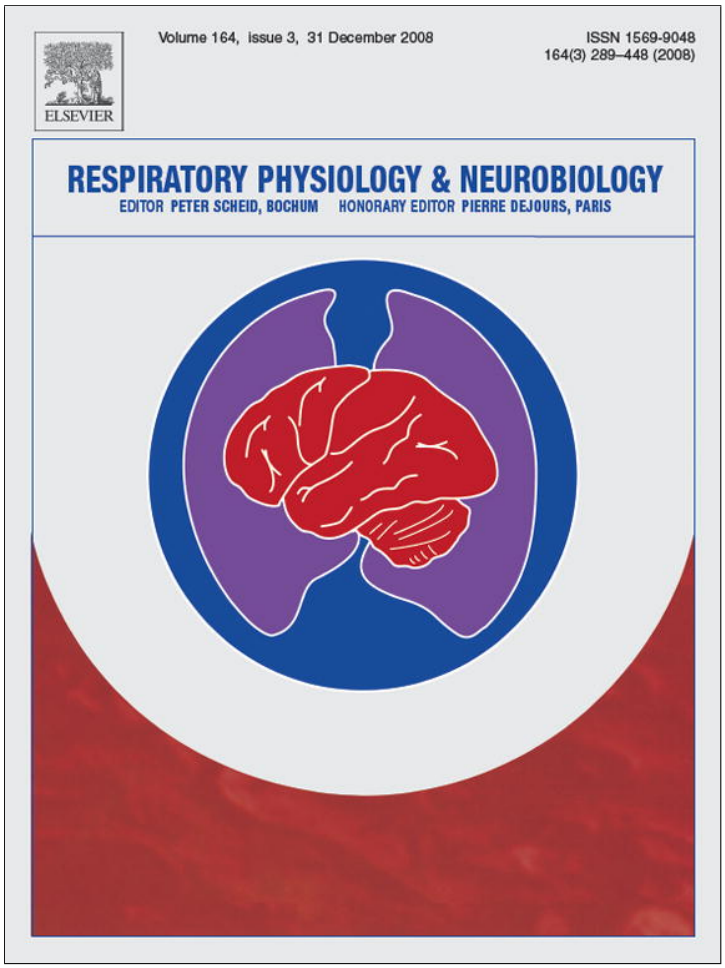

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# Respiratory rhythm of brainstem–spinal cord preparations: Effects of maturation, age, mass and oxygenation

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ABSTRACT

We examined the effect of age, mass and the presence of the pons on the longevity (length of time spontaneous respiratory-related activity is produced) of brainstem–spinal cord preparations of neonatal rodents (rats and hamsters) and the level of oxygenation in the medulla respiratory network in these preparations. We found the longevity of the preparations from both species decreased with increasing postnatal age. Physical removal of the pons increased respiratory frequency and the longevity of the preparation. However, tissue oxygenation at the level of the medullary respiratory network was not affected by removal of the pons or increasing postnatal age (up to postnatal day 4). Taken together, these data suggest that the effect of removing the pons on respiratory frequency and the longevity of brainstem–spinal cord preparations with increasing postnatal age are primarily due to postnatal development and appear to be unrelated to mass or changes in levels of tissue oxygenation.



## 1. Introduction

The respiratory network, including the centers required for the neurogenesis of respiratory rhythm, must be sufficiently well developed at the time of birth to cope with the immediate requirements of respiration. Current evidence, however, indicates that the system continues to undergo substantial postnatal growth and maturation (Liu and Wong-Riley, 2001; Dutschmann et al., 2004; Kron et al., 2007). We have been particularly interested in the postnatal changes that occur in cold tolerance and the ability of the respiratory system to restart (autoresuscitate) in neonatal rodents following hypothermic respiratory arrest (Mellen et al., 2002; Tattersall and Milsom, 2003; Zimmer and Milsom, 2004). We have attempted to address these questions using brainstem–spinal cord (*en bloc*) preparations from neonatal rodents (rats and hamsters) of various ages with the pons intact (Pontomedullary preparations) and with the pons removed (Medullary preparations). In attempting to interpret our data we have been confronted by the need to dissociate the effects of increasing tissue mass from postnatal growth that may result in hypoxia at the respiratory rhythm generators from the effects of postnatal maturation.

The *en bloc* preparation has been widely used in the study of respiratory rhythmogenesis (Suzue, 1984; Hilaire et al., 1989; Smith et al., 1990; Errchidi et al., 1991; Ito et al., 2002; Viemari et al., 2003; Guimaraes et al., 2007). Although it is commonly acknowledged that older preparations have reduced viability, this is generally assumed to be due to the effects of increasing mass which reduces oxygen diffusion from the superfusate to the respiratory neurons. However, the same trend in reduced viability occurs in neonatal rodents of very different sizes (rats vs. mice) (Errchidi et al., 1991; Viemari et al., 2003), suggesting that oxygen diffusion may not be the primary cause for the reduced longevity of the *en bloc* preparation. Although, one may argue that given the increase in mass specific metabolic rate seen with decreasing size *in vivo* in mammals, the smaller mouse tissue may have a higher metabolic rate than rat tissue resulting in similar $P_{O_2}$ profiles within the medulla. Also while numerous studies have shown that preparations containing the pons have lower respiratory frequencies and this is generally attributed to descending inhibition arising from the pons (Hilaire et al., 1989; Errchidi et al., 1991; Ito et al., 2002; Viemari et al., 2003; Guimaraes et al., 2007), part of this effect may be due to increased mass and reduced oxygenation at the medullary respiratory rhythm generators. As a result of these observations, the present study was designed to attempt to dissociate the effects of age, mass, postnatal development and levels of oxygenation, on the changes that occur in respiratory frequency and temporal longevity of brainstem–spinal cord preparations from neonatal rodents with and without the pons.

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## 2. Methods

All experiments were performed with prior approval from the University of British Columbia Animal Care Committee, under the guidelines of the Canadian Council for Animal Care (CCAC).

### 2.1. Brainstem–spinal cord preparation

Neonatal Sprague–Dawley rat pups (0–8 days postnatal) or Syrian (Golden) hamster pups (0–8 days postnatal) were deeply anaesthetized with 2–4% halothane or isoflurane until the absence of a withdrawal reflex from toe pinch. The brainstem and spinal cord were isolated *en bloc* (preparation previously described by Suzue, 1984) at room temperature while submerged in artificial cerebral spinal fluid (aCSF, 1.5 mM $CaCl_2$, 113.0 mM NaCl, 3.0 mM KCl, 1.2 mM $NaH_2PO_4$, 30.0 mM $NaHCO_3$, 30.0 mM D-glucose) bubbled with 95% $O_2$–5% $CO_2$, to obtain a pH of 7.4. The brainstem was trimmed rostrally at one of two locations: (1) just rostral of the pons at the level of the superior cerebellar peduncles for pons–medulla–spinal cord (Pontomedullary) preparations or (2) the rostral end of the pyramids and just rostral of the caudal cerebellar artery for medulla–spinal cord (Medullary) preparations. The spinal cord was transected caudal to the seventh cervical root and the entire *en bloc* preparation was transferred and pinned ventral side up on a stainless steel grid in a plexiglass recording dish. The stainless steel grid allows for simultaneous superfusion of the preparation over both the ventral and dorsal surfaces. The preparation was continuously superfused with oxygenated aCSF maintained at 27 °C by a Lauda water bath (Model RC6) and monitored using a thermocouple placed in the bathing fluid in the chamber. The flow rate of the aCSF was maintained between 5 and 9 ml/min. Whole nerve activity was recorded from C1 or C4 cervical rootlets via a glass suction electrode, amplified (20–50 K), filtered (100 Hz to 3 kHz) and recorded (2000 samples/s) using a Windaq data acquisition system (DI200; DataQ Instruments, Akron, OH, USA).

### 2.2. Experiment 1: longevity of respiratory-related motor output of en bloc preparations at different ages

To examine the effect of postnatal age, brain mass and the presence of the pons on the longevity of the *en bloc* preparations, Pontomedullary and Medullary preparations from both rats and hamsters at specific postnatal days were divided into five age groups: P0 (day of birth), P2 (2 Postnatal days), P4, P6 and P8 ($n$ = 5–9, in each group). Fictive breathing was recorded from C1 or C4 nerve rootlets via a glass suction electrode and the preparations were maintained in oxygenated aCSF at 27 °C at a constant flow rate until nerve activity ceased. Wet weights (mass) of the transected pons and the medulla with spinal cord (up to C7) were collected from brains of rats and hamsters after experimentation. The total mass was obtained by adding the pons and medulla/spinal cord masses together. The tissue was removed from the experimental chamber and blotted dry with kimwipes and weighed immediately.

### 2.3. Experiment 2: chemical inhibition of the pons vs. pontine removal or transection

We examined the effect of pontine influence on respiratory rhythm at the ages of P0, P2 and P4 ($n$ = 7–11 per age per treatment). Only preparations from rat pups up to P4 were used in this series of experiments as preparations from rat pups older than P4 did not last long enough for the protocol, as determined from results from Experiment 1. Pontomedullary preparations were prepared as described above and placed in an acrylic recording dish consisting of two chambers joined by a narrow gap. The preparation was placed in the chamber with the caudal cerebellar artery at the narrow gap (Fig. 1A). The gap between the two chambers was packed with Vaseline (grease gap) to completely separate the two

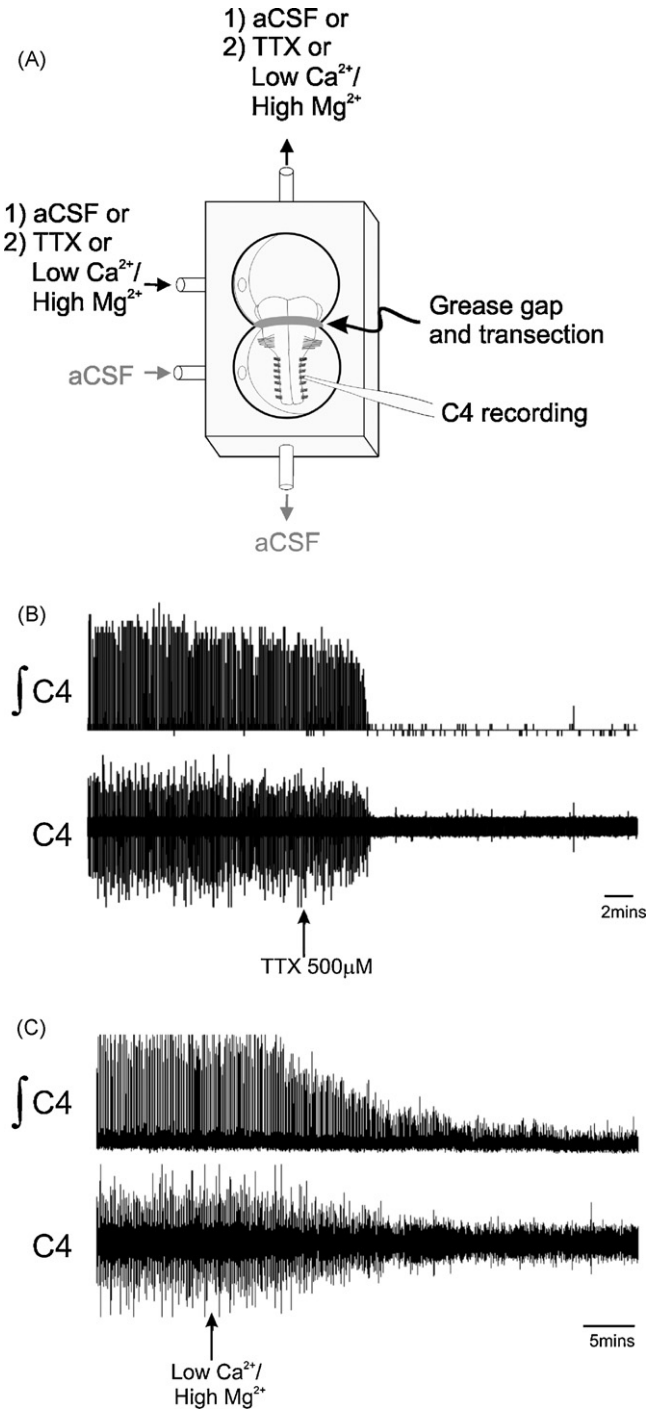


**Fig. 1.** (A) Schematic illustration of the double perfusion chamber used for separate superfusion of the pons and medulla/spinal cord. The vaseline (grease gap) was placed at the level of the caudal cerebellar artery and fictive respiratory bursts were recorded from C4 nerve rootlets. (B) Preliminary experiments superfusing tetrodotoxin (TTX, 500 (M) over the medulla and spinal cord resulted in cessation of burst activity within 8 min. (C) Superfusion of artificial cerebral spinal fluid (aCSF) containing 0.2 mM $Ca^{2+}$ and 5 mM $Mg^{2+}$ (low $Ca^{2+}$/high $Mg^{2+}$) over the medulla and spinal cord resulted in a gradual decrease in burst amplitude until C4 bursts could not be detected after 30 min. Arrows indicate when the superfusate was switched from regular aCSF to the treatment indicated.

chambers, with one chamber containing the pons and the other containing the medulla and spinal cord. Each chamber had its own perfusion circuit allowing for separate superfusion of the pons and medulla (Fig. 1A). Fictive breathing was recorded from C4 rootlets using a glass suction electrode.

The preparation was allowed to stabilize for 20–30 min, with both chambers superfused with oxygenated aCSF maintained at 27 °C, as described above. The integrity of the grease gap was tested by addition of a small amount of dye (~50–100 (l of 1% Fast Green FCF w/v, into 200 ml aCSF, resulting in equivalent concentration of ≤0.0005%) into each chamber separately to check for leaks during the stabilization period. At the end of the stabilization period, the superfusate to the pontine chamber was replaced with one containing either (1) 0.2 mM $Ca^{2+}$ and 5 mM $Mg^{2+}$ (low $Ca^{2+}$/high $Mg^{2+}$) for 45–60 min (pH 7.4), or (2) tetrodotoxin (TTX, 500 (M, pH 7.4) for 15 min. In preliminary experiments, superfusion of the medulla and spinal cord with TTX (500 (M) resulted in a rapid and sudden cessation of C4 activity approximately 8 min after the addition of TTX (Fig. 1B). Based on this data, the time course of 15 min of TTX exposure was determined to be sufficient to inhibit neuronal activity. Similarly, in preliminary experiments, superfusion of the medulla and spinal cord with low $Ca^{2+}$/high $Mg^{2+}$ superfusate abolished C4 discharge progressively over 30 min (Fig. 1C). Based on this data, a time course of 45 min superfusion of the pons was chosen as sufficient to inhibit neuronal activity. After superfusion with TTX or low $Ca^{2+}$/high $Mg^{2+}$, the pontine superfusate was switched back to regular aCSF for 20 min to ensure that all drug was washed out of the pontine chamber and tubing. Following washout of TTX or low $Ca^{2+}$/high $Mg^{2+}$, the grease gap was removed and the pons was manually transected using a razor blade at the same level as the grease gap. In experiments with TTX, the pontine tissue was removed from the perfusion chamber, while in experiments using low $Ca^{2+}$/high $Mg^{2+}$ superfusate, the pontine tissue mass was not removed following transection but left *in situ* in the chamber. This was done to examine the effect of pontine removal vs. pontine transection (descending inputs severed but without altering total tissue mass in the perfusion chamber). The preparation was again allowed to stabilize and fictive breathing was recorded for 30 min. At the end of the experiment, the preparation was removed from the chamber and fixed in 10% neutral buffered formalin followed by cryoprotection in 30% sucrose for later histological verification of the transection level.

### 2.4. Experiment 3: oxygenation of the medulla

*En bloc* preparations were prepared as described above with the pons intact and transferred to an acrylic chamber, superfused with oxygenated aCSF maintained at 27 °C and at a constant flow rate. The preparation was always orientated in the same direction with respect to the flow of the superfusate, such that the superfusate flowed over the pons prior to the medulla and spinal cord. Fictive breathing was recorded from C4 nerve rootlets throughout the experiment to ensure that the preparation was rhythmically active. The preparation was allowed to stabilize for 30 min before measurement of $P_{O_2}$ began. All preparations maintained fictive breathing throughout the experimental protocol.

Measurement of $P_{O_2}$ was performed using a commercially available fiber-optic microsensor (Optode, OxyMicro System, World Precision Instruments) with a tapered tip diameter of ~30 (m that allowed for high spatial resolution. Prior to each experiment, the Optodes were calibrated for two points: water-vapor saturated air and oxygen-free solution. The measurement for water-vapor saturated air was obtained by placing wet cotton wool in a container with two access holes for the introduction of the Optode and temperature sensor. The system was allowed to equilibrate for >10 min, to ensure water-vapor saturation. Oxygen-free solution for calibration was obtained by bubbling 10 ml of aCSF with 100% nitrogen for >10 min, in a small container with two access holes for the introduction of the Optode and temperature sensor. $P_{O_2}$ measurements were taken every 5 s and were acquired directly by the data acquisition software. All measurements were temperature corrected.

#### 2.4.1. Effect of the presence of the pons on oxygenation of the medulla

$P_{O_2}$ measurements were taken in the medulla in both Pontomedullary ($n = 6$) and Medullary ($n = 6$) preparations from P4 rat pups at three mediolateral tracts. $P_{O_2}$ was also measured at the approximate level of the PreBötC (midline + 1.0 mm, depth 400 (m) in P2 Pontomedullary ($n = 7$) and P2 Medullary ($n = 8$) preparations. The Optode was mounted in a stereotaxic frame in an electrode holder attached to a hydraulic microdrive to enable fine control in the vertical plane. Using a dissecting microscope, the tip of the Optode was placed rostrocaudally at the level of the rostral most hypoglossal nerve rootlet, corresponding to the level containing the preBötC (Smith et al., 1990; Ruangkittisakul et al., 2007). The Optode was then placed on the ventral surface of the brainstem at one of three mediolateral co-ordinates: 0.5, 1.0 and 1.5 mm lateral from midline, chosen at random to minimize the effect of sequence on the $P_{O_2}$ measurements. The Optode was placed 1.0 mm above the ventral surface of the brainstem and $P_{O_2}$ measurements began. The surface of the brainstem was confirmed by observing the tip of the Optode as it was advanced into the tissue. $P_{O_2}$ measurements were taken every 200 (m in the superfusate and every 100 (m in the brainstem until a $P_{O_2}$ of 0 Torr was recorded. $P_{O_2}$ measurements were taken at 5 s intervals and allowed to equilibrate for at least 1 min at each depth, a time sufficient for the $P_{O_2}$ measurements to stabilize. At the completion of each tract, the Optode was retracted and moved to a different mediolateral coordinate on the same side of the brainstem until measurements were obtained from all three mediolateral tracts. The pons was then removed and the measurements repeated on the other side of the medulla.

At the conclusion of the experiment, the tissue was fixed in 10% neutral buffered formalin and cryoprotected in 30% sucrose for subsequent histological processing to verify the level of transection.

### 2.5. Histology

For histological analysis, brainstems were fixed in 10% neutral buffered formalin prior to cryoprotection in 30% sucrose. Coronal cryosections (50 (m) were cut for analysis of the level of brainstem transection. The slide-mounted sections were counterstained in 0.5% neutral red, dehydrated in increasing concentration of alcohol, cleared in xylene and coversliped with mounting media (Permount, Fisher Scientific). Counterstained sections were examined using a Zeiss Axioskop under standard brightfield conditions.

### 2.6. Data and statistical analysis

All data were recorded online using commercially available data acquisition hardware and software (DI200 analog-to-digital converter, Windaq software, DataQ, Akron, OH, USA) and analyzed offline. Raw nerve recordings were full wave rectified and integrated for measurements of burst frequency, determined as bursts per minute and burst amplitude measured in arbitrary units and normalized to baseline.

In Experiment 1, the time from the start of recording until discharge from cervical rootlets ceased (time to last burst) was

recorded for preparations from P0 to P8 pups. For simplicity, we refer to the time to last burst as 'longevity' for the remainder of the manuscript. Fictive breathing frequency and amplitude were also measured in preparations from P0 to P4 pups for a 2-min period every 30 min, beginning at the start of recording (Time 0) and continuing until discharge stopped. A two-way ANOVA on ranks was used to examine the effect of age and preparation type (Factors: age and presence of pons) on longevity (not normally distributed data). A two-way repeated measures ANOVA was used to examine the effect of age and time (Factors: age and time, Repeated factor: time) on both the consistency of frequency and amplitude over time within each type of preparation of ages P0–P4. The starting frequency at different ages within each preparation type was compared using a one-way ANOVA and the difference in starting frequency between preparation types within the same age was compared using a *t*-test. The masses of brainstems at different ages within each species were compared using a one-way ANOVA.

In Experiment 2, burst frequency was measured over a period of 5 min at three different time points: (1) at the end of the 30 min stabilization period (baseline), (2) the last 5 min of TTX or low $Ca^{2+}$/high $Mg^{2+}$ and (3) 30 min after pontine transection or removal. Data from each age group was combined and compared between conditions using one-way repeated measures ANOVA.

In Experiment 3, $P_{O_2}$ measurements at each depth were taken as the average of all values recorded over the entire duration that the Optode remained at that depth. $P_{O_2}$ measurements from 800 (m above the surface of the medulla to 800 (m below the surface, or when $P_{O_2}$ reached 0 Torr, were used for statistical analysis. The effect of depth and preparation type on $P_{O_2}$ at each mediolateral level was examined using two-way repeated measures ANOVA to compare the presence of the pons and depth (Factors: pons and depth, Repeated Factor: depth). The effect of age and the presence on the pons on $P_{O_2}$ in the medulla was tested using a two-way ANOVA (Factors: age and presence of the pons).

A Student Newman–Kuel's post hoc analysis was performed following the ANOVA where appropriate. $P < 0.05$ was considered significant in all cases.

## 3. Results

### 3.1. Experiment 1: longevity and respiratory-related motor output of en bloc preparations

#### 3.1.1. Effect of age and pons on the longevity of en bloc preparations

To examine the viability of the *en bloc* preparations at different postnatal ages we recorded the longevity (time to last burst) of rat and hamster preparations with the pons intact (Pontomedullary) and the pons removed (Medullary) (Fig. 2). In rats, increasing age significantly decreased longevity of the *en bloc* preparations ($P < 0.001$). P0 and P2 Pontomedullary preparations had similar longevity (354.9 ± 37 and 333.8 ± 65 min, respectively) but longevity significantly decreased with increasing postnatal age (Fig. 2A). In rat Medullary preparation, the longevity of P2 preparations was significantly greater than all other preparations. The presence of the pons also had a significant effect on the longevity of the preparation ($P < 0.001$), with pontine removal leading to significantly prolonged longevity in P2 and P4 preparations (Fig. 2A).

In hamster *en bloc* preparations, age also had a significant effect on the longevity of the preparations ($P < 0.001$). The longevity of P0 and P2 hamster Pontomedullary preparations was similar (600.0 ± 118 min vs. 756.0 ± 112 min) and significantly longer than preparations from older pups (Fig. 2B). In hamster Medullary preparations, the longevity of P2 preparations was significantly longer than all other ages. In hamster preparations, the presence of the pons did not affect the longevity of the *en bloc* preparations, except in P4 hamster preparations where the pons reduced the longevity of the preparation by half (P4 Medullary: 530.5 ± 74.1 min vs. P4 Pontomedullary: 208.9 ± 67.1 min, Fig. 2B).

#### 3.1.2. Effect of brain mass on longevity of en bloc preparations

In Figs. 3 and 4, the data shown in Fig. 2 are plotted as a function of mass rather than age to examine the relationship between these two variables. Since mass of both pons and medulla increased with postnatal age in both hamsters and rats (Table 1), the relationship between longevity and mass (Fig. 3) was similar to the

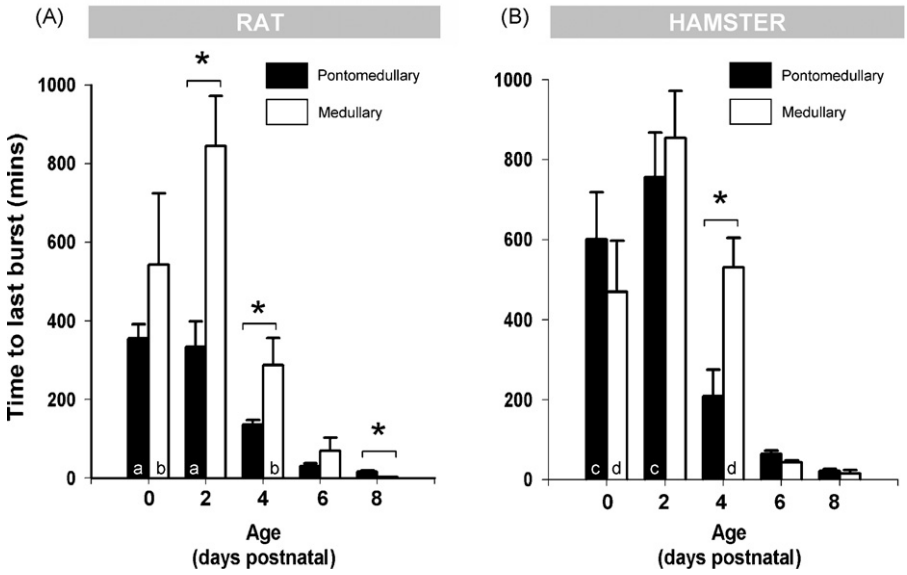


**Fig. 2.** Effect of postnatal age on longevity of rat (A) and hamster (B) Pontomedullary and Medullary preparations. (A) Time to last burst (longevity) for rat Pontomedullary and Medullary preparations decreased with postnatal age beyond P2. Pontomedullary preparations from P2 and P4 pups had shorter longevity than Medullary preparations of the same age. (B) Time to last burst (longevity) for hamster Pontomedullary and Medullary preparations decreased with postnatal age. Pontomedullary preparations from P4 hamster pups also had significantly reduced longevity. *$P < 0.05$, Pontomedullary vs. Medullary within the same age in the same species. Same letter indicates no statistical difference between the ages within the same preparation type in each species. Two-way ANOVA, followed by Student Newman–Kuel's post hoc test.

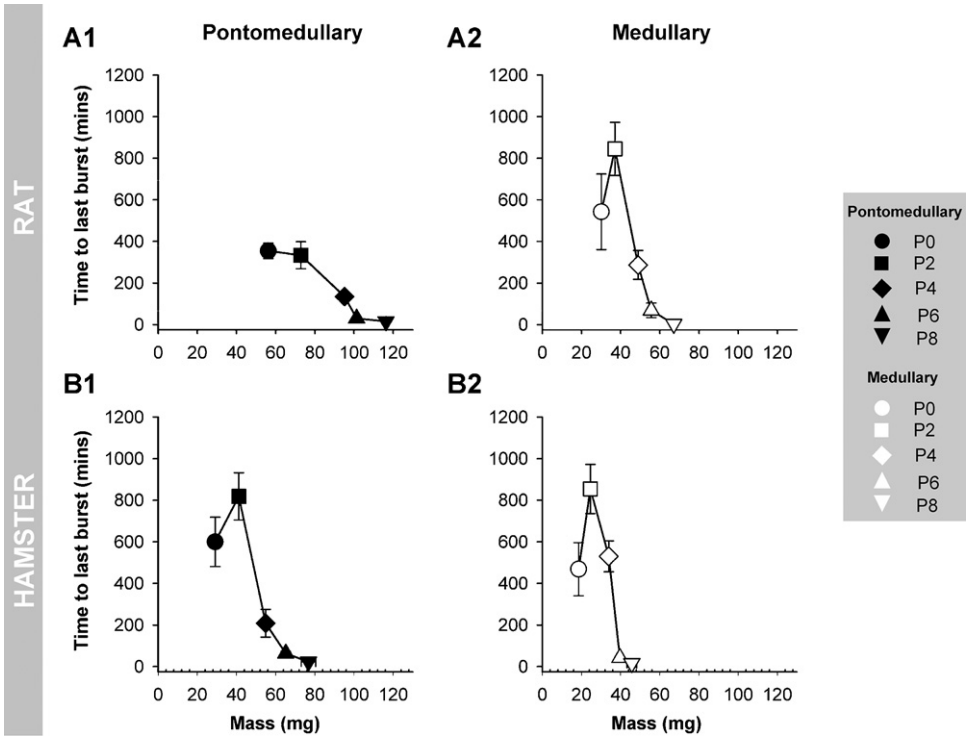


**Fig. 3.** Increasing mass correlated with a decrease in time to last burst (longevity) in rat Pontomedullary (A1), rat Medullary (A2), hamster Pontomedullary (B1) and hamster Medullary (B2) preparations. There was also a correlation between the mass, preparation type and time to last burst in both rats and hamsters.

relationship between longevity and age (Fig. 2). In rat and hamster preparations, both with and without the pons, longevity was inversely correlated to mass (Fig. 3). However, the lowest mass did not have the greatest longevity, as P2 preparations had the greatest longevity, although the mass of P2 preparations was greater than P0 preparations (Fig. 3).

Removing the pons reduced the mass of the preparation but the effect on longevity was variable. In general, the longevity of rat *en*

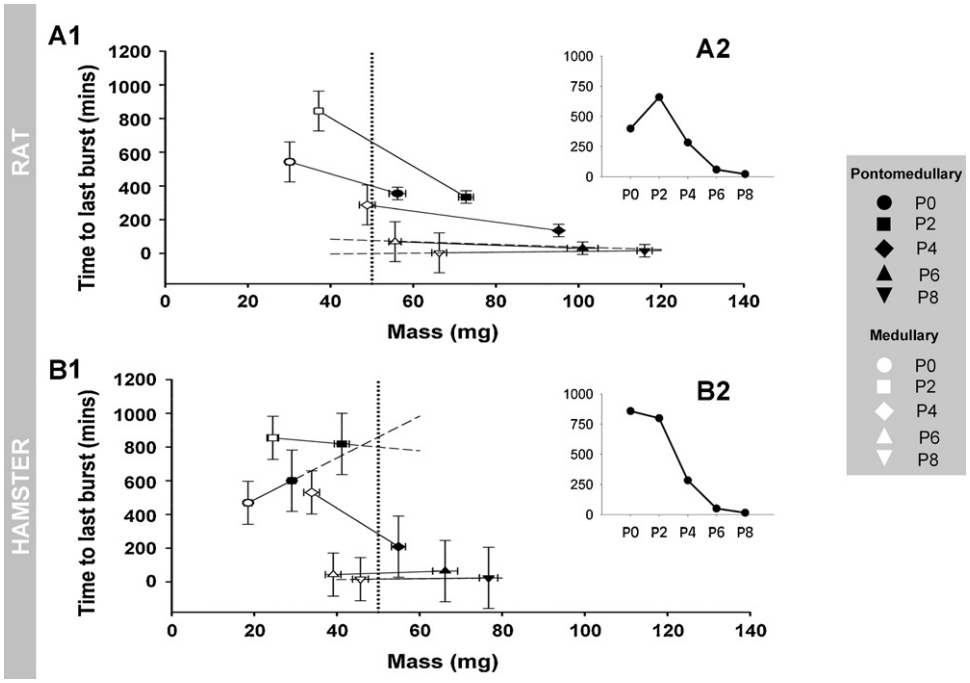


**Fig. 4.** (A1) In rats, Pontomedullary preparations (closed symbols) had greater mass and shorter time to last burst compared to age matched Medullary preparations (open symbols). (B1) In hamsters, the relationship between preparation type and longevity was more variable depending on the age of the preparation. By plotting the estimated longevity of a hypothetical 50 mg preparation taken from the regression line between Pontomedullary and Medullary preparations for each age group in rats and hamsters, it appears that rat preparations from postnatal day 2 had the greatest longevity independent of mass (A2), while hamster P0 and P2 preparations lasted longer than older preparations (B2). Vertical dashed line in (C1) and (D1) indicates 50 mg.

**Table 1**
Mass of brainstem (mg) of rat and hamster neonates at different postnatal ages.

| Postnatal age | Rat | | | | Hamster | | | |
|---|---|---|---|---|---|---|---|---|
| | Pons | Medulla | Total | *n* | Pons | Medulla | Total | *n* |
| 0 | 26.0 ± 1.2 | 30.1 ± 0.9 | 56.2 ± 2.0 | 8 | 10.6 ± 0.7 | 18.5 ± 0.9 | 29.1 ± 0.9 | 8 |
| 2 | 35.8 ± 1.7* | 37.1 ± 0.7* | 72.8 ± 1.8* | 10 | 16.7 ± 9.0* | 24.5 ± 1.4* | 41.2 ± 1.8* | 8 |
| 4 | 46.4 ± 1.6* | 48.9 ± 1.9* | 95.2 ± 2.7* | 10 | 21.0 ± 1.1* | 33.9 ± 1.9* | 54.9 ± 1.7* | 8 |
| 6 | 45.4 ± 1.5* | 55.6 ± 0.8* | 101.0 ± 1.9* | 13 | 27.1 ± 3.9* | 39.1 ± 0.9* | 66.2 ± 4.1* | 5 |
| 8 | 49.9 ± 1.1* | 66.3 ± 1.4* | 116.0 ± 1.9* | 8 | 31.0 ± 2.0* | 45.7 ± 2.4* | 76.7 ± 3.7* | 7 |

All measurements are expressed in milligrams (mg).
* $P < 0.05$, compared to previous age, within the same tissue type. One-way ANOVA, followed by Student Newman–Kuel's post hoc test.

*bloc* preparations increased with removal of the pons, although this effect waned after P4 (Figs. 2A and 4A). In hamster preparations, the effect of the pons on longevity was less pronounced. Removing the pons significantly increased longevity only in P4 preparations (Figs. 2B and 4B).

We wondered whether the effect of the pons and age on the longevity of the *en bloc* preparation was simply the result of adding mass to the preparation. To address this issue, we calculated the longevity of preparations with a hypothetical mass of 50 mg from the regression lines between mass and longevity for Pontomedullary and Medullary preparations at each postnatal age for rats and hamsters (Fig. 4A1 and B1). Note that in so doing we are adjusting more for difference in mass at the rostral end of the preparation than for mass geometrically distributed throughout the medulla. With this caveat in mind, in rats, a hypothetical 50 mg preparation at P2 had the greatest estimated longevity and would last more than 200 min longer than P0 preparations of the same size (Fig. 4A2) with the estimated longevity declining sharply with increasing age after P2. In hamsters, hypothetical 50 mg preparations from P0 and P2 pups were estimated to survive for similar durations (Fig. 4B2). Thus, mass (mainly of the pons) was not a primary impediment to longevity, but rather age and postnatal development were the predominant contributing factors in the decline in longevity of the *en bloc* preparation.

### 3.1.3. Effect of age on respiratory-related motor output of en bloc preparations

We analyzed the frequency and amplitude of respiratory-related burst discharge of P0, P2 and P4 rat and hamster *en bloc* Pontomedullary and Medullary preparations over the first 4 h of recording (Fig. 5). Four hours was chosen as this is the length of most studies performed using *en bloc* preparations both in this lab and in the published literature. In rat Pontomedullary preparations, although P2 preparations had a slightly lower starting frequency (Table 2), no statistical difference was found in the burst frequency over the 4 h duration despite the appearance of considerable variation (Fig. 5A1). It should be noted that P4 Pontomedullary preparations stopped bursting after 150 min (Fig. 5A1). In rat Medullary preparations, the starting frequency was similar between the ages (Table 2) and burst frequency also remained consistent with little change over the 4 h period (Fig. 5A2). In rat *en bloc* preparations, the presence of the pons reduced the starting frequency of P2 but had no effect on P0 and P4 preparations (Table 2). The respiratory related frequency of Pontomedullary preparations had greater variability over time (Fig. 5A1 vs. Fig. 5A2), possibly indicating reduced stability in preparations with the pons attached.

The starting frequency of hamster Pontomedullary preparations was also similar between all three age groups (Table 2), with a slightly lower frequency in P2 preparations. Burst frequency was also consistent over the 4 h duration examined (Fig. 5B1). In hamster Medullary preparations, the starting frequency was significantly lower in P2 preparations compared to P0 and P4 preparations (Table 2). The discharge frequency over time was more variable between the ages, with both P0 and P2 preparations increasing discharge frequency initially (Fig. 5B2). In the hamster *en bloc* preparation the presence of the pons reduced starting frequency at all three postnatal ages examined (Table 2).

In addition to frequency, we also examined the amplitude of the respiratory-related bursts over the first 4 h of recording. There were no significant changes in amplitude over time in any type of preparation, although there was greater variability in P4 preparations of both species, with and without the pons (Fig. 5C and D).

Therefore, despite some differences in starting frequencies, all preparations from both species, with or without the pons, from the ages P0 to P4 were consistent over a 4-h period.

**Table 2**
Starting fictive respiratory frequency (bursts/min) in rat and hamster *en bloc* preparations at different postnatal ages.

| Preparation type | | Postnatal age | | |
|---|---|---|---|---|
| | | P0 | P2 | P4 |
| Rat | Pontomedullary | 7.1 ± 0.1 (6) | 6.6 ± 0.1*,† (6) | 7.3 ± 0.1 (6) |
| | Medullary | 8.7 ± 1.8 (6) | 11.2 ± 0.9 (6) | 9.0 ± 1.5 (6) |
| Hamster | Pontomedullary | 4.9 ± 1.5 (6) | 1.7 ± 0.4 (5) | 5.0 ± 1.0 (6) |
| | Medullary | 12.9 ± 1.2† (6) | 8.1 ± 1.3*,† (6) | 11.2 ± 0.9† (6) |

All values are expressed as bursts/min.
Number in parentheses indicates number of observations in each group.
* $P < 0.05$ compared to other ages within the same preparation type in the same species. One-way ANOVA, followed by Student Newman–Kuel's post hoc test.
† $P < 0.05$ compared within the same age between preparation types in the same species, *t*-test.

### 3.2. Experiment 2: chemical and physical removal of the pons

As observed in Experiment 1, the presence of the pons inhibited the starting burst frequency in P2 rats (Table 2). Thus, we examined whether this effect was due to descending pontine inputs or to the removal of pontine tissue mass. We utilized a split chamber (Fig. 1A) to compare the effect of chemical inhibition of the pons (tissue mass still present but non-functional) vs. physical removal of the pons. Physical removal of the pons had no effect on P0 preparations but significantly increased burst frequency in both P2 and P4 preparations (Fig. 6A and B). Incubation of the pons with TTX had no effect on burst frequency in preparations of any age (Fig. 6A). Similarly, incubation of the pons in low $Ca^{2+}$/high $Mg^{2+}$ did not affect burst frequency in P0 or P2 preparations, but increased burst frequency in P4 preparations (Fig. 6B). The effect of physical transection was always greater than the effect of chemical inhibition.

Histological examination of the transection at the conclusion of the experiment showed that transection was at the level of the caudal cerebellar artery (Fig. 6C) and bisected the preparation either at the rostral pole of the facial nucleus or just rostral of the facial nucleus (Fig. 6D). This would have preserved the parafacial nucleus within the medullary portion of the preparation.

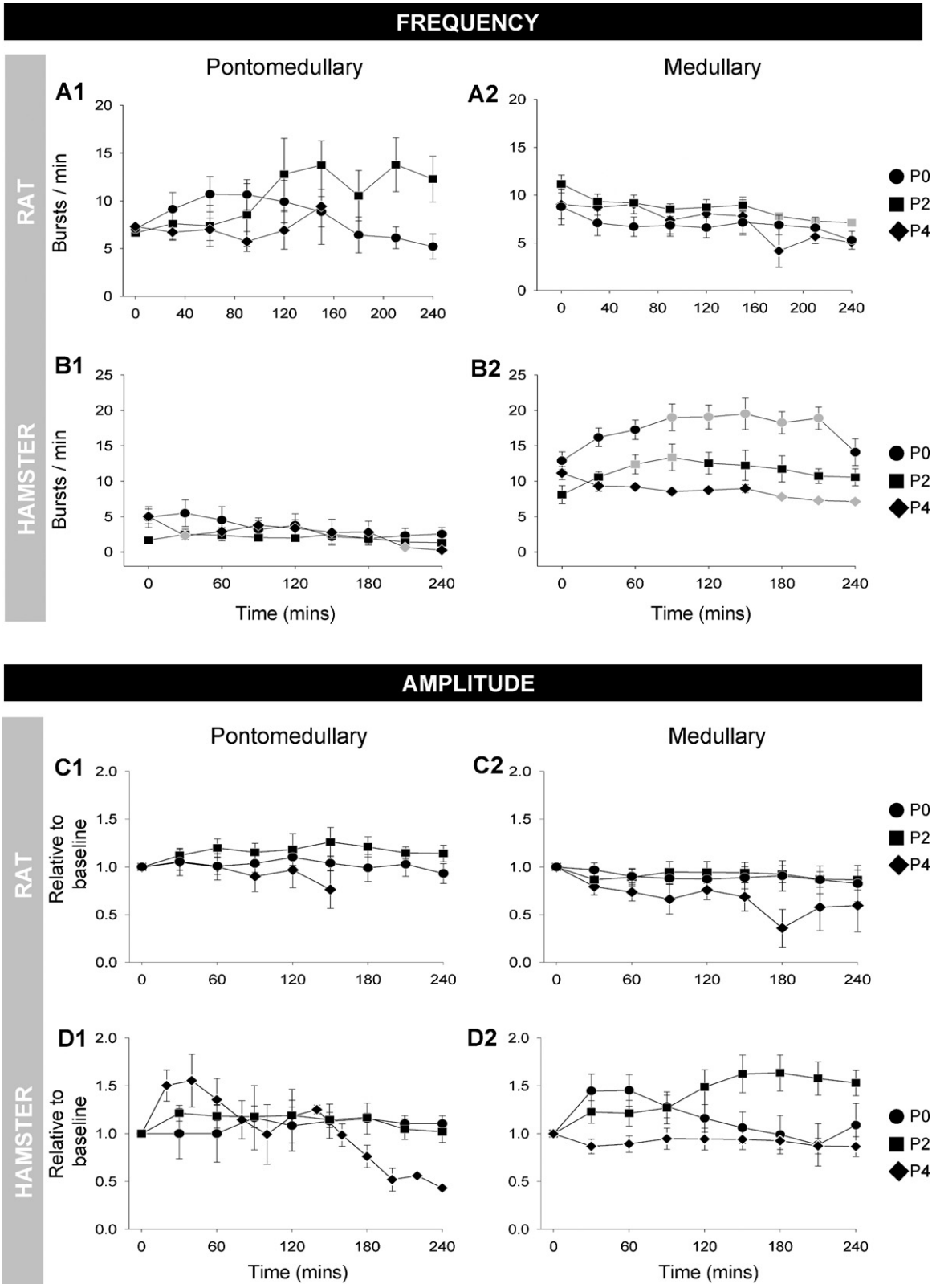


**Fig. 5.** Burst frequency of rat Pontomedullary (A1) and rat Medullary (A2) preparations was consistent over the first 4 h of recording at different postnatal ages. (B1) Hamster Pontomedullary preparations had consistent burst frequency over the 4 h period at all three ages. (B2) Hamster Medullary preparations had differing patterns of burst frequency over the 4 h period depending on the age of the preparation. Burst amplitude remained consistent in the rat Pontomedullary (C1), rat Medullary (C2), hamster Pontomedullary (D1) and hamster Medullary (D2) preparations for all three ages examined. Gray symbols denote significant difference from time 0 ($P < 0.05$, one way repeated measures ANOVA, followed by Student Newman–Kuel's post hoc test).

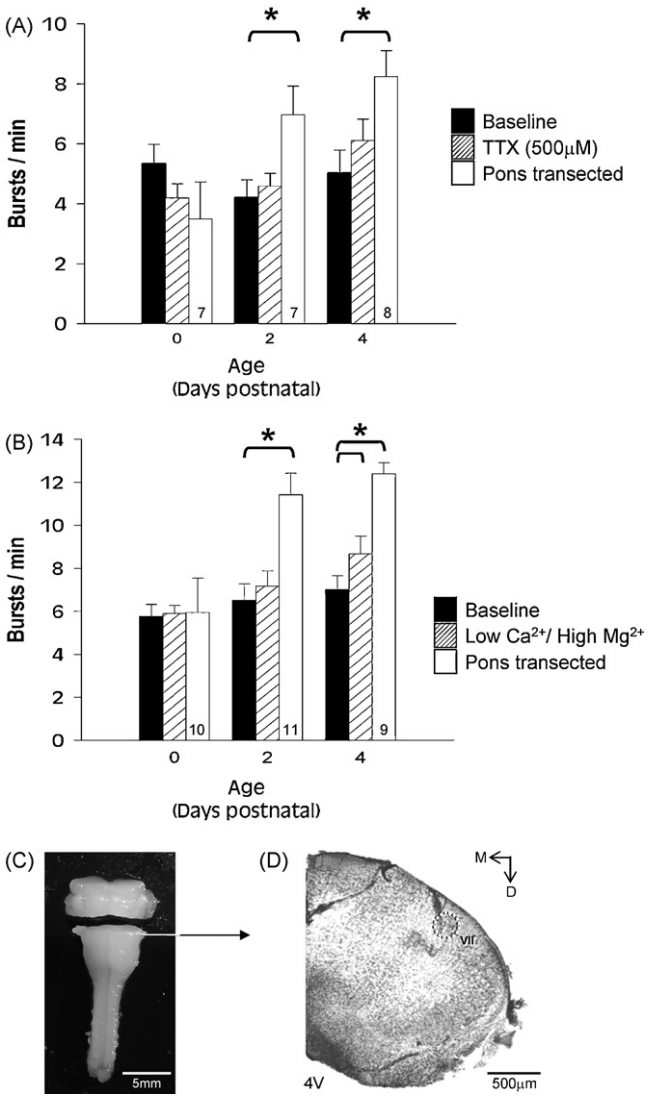


**Fig. 6.** (A) Quantitative changes in burst frequency following chemical inhibition of the pons by superfusion with TTX and pontine transection of P0, P2 and P4 rat preparations. (B) Quantitative changes in burst frequency following pontine superfusion with low $Ca^{2+}$/high $Mg^{2+}$ and pontine transection of P0, P2 and P4 rat preparations. (C) Photograph of an *en bloc* preparation showing the level of the pontine transection, and (D) a representative photomicrograph of a hemisection at the cut surface on the medullary side, showing the rostral pole of the facial nucleus (VII) and the location of the fourth ventricle (4 V). Arrows indicate direction for dorsal surface (D) and midline (M). *$P < 0.05$, one-way repeated measures ANOVA, followed by Student Newman–Kuel's post hoc test. Numbers in the bars indicate number of preparations in each age group.

### 3.3. Experiment 3: oxygenation of medulla

The results of Experiment 2 did not allow us to clearly determine whether the inhibition of burst frequency by the pons was due to descending pontine inputs or due to the presence of the pontine tissue mass. To better examine whether the effect of age, mass or presence of the pons on burst frequency was due to alteration in tissue oxygenation in the medulla, we directly measured $P_{O_2}$ in the medulla in various preparations.

#### 3.3.1. Effect of pons on $P_{O_2}$ in the medulla

Removal of pontine inputs by chemical and physical transection influenced the frequency of respiratory-like activity in P4 preparations. We measured the $P_{O_2}$ medulla of P4 preparations with and without the pons attached. The $P_{O_2}$ in the superfusate (bubbled with 95% $O_2$) at the start of each set of measurements was 554.8 ± 5.8 Torr ($n = 12$) and began to decrease 300–400 (m above the surface of the brainstem, becoming significantly different to the bulk superfusate $P_{O_2}$ 200–300 (m above the surface of the brainstem (Fig. 7A). This boundary layer effect resulted in an average $P_{O_2}$ at the surface of the brainstem of 273.9 ± 12.4 Torr. The presence of the pons had no effect on the $P_{O_2}$ measured at the surface at any of the mediolateral co-ordinates (Fig. 7A and B). The $P_{O_2}$ in the medulla decreased significantly with greater depth in the brainstem at all mediolateral co-ordinates in both Pontomedullary and Medullary preparations ($P < 0.001$). The presence of the pons had no effect on the $P_{O_2}$ levels at any depth within the medulla, regardless of the mediolateral co-ordinates (Fig. 7B). $P_{O_2}$ reached zero between 600 and 700 (m below the ventral surface of the brainstem in both Pontomedullary and Medullary preparations.

To better examine the relationship between locations within the medulla and $P_{O_2}$, the average of measurements taken at the three mediolateral tracts within Pontomedullary and Medullary preparations (shown in Fig. 7) were used to calculate a regression plot. This regression plot was then overlaid on a drawing of a representative neonatal rat brainstem section illustrating the location of respiratory nuclei of interest, as shown in Fig. 8. Regression analysis demonstrated a layer of hyperoxygenated tissue throughout the mediolateral aspect of the medulla, with $P_{O_2}$ decreasing to anoxic levels with increasing distance from the surface, reaching 0 Torr at the level of Nucleus Ambiguus. The pattern of oxygenation was the same in both Pontomedullary and Medullary preparations. $P_{O_2}$ at the PreBötC in both Pontomedullary and Medullary preparations were similar and ranged between 50 and 140 Torr (Fig. 8).

**Table 3**
Effect of age on $P_{O_2}$ (Torr) within the medulla at the level of the PreBötzinger Complex of rat *en bloc* preparations from pups of different postnatal ages.

| Age | Preparation type | |
|---|---|---|
| | Pontomedullary | Medullary |
| P2 | 54.2 ± 11.1 (7) | 37.8 ± 14.0 (8) |
| P4 | 42.1 ± 17.3 (6) | 40.1 ± 3.8 (6) |

All measurements are shown in Torr. Number in parentheses indicates number of preparations in each group.

#### 3.3.2. Effect of age on $P_{O_2}$ at the PreBötC

Measurement of $P_{O_2}$ at a single location (1.0 mm from midline, 400 (m depth, the approximate level of the PreBötC) was similar between P2 and P4 Pontomedullary and Medullary preparations (Table 3). $P_{O_2}$ at the PreBötC in both Pontomedullary and Medullary preparations, regardless of age, were similar and ranged between 38 and 54 Torr (Table 3).

## 4. Discussion

While it is widely recognized that the longevity of the *en bloc* preparation decreases with postnatal age, this study is the first to quantify the relationship between longevity, age and mass of the preparations, both with the pons-intact and pons-removed in rats and hamsters. We found that postnatal development rather than mass appeared to be the major factor determining the longevity of the *en bloc* preparations. This was re-enforced by the similar relationships between longevity and age in both rats and hamsters despite significant differences in brain mass between the species. We also found that pontine inhibition of medullary respiratory rhythmogenesis increased with postnatal age and was not due to changes in oxygenation at the rhythm generating sites in the medulla. In *en bloc* rat preparations, regardless of mass, or pres-

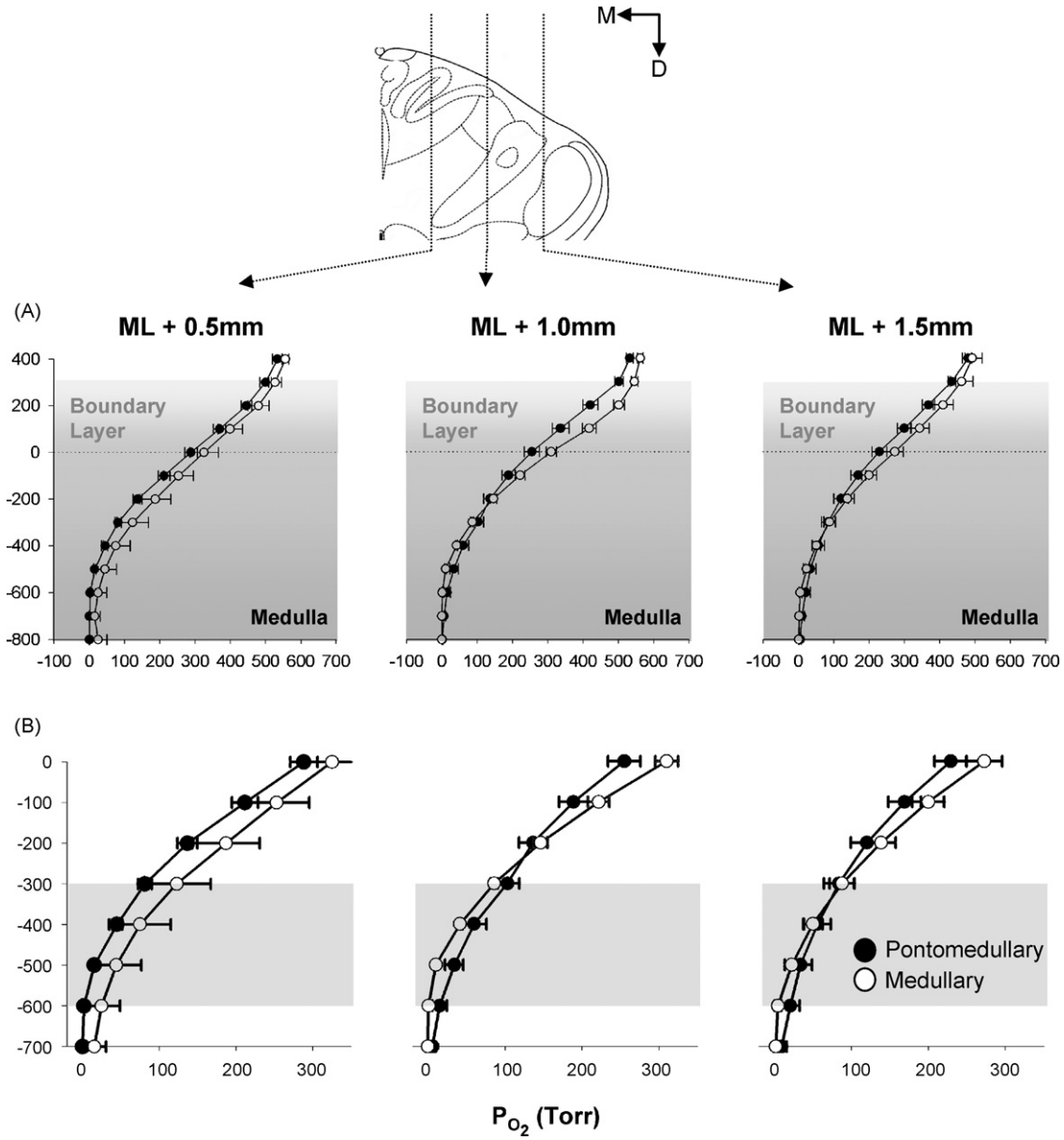


**Fig. 7.** Measurement of $P_{O_2}$ (Torr) in the medulla of rat Pontomedullary and Medullary preparations shows a boundary layer of 300 (m over the ventral surface of the brainstem where $P_{O_2}$ decreased from superfusate levels at all three mediolateral tracts (A). Increasing depth in the medulla resulted in decreasing $P_{O_2}$ until 0 Torr was reached between 600 and 700 (m below the ventral surface. No difference in $P_{O_2}$ levels was found between the Pontomedullary and Medullary preparations at any depth. No difference was found between the $P_{O_2}$ at matching depths in different mediolateral co-ordinates in either preparation types. Panel B shows expanded profiles for $P_{O_2}$ from the surface of the medulla to a depth of 700 (m. The shaded area in panel B indicates the reported depth of respiratory neurons in the medulla of the *en bloc* preparation.

ence of the pons, or age (from P2 to P4), the $P_{O_2}$ at the approximate level of the PreBötzinger complex at 27 °C was similar and within the physiological range, indicating sufficient oxygenation of the medullary respiratory network.

### 4.1. The effect of age and mass on the en bloc preparation

We were interested in evaluating the effect of age-related changes in mass vs. developmental effects on the *en bloc* preparation. Our study provides a systematic examination of the well-established and widely observed phenomenon that longevity (time to last burst) of the *en bloc* preparation decreases with developmental age. This reduced longevity has generally been attributed to increasing tissue mass with age and a corresponding increase in diffusion distance for oxygen and thus, reduced oxygen availability to the medullary respiratory network. A number of noteworthy observations from our study suggest that the reduced longevity is likely due to developmental and maturational changes, rather than just increasing size. First, during the first two postnatal days, longevity increased despite increasing total mass. Secondly, while there was an inverse correlation between tissue mass and longevity after P2, the $P_{O_2}$ at the level of the ventral respiratory column (VRG) in the medulla, including the preBötzinger Complex (PreBötC), did not change with increasing age. Lastly, both hamster and rat preparations exhibited a similar relationship between age and longevity despite the larger mass of rat brainstems (Table 1). Equally, preparations from rats and hamsters at different postnatal ages with similar mass did not have the same longevity. For example, the masses of P8 hamster Medullary preparations (45.7 mg) and P4 rat Medullary preparations (46.4 mg) are similar, however, the rat Medullary

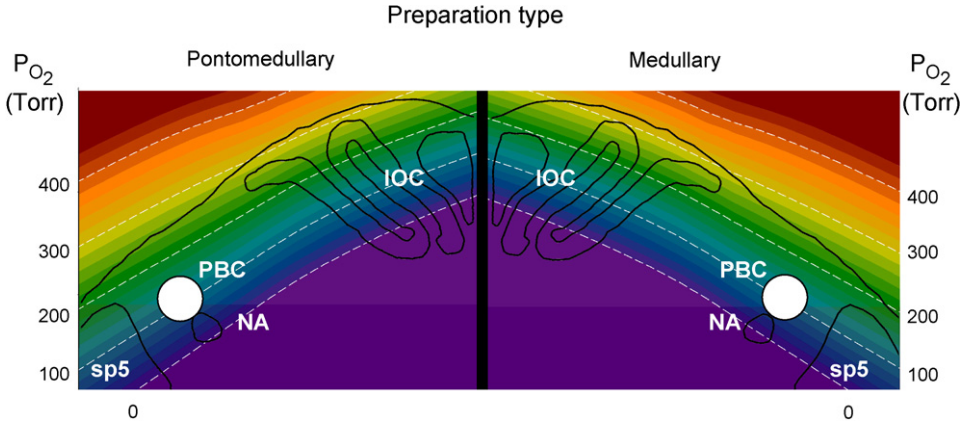


**Fig. 8.** Psuedocolored plot of $P_{O_2}$ level within the medulla in Pontomedullary and Medullary preparations overlaid on a coronal schematic representation of a neonatal rat medulla. The location of the PreBötzinger Complex (PBC) is indicated in white. There is no difference in the oxygenation profile between the two preparations with similar levels of $P_{O_2}$ within the PBC (50–140 Torr). *Abbreviations*: IOC, inferior olivary complex; NA, nucleus ambiguous; PBC, pre-Bötzinger complex; ROb, raphé obscurus.

preparations produced spontaneous fictive respiratory activity for almost 5 h, while P8 hamster Medullary preparations lasted only 16 min. Rats and hamsters are developmentally equivalent by P8, even though hamsters are born more altricial to rats (Finlay and Darlington, 1995; Clancy et al., 2001, 2007). Thus, despite the similar mass between the P8 hamster Medullary preparations and the P4 rat Medullary preparation, the younger preparation has greater viability. Taken together, these observations suggest that postnatal developmental is the primary determinant of longevity of the *en bloc* preparation and the decreased longevity of *en bloc* preparations with age is not due to limitation of oxygen diffusion due to larger size.

### 4.2. The effect of the pons on the en bloc preparation

In the present study, we report that the pons reduced longevity and inhibited burst frequency on the *en bloc* preparation. This is in agreement with numerous reports of increasing respiratory frequency following pontine removal in the *en bloc* preparation (Hilaire et al., 1989; Smith et al., 1990; Errchidi et al., 1991; Tanabe et al., 2005). In our study, this change in frequency following transection was not due to removal of the parafacial respiratory group, a proposed second respiratory rhythm generator (Onimaru and Homma, 2003; Janczewski and Feldman, 2006). Our level of transection consistently left the facial nucleus and therefore, the adjacent pFRG intact within the medullary preparation (Ruangkittisakul et al., 2007). In addition, chemical inhibition of the pons in P4 preparations produced similar results to physical transection (Fig. 6B), suggesting that the increase in burst frequency was not due to removal of pontine tissue mass, but rather the removal of pontine inputs. Furthermore, the removal of the pons did not alter the level of oxygen in the medulla (see next section). Together, these observations support the conclusion that the pons provides direct descending inhibitory inputs to the medulla (Hilaire et al., 1989; Errchidi et al., 1991).

While pontine inhibition produced similar results to physical transection, chemical inhibition was always less effective than physical transaction. The reason for this is not clear. As the level of the grease gap isolating the chemical inhibition to the pons was identical to the level of transection, the tissue that was bathed by the pontine superfusate was identical to that removed by transection. It is possible that the chemicals did not penetrate to the deeper structures in the pons. Or the concentrations of TTX or $Ca^{2+}/Mg^{2+}$ may have been insufficient to completely inhibit neuronal activity throughout the pons. Preliminary experiments showed that the concentrations of TTX and low $Ca^{2+}$/high $Mg^{2+}$ superfusate were capable of silencing the medulla when applied to the medulla side of the split bath, therefore, the same concentrations of chemical was presumed to be sufficient to silence the neuronal activity of the pons. However, as the medullary structures involved in respiratory rhythmogenesis are generally more superficial than the pontine sites known to modulate respiratory activity, these medullary regions may have had greater accessibility to the drugs. Another possibility is that the grease covered some of the pontine ventral surface limiting the access of the superfusate (and drug) to this small portion of the pons, especially regions located close to the ventral surface, such as the A5 region which is known to participate in modulation of respiratory rhythm (Errchidi et al., 1991; Hilaire et al., 2004). The potential pontine mechanisms involved in providing the inhibition to medullary respiratory networks were not examined in the current study but will be the focus of a future study.

### 4.3. Tissue oxygenation in the medulla of the en bloc preparation

Another objective in the present study was to examine the effect of age, mass and the presence of the pons on oxygenation of the medulla in the *en bloc* preparation. It was possible that the effects of pontine removal on longevity and burst frequency were due to increased oxygenation to the medullary respiratory rhythm generators as pontine inhibition was not as effective as pontine removal. Our data, however, show that $P_{O_2}$ measurements in the medulla were unaffected by age or the presence of the pons. In particular, the $P_{O_2}$ in the preBötzinger Complex (PreBötC) did not change with increasing age. The distance between the ventral surface and the ventral edge of the Nucleus Ambiguus was roughly constant between P0 and P4 (540–580 (m), placing the PreBötC within a well-oxygenated region of the medulla.

A number of postnatal developmental events may contribute to the reduced longevity of the *en bloc* preparations. Current evidence indicates the appearance of myelin in the brainstem from birth (in spinal trigeminal tract) to appearance after the first postnatal week (in pyramidal tract) (Hamano et al., 1998). Myelin continues to develop over early postnatal period reaching mature levels at different rates in different regions (Hamano et al., 1998; Butt and Berry, 2000). This postnatal increase in myelin may alter the diffusion of oxygen through the tissue, increasing the distance between

the ventral surface and the PreBötC or alter metabolism of the brainstem. Since the oxygenation of the medullary respiratory network did not change over this period (P0–P4), our data suggest that the reduced longevity of the brainstem–spinal cord preparation is not due to events that alter $O_2$ tissue diffusion by developmental events such as postnatal myelination.

$P_{O_2}$ measurements in the medulla in our study are in agreement with earlier study (Brockhaus et al., 1993) and the $P_{O_2}$ at the PreBötC is comparable to data obtained *in vivo*. Several studies have demonstrated oxygen tension in different brain regions ranging from 2 to 38 Torr under normoxic conditions in *in vivo*, anaesthetized rats (Sick et al., 1982; Nwaigwe et al., 2000; Hou et al., 2003; Hare et al., 2006). Compared to these *in vivo* values, the superficial regions (up to 400 (m) of the *en bloc* preparations are hyperoxic, while the $P_{O_2}$ in regions between 400 and 600 (m from the surface, including the respiratory neurons of the VRG, are similar to those observed *in vivo*.

Although the VRG is well oxygenated, regions located deeper in the medulla, such as the midline raphé, will be situated within an anoxic region. The role of the midline raphé in respiratory rhythm modulation is well established (Li et al., 2006) and excitatory drive from this region is essential for respiratory rhythmogenesis in the brainstem slice preparation (Pace et al., 2007). The *en bloc* preparation continuously produces rhythmic motor output for many hours with spontaneously active respiratory neurons within the VRG (Suzue, 1984; Smith et al., 1990) indicating that the respiratory network is functional in these preparations. This suggests that deep regions such as midline raphé is not required for generation of respiratory rhythm in *en bloc* preparations. However, it is not clear whether the roles of these sites increase during postnatal development.

One criticism of oxygen probes is that the physical introduction of the sensor into the brainstem may provide a conduit around the electrode for the highly oxygenated superfusate to reach the deeper depths of the tissue, leading to an overestimation of the $P_{O_2}$ level. To minimize this possibility, we used optical electrodes with a small diameter (30 (m) to minimize the size of the opening in the tissue and reduce the likelihood of damage and any inadvertent introduction of the highly oxygenated aCSF to the depths of the tissue. This effect of introducing the electrode would be similar regardless of age and the presence of the pons and thus, does not alter our conclusion that $P_{O_2}$ within the medulla is unaffected by presence of the pontine tissue mass or age. Furthermore, $P_{O_2}$ measurements from our study are in agreement with those previously reported by Brockhaus et al. (1993) supporting the $P_{O_2}$ levels measured in our study.

### 4.4. Conclusion

This study demonstrates that postnatal development has a profound effect on the longevity of the *en bloc* preparation. In addition, our data support earlier reports of direct pontine inhibition of respiratory rhythm and adds that this pontine inhibition increases with postnatal development. Finally, we show that, under the conditions used in the current study, the VRG is well oxygenated and the $P_{O_2}$ at the PreBötzinger Complex at 27 °C is within the physiological range of $P_{O_2}$ *in vivo* and is unaffected by age or the presence of the pons in *en bloc* preparations from rats. Taken together, these data suggest that the effects of postnatal age or pontine removal on respiratory rhythmogenesis in the *en bloc* preparation are not due to reduced availability of oxygen at the medullary respiratory network. The exact manner by which postnatal development reduces the longevity of the *en bloc* preparation and the extent to which this also applies to even older preparations, remains to be determined.

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