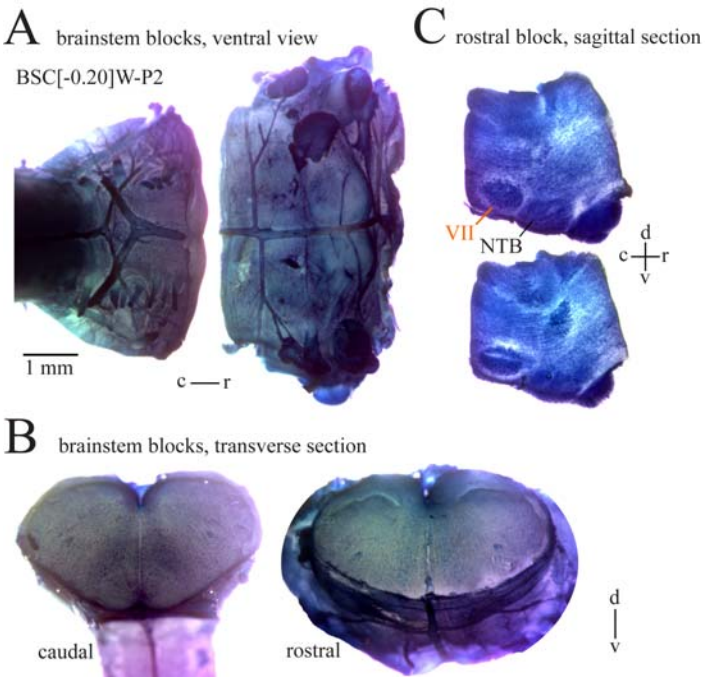
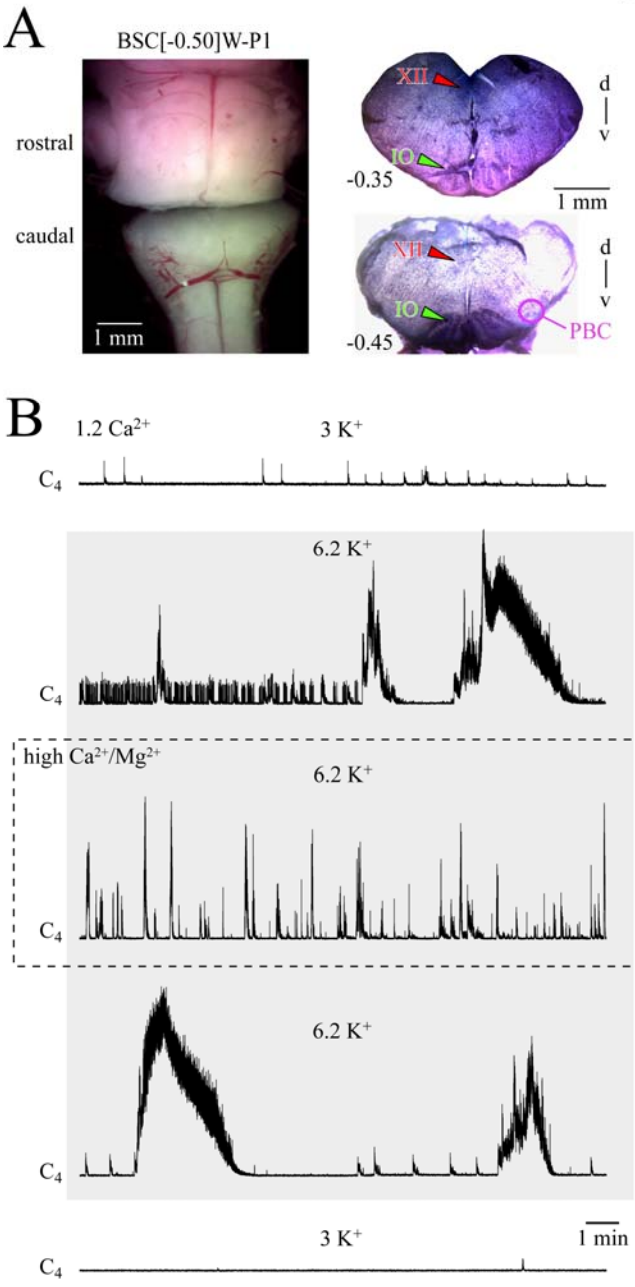

A brainstem blocks, ventral view
BSC[-0.20]W-P2
1 mm
c — r
C rostral block, sagittal section
VII
NTB
d
c r
v
B brainstem blocks, transverse section
caudal
rostral
d
v

Fig. S4


A
BSC[-0.50]W-P1
rostral
caudal
1 mm
XII
IO
-0.35
d
v
1 mm
XII
IO
PBC
-0.45
d
v
B
1.2 Ca2+
3 K+
C4
6.2 K+
C4
high Ca2+/Mg2+
6.2 K+
C4
6.2 K+
C4
3 K+
1 min
C4