Published in final edited form as:

J Neurosci Methods. 2007 March 15; 160(2): 256–263.

Efficient measurement of endogenous neurotransmitters in small localized regions of central nervous systems *in vitro* with HPLC

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Abstract

High performance liquid chromatography (HPLC) is widely used to determine neurotransmitter concentrations in the central nervous system (CNS). Finding the optimal methods to sample from CNS tissue poses a challenge for neuroscientists. Here we describe a method that allows assay of neurotransmitters (or other chemicals) in small regions (down to 180 μm in diameter) in *in vitro* preparations concurrently with electrophysiological recordings. The efficiency for measuring small amounts of chemicals is enhanced by a sample collecting pipette with filter paper at the tip that makes close contact with the target region in CNS tissue. With a wire plunger in the calibrated pipette controlled by a microsyringe pump, there is virtually no dead volume. Samples in a volume of 10 μL (taken, e.g., at 2 $\mu\text{L}/\text{min}$ over 5 minutes) can be injected into a HPLC machine with microbore columns. We demonstrate the effectiveness of this method by measuring acetylcholine (ACh) in the ventral horn and its surrounding areas of the spinal cord in *en bloc* brainstem-spinal cord preparations. In control conditions, endogenous ACh levels in these regions were detectable. Application of neostigmine (an inhibitor of acetylcholinesterases (AChEs)) increased ACh concentrations, and at the same time, induced tonic/seizure-like activity in efferent motor output recorded from cervical ventral nerve roots. Higher ACh concentrations in the ventral horn were differentiated from nearby regions: the lateral and midline aspects of the ventral spinal cord. In addition, ACh in the preBötzinger Complex (preBötC) and the hypoglossal nucleus in medullary slice preparations can also be measured. Our results indicate that the method proposed in this study can be used to measure neurotransmitters in small and localized CNS regions. Correlation between changes in neurotransmitters in target regions and the neuronal activities can be revealed *in vitro*. Our data also suggest that there is endogenous ACh release in spinal ventral motor columns at 4th cervical (C4) level that regulates the respiratory-related motor activity.

Keywords

Neurotransmitter measurement; HPLC; Acetylcholine; ventral motor columns of the spinal cord; preBötzinger Complex; brain slice; *in vitro*; respiratory modulation

1. Introduction

Quantitative measurement of neurotransmitters, hormones or their metabolites in specific regions in CNS provides important information linking regionally specific chemical events to

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CNS functions. In this regard, HPLC is extensively used to determine the concentrations of various neurotransmitters, including ACh, amino acids and dopamine (Bianchi et al., 2003; Gobert et al., 2003; Hernandez et al., 2003; Kapoor et al., 1990; Potter et al., 1983) in CNS. Determining the optimal sampling methods from CNS tissue poses a significant challenge. Sampling by microdialysis is one method used *in vivo* (Delgado et al., 1984; Fillenz, 2005; Kapoor et al., 1990). However, the relatively large size of the microdialysis probe (although the diameter of these probes can be made as small as 0.24 mm, the length of the membrane for chemical exchanges is typically ≥ 1 mm (CMA microdialysis Inc. MA, USA)) puts a significant limit on the spatial resolution of sampling. In addition, significant dead space volume in the microdialysis system limits the efficiency and accuracy of measurements. Alternatively, push-pull perfusion can be used and is reported to improve the recovery rates for dopamine and its metabolites from rat brain *in vivo* (Myers et al., 1998). Under *in vitro* conditions, samples from brain slices can be collected from the outflow of a perfusion system (Bianchi et al., 1999; Greer et al., 1992) concurrently with electrophysiological recordings or other physiological experiments. However, this method cannot provide information about the regional distribution of molecules of interest. The push-pull method can be used in *in vitro* conditions to allow sampling from and drug application to small regions of a brain slice in an interface chamber (West et al., 1992). However, the peristaltic pump connected to the push-pull cannula for application of drugs and sampling of fluid introduces a significant dead volume. An alternative approach (Roisin et al., 1991) uses a cannula positioned above the target region of a slice with which the spatial localization and the efficiency of sampling are limited by the distance between the tip of the cannula and the slice surface (~ 100 μm), and also by the dead volume.

Here we describe a method that allows assay of neurotransmitters or other chemicals in regions down to 180 μm in diameter *in vitro* that can be done concurrently with electrophysiological recordings. The efficiency for measuring small amounts of neurotransmitters is enhanced by close contact of the collection pipette tip with the region of interest and by a sample collecting system with virtually no dead volume. To demonstrate the application of this method, we used an *en bloc* brainstem-spinal cord preparation from neonatal rats which contains the preBötC (the proposed primary site for respiratory rhythm generation) (Feldman and Del Negro, 2006; Smith et al., 1991) in the rostroventral medulla and the phrenic nucleus (phrenic motoneurons innervate the diaphragm) in the ventral horn of upper cervical spinal cord. We also use a medullary slice preparation which contains the preBötC and hypoglossal nucleus. These preparations generate respiratory rhythm which can be recorded from the ventral roots of spinal nerves (Smith and Feldman, 1987; Suzue, 1984) and from the hypoglossal nerve (XIIIn) (Smith et al., 1991) *in vitro*. We measured the endogenous ACh levels under control conditions or following inhibition of AChEs by pharmacological agents and correlated the changes in ACh levels to simultaneously recorded neuronal activities. We also measured the ACh levels in a few neighboring regions to illustrate if the proposed method can differentiate the ACh levels in these regions in the spinal cord *in vitro*.

2. Materials and Methods

2.1. *En bloc* brainstem-spinal cord and slice preparations

All animal experiment protocols were in accordance with The National Institute of Health (USA) Guide for Care and Use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee. Neonatal Sprague-Dawley rats (P0–P3) were anesthetized with isoflurane and then promptly decerebrated. The cerebellum was removed and the brainstem-spinal cord was isolated. For the *en bloc* brainstem-spinal cord preparation, transverse cuts were made rostrally at the pontomedullary junction and caudally at cervical spinal cord between the 6th and 7th cervical (C6 and C7) nerve roots. For medullary slice preparations, the brainstem-spinal cord was mounted in the specimen vise of a Vibratome™

(VT 100, Technical Products International, Inc., MO, USA) oriented vertically with rostral end upward. The brainstem was sectioned serially in the coronal plane under a dissection microscope until landmarks at the rostral boundary of the preBötC were visible. One transverse slice (500–650 μm thick) was cut (Fig. 3). The preparation was transferred to a recording chamber and stabilized. The dissection and slicing were performed in an artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2 - 5% CO_2 at room temperature. The ACSF contained (in mM) 128 NaCl, 3.0 KCl, 1.5 CaCl_2 , 1.0 MgSO_4 , 23.5 NaHCO_3 , 0.5 NaH_2PO_4 and 30 glucose. During electrophysiological recording, the preparation was continuously superfused ($2 - 3 \text{ ml min}^{-1}$) with ACSF equilibrated with 95% O_2 - 5% CO_2 and maintained at $27 \pm 0.5^\circ\text{C}$ with an inline heater (Warner Instruments Inc. CT, USA). For slice preparations, ACSF with elevated KCl (9 mM) was used as it maintains a more regular frequency and long-lasting rhythm. Both the slice and the *en bloc* brainstem-spinal cord preparations generate respiratory rhythm *in vitro* which are similar in frequency and in temporal pattern (Smith et al., 1991; Suzue, 1984).

2.2. Sample Collection

The sample collection system consisted of a sample collection pipette with a plunger, a pipette holder, a micropump (UMP2, World Precision Instrument, FL, USA) controlled by a controller (Micro 4TM, World Precision Instrument, FL, USA) and a micromanipulator that held the micropump at an angle toward the recording chamber (Fig. 2). To make the sample collection pipette, a calibrated glass pipette (Wiretrol I, 25 μL , provided with wire plungers or Wiretrol II, 25–50 μL , wire plungers with Teflon tip are provided; Drummond Scientific, PA, USA) was pulled with a micropipette puller (Model P-97, Sutter Instrument Company, CA, USA) and cut with a diamond knife to a desired tip size (down to 180 μm ID, Fig. 1 A). There is no upper limit. We have tried up to 600 μm). Filter paper (Cellulose filter, Qualitative grade 4, Whatman plc., UK) was cut into triangles with one acute angle ($10-15^\circ$). The acute angle of the filter paper was inserted into the pipette tip with the aid of a fine forceps under a dissection microscope (Fig. 1 B). The filter paper outside the pipette tip was then cut (Fig. 1 C). The filter paper left at the tip thus formed a mechanical sieve allowing suction of fluid without damaging the tissue. The piece of filter paper set up in this way is mechanically stable at the pipette tip when it is wet. We tested dozens of these collection pipettes, and in no case did the filter paper fall off the tip when ejecting sample or get sucked into the pipette when pulling the plunger to collect samples. A collection pipette can be used repeatedly if one collects multiple samples in the same experimental conditions.

The sample collection pipette was placed in a pipette holder and its tip pressed on the surface of the preparation over the target area, visualized with the aid of a microscope. Fluid samples were collected by pulling the plunger at a rate equivalent to 2 $\mu\text{L/min}$ with a micropump mounted on a micromanipulator. The plunger also prevented ACSF from getting into the pipette by capillary action when the pipette entered the perfusate. The collected sample (10 μL) was ejected (by pressing the plunger) into a 0.5 mL general-purpose microcentrifuge tube and kept at -20°C before HPLC analysis. It took 5 min to collect each 10- μL fluid sample. It took 2 to 3 min to eject the fluid and to fast forward the pump to its initial position for next collection and/or to change pipette. Thus we took 8 ± 1 min as the interval between samples (temporal resolution). Since the concentrations of chemicals in the 10 μL sample are the means of the concentrations during the 5-min collection time, we took the midpoint, i.e., 2.5 min from start of collection, as the time point of collection (Fig. 3 Ad and B).

2.3. HPLC and Electrochemical Detection (HPLC-EC) of ACh

We assayed ACh in this study. Pre-drug control samples were kept in tubes containing 1 μL of 100 μM neostigmine and 0.3% acetic acid to prevent ACh from hydrolyzing. Samples collected during application of neostigmine were kept in tubes containing 1 μL 0.3% acetic acid only. Samples (in volume of 11 μL) were thawed and filtered with 0.22 μm centrifugal filter unit

(Ultrafree-MC, Millipore Co. MA, USA). The filter membrane were pretreated with standard diluent (0.3% acetic acid, pH adjusted to 5.0 with NaOH) to minimize sample absorption. Some of the 11 μ L fluid sample may be lost during preparation including filtering, therefore, 10 ± 1 μ L fluid was injected into a HPLC machine (BAS 200, Bioanalytical Systems Inc. West Lafayette, IN) through a Rheodyne injection valve with a 20 μ L-loop. The samples first passed through a microbore ACh/Choline (Ch) analytical column (cation exchange column) and then through a microbore ACh/Ch immobilized enzyme reactor (Bioanalytical Systems Inc. West Lafayette, IN) (Damsma et al., 1985). The mobile phase contained 50 mM Na_2HPO_4 , 0.5 mM EDTA- Na_2 and 0.005 % ProclinTM (a bacteriocide, Bioanalytical Systems Inc. West Lafayette, IN), pH adjusted to 8.50 ± 0.05 with H_3PO_4 , and ran at a flow rate of 0.10 ml/min. ACh and Ch can be readily separated under these conditions and were detected by a platinum electrode at a potential of +500 mV or a peroxidase redox polymer wired enzyme electrode at a potential of +100 mV versus an Ag/AgCl reference electrode. ACh (and Ch if needed) was quantified by comparing the amplitude of chromatographic peaks to a standard curve (linear fitted line) generated by injecting known concentrations of ACh and Ch (10–10,000 fmol) into the HPLC machine.

2.4. Electrophysiological Recordings

Respiratory-related rhythmic motor activity was recorded from the cut ends of the XIIIn (slice preparation) or C4 ventral roots (*en bloc* brainstem-spinal cord) with a suction electrode (Fig. 3 and Fig. 5A). Since we collected fluid samples from both left and right ventral horn at C4 level in some experiments, we sometimes recorded from C2 or C3 nerve roots instead of C4; all of these cervical ventral roots have respiratory-related rhythmic activity. The signal was amplified ($\times 20,000$) and bandpass filtered (1 – 1000 Hz) with an amplifier (P5 series, GRASS instruments Co., MA, USA).

Neurons within 100 μ m of the slice surface were visualized with an infrared-differential interference contrast microscope (Axioskop2, Carl Zeiss MicroImaging, Inc., Göttingen, Germany). The respiratory neurons we recorded fired in phase with the inspiratory bursts of XIIIn rhythmic motor output and were located ventral to the nucleus ambiguus in the preBötC. Patch electrodes were pulled from thick wall (0.32 mm) borosilicate glass with tip size 1 – 1.5 μ m (resistance: 4–6.5 M Ω). The electrode filling solution contained (in mM) 140 K-gluconate, 5.0 NaCl, 0.1 CaCl_2 , 1.1 EGTA, 10 HEPES and 2.0 ATP (Mg^{2+} salt), pH adjusted to 7.25 with KOH. Intracellular signals were amplified and low pass-filtered at 2 KHz with a patch-clamp amplifier (MultiClamp 700A, Axon Instruments, Inc., CA, USA).

2.5. Data analysis

Signals from nerve recordings and whole-cell patch-clamp for the slice preparation were digitized at 10 KHz sampling frequency with DIGIDATA 1440A and software CLAMPEX 10 (AXON Instruments/Molecular devices Co., CA, USA) on a Pentium-based computer. The two channels of signals were saved as data files for further analyses off-line.

To determine the amplitude of the respiratory-related motor activities recorded from cervical ventral roots, the signal was digitally integrated by full-wave rectification and low-pass-filtering with a time constant of 40 ms using a data analysis software package (DataView V4.1, W. J. Heitler, University of St. Andrews, UK). The amplitude of the inspiratory bursts was measured from the averaged envelope of 10 consecutive respiratory cycles for each preparation. The start time and duration of seizure-like activity were measured at 20% of peak amplitude of the integrated trace. Data were expressed as mean $\pm$ SD in the text and mean $\pm$ SE in the figures. Repeated measures ANOVA (for more than two groups of data) was used to test the statistical significance between grouped data. The procedure MIXED in the data analysis

software package SAS (V 9.1, SAS institute Inc. Cary, NC, USA) was used for these analyses. In all analyses, $p \leq 0.05$ was the criterion for statistical significance.

3. Results

To demonstrate the usefulness and effectiveness of the proposed method, we examined endogenous ACh release in the phrenic nucleus that may modulate respiratory motor output. We recorded respiratory rhythmic activity from one of the C2 – C4 ventral roots and collected fluid samples from the surface of the ventral horn of the C4 spinal cord from the *en bloc* brainstem-spinal cord preparation (Fig. 3 A). Fig. 3 Aa shows a standard HPLC trace of 100 fmol/10 μ L ACh and Ch (10nM each in 10 μ L of standard diluent). A sample from the ventral horn shows a big Ch peak and a small ACh peak in control conditions (Fig. 3 Ab). Bath application of neostigmine (10 – 20 μ M) induced an increase in the ACh peak (Fig. 3 Ac). Fig. 3 Ad shows an example of changes in ACh concentration in a series of samples collected in parallel with C4 nerve recording from the same preparation. Neostigmine induced intense seizure-like activities in the ventral roots (Fig 3 A) with a delay of 2.73 ± 0.73 min ($n = 7$) from the time of neostigmine application. The amplitude of integrated seizure-like activities was 23.0 ± 11.7 μ V (compared with 18.3 ± 7.1 μ V, the amplitude of rhythmic inspiratory bursts in pre-drug control conditions). The first seizure-like activity lasted 2.96 ± 0.91 min and these activities often occurred repeatedly. Summary data ($n = 3-6$, Fig. 3 B) shows that ACh levels were stable at 87 to 111 fmol/10 μ L in control conditions. Application of neostigmine increased ACh concentrations to 189.3 ± 83.8 fmol/10 μ L, which corresponds to the starting time of seizure-like activity in ventral roots. These data were analyzed with a repeated measures ANOVA model. Asterisks “*” in Fig. 3 B indicate significant differences ($p \leq 0.05$) in ACh levels between the time points in the presence of neostigmine and the time prior to neostigmine application (4th column).

Motoneurons in the ventral horn motor columns of the spinal cord receive cholinergic synaptic inputs (Barber et al., 1984). These motoneurons are contacted by terminals immunoreactive for the vesicular ACh transporter (Schafer et al., 1998). The ventral horn motor columns are also rich in AChEs suggesting cholinergic transmission in these areas (Navaratnam and Lewis, 1970). Using the modified copper thiocholine method (Ma et al., 2001), we found that the middle and lateral motor columns of ventral spinal cord are heavily stained by AChEs in neonatal rats (Fig. 4 A). Note the differences in gray matter distribution between neonatal and adult spinal cord. Ventral motor columns are very close to the ventral surface in neonatal rats (Lindsay et al., 1991). To test whether the proposed method for collecting fluid samples can be localized and differentiate regional differences in ACh levels, we collected samples from left lateral side, the left ventral horn, the ventral midline, the right ventral horn and right lateral side of the spinal cord at C4 level (Fig. 4 A). HPLC analysis showed that, in control conditions, ACh level in left ventral horn was 122.8 ± 46.8 fmol/10 μ L which was significantly higher than 39.5 ± 13.7 fmol/10 μ L in left lateral side of C4 spinal cord. In the presence of neostigmine (10 – 20 μ M, bath application), the ACh levels in the left ventral horn increased to 175.4 ± 96.0 fmol/10 μ L and the right ventral horn, 175.6 ± 76.3 fmol/10 μ L; both significantly higher than 85.5 ± 43.8 fmol/10 μ L in the left lateral side and 86.7 ± 56.7 fmol/10 μ L in the right side, respectively. ACh levels in the left and right ventral horns were also significantly higher than 106.2 ± 47.9 fmol/10 μ L in the midline (Fig. 4 B; each column were averages of 4 – 11 samples). These data were analyzed in a repeated measures ANOVA model. In Fig. 4 B, asterisk symbols “*” indicate significant differences ($p \leq 0.05$) in ACh levels between the ventral horn (either left or right) and ipsilateral side at the same experimental conditions (either control or in the presence of Neo). Cross symbols “†” indicate significant differences ($p \leq 0.05$) in ACh levels between the ventral horn (either left or right) and the midline.

To examine whether the method could detect endogenous ACh in regions as small as the preBötC and the hypoglossal nucleus in a brain slice of neonatal rat, and whether ACh in these regions modulates the excitability of respiratory neurons, we whole-cell voltage-clamped preBötC inspiratory neurons (-65mV) while recording XIIIn activity. A pipette was positioned on the contralateral preBötC for sampling fluid (Fig. 5 A). 29 ± 11.9 fmol of ACh in the preBötC and 38 ± 11.2 fmol of ACh in the hypoglossal nucleus were detected under control conditions ($n = 4$, Fig. 5 B). Bath application of neostigmine ($10\text{--}20\text{ }\mu\text{M}$) elevated ACh levels to 105.6 ± 121.3 and 135.1 ± 149.8 in preBötC and XII nucleus respectively. Repeated measures ANOVA was used to analyze these data and the increase in ACh levels in XII nucleus was statistically significant ($p \leq 0.05$) compared with the control (Fig. 5 B). Neostigmine also induced an inward current that excited inspiratory neurons, increased the respiratory frequency and amplitude as well as induced tonic activities (indicated by arrows) in XIIIn (Fig. 5 C).

4. Discussion

We developed a sampling system that collects fluid samples efficiently from small and localized CNS regions *in vitro* for HPLC measurement of neurotransmitters (or other chemicals). This method can be used concurrently with other physiological techniques such as stimulation, electrophysiological recording and pharmacological agent applications. Thus, this method can provide information regarding correlations between regional chemical events and changes in neuronal activity. This method is straightforward to implement and reliable. We showed endogenous ACh release in the ventral horn including the phrenic nucleus that played a role in regulation of respiratory-related motor output. Inhibition of AChEs induced significant increases in concentrations of ACh in the phrenic nucleus, associated with (and likely causal to) the enhancement of the neuronal excitability which results in intense tonic/seizure-like activities.

The origins of the cholinergic synaptic contacts to the ventral motor nuclei are likely from cholinergic neurons in the spinal cord (Woolf, 1991). ACh was also detected in the preBötC and in the hypoglossal nucleus in the medullary slice preparation under control conditions. Application of neostigmine elevated ACh levels and at the same time, changes in electrophysiological signals consistent with our previous results with physostigmine were observed (Shao and Feldman, 2005). These experiments with a limited number of samples are for illustration of application of this method in brain slices. Detailed studies will need to be performed, which is beyond the scope of this study, to make more definitive conclusions.

A limiting factor of this sample fluid collection system is that the resistance of the pipette tip prevents enough fluid from being sucked into the pipette by the negative pressure generated by pulling the plunger. For instance, if the tip diameter is $<180\text{ }\mu\text{m}$, collecting $10\text{ }\mu\text{L}$ of fluid is very difficult. The piece of filter paper at the pipette tip acts as a coarse sieve; it does not generate much additional resistance at $\sim 200\text{ }\mu\text{m}$ tip diameter. Although silica gel and some polymer materials are porous, when we tried to put silica gel or form polymerized acrylamide at the tip as described for microcolumn-HPLC (Li et al., 1994), they rarely worked with our fluid collection conditions. Too much additional resistance is generated from the small pores in these materials. The air tightness of the plunger is another important factor that affects generation of the negative pressure necessary for suctioning. Occasionally, we saw bubbles leaked into the pipette through the surface of the plunger in contact with the inner wall of the pipette during collection of fluid samples. Subsequently, not enough negative pressure was generated in the pipette. A layer of vacuum grease (Dow Corning Co., MI, USA) applied on the plunger improved the air tightness and improved the suctioning for collection of the fluid.

The temporal resolution for collecting fluid samples was 8 ± 1 min in this study. Part of the time between collections was taken up by manual operation of our micromanipulator that held

the micropump and the unsatisfactory fast-forward operation of the micropump controller. The temporal resolution could be improved by using a more automatic micromanipulator and by computer-controlled fast forward function of the syringe pump.

Like microdialysis and push-pull perfusion, the fluid sample collected with the pipette described in this study is not primarily interstitial fluid of the nervous tissue. The fluid sample in the pipette is primarily the perfusate (typically ACSF) which mixes and exchanges chemicals with the interstitial fluid during the collection process. Therefore, the absolute concentrations of neurotransmitters in the interstitial fluid or extracellular compartment were not directly determined. But relative amounts of chemicals during experimental manipulations can be measured more accurately with the proposed method due to absence of a membrane between the collection fluid and the interstitial fluid, and also due to the minimized dead volume in the fluid collection system.

The regions of interest should be beneath or close to the surface of the preparation. If the regions of interest are just beneath the surface of the preparation such as the preBötC and hypoglossal nuclei in our slice preparation, we collect sample fluid by putting the collection pipette tip at the slice surface over the region of interest. Some fibers of the piece of filter paper stick out from the pipette tip allowing a small space for the bath solution to exchange with the extracellular compartment of the target area and to get into the pipette. If the regions are close to the surface, we insert the pipette into the tissue and then back the pipette up for several micrometers, leaving space for continual diffusion between the interstitial fluids in the target region and the perfusate.

This method can be used for measuring variety of neurotransmitters or chemicals in different CNS regions. Combined with electrophysiological recordings, possible links between transmitter events and neuronal or system effects can be revealed. It provides a powerful tool to understand the cellular and molecular mechanisms underlying CNS system functions and pathophysiological processes.

Acknowledgements

This work was supported by Tobacco-Related Disease Research Program (California) grant 13QT-0164 and NIH Grant HL40959.

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Fig. 1. Making fluid collection pipette. (A) A Wiretrol glass pipette (Drummond Scientific, PA) was pulled with a pipette puller and the tip was cut with a diamond knife to a desired tip size (ID 180–600 μm). Inset: a pipette tip ~200 μm. (B) Filter paper was cut into triangular pieces with acute angles. One piece was positioned in the pipette tip with the aid of a fine forceps under a dissection microscope. The larger part outside the pipette tip was cut. (C) The paper left at the tip formed a sieve that prevented the tissue from being sucked into the pipette and being damaged while collecting fluid. The scale for (B) and (C) is 1 mm per division.

Fig. 2.

Fluid collection system assembly. The collection pipette was tightened in a pipette holder that was held with the syringe clamps on the micropump (UMP2, World Precision Instrument, FL). The plunger button holder of the micropump pulled the plunger and the withdrawal speed was control by a Micro 4TM controller that produced a suction volume of 2 μ L/min. The micropump was mounted on a micromanipulator that was 45° toward the chamber plane.

Fig. 3.

(A) Collecting fluid samples from an *en bloc* brainstem-spinal cord preparation and recording C4 nerve activity. The two dashed lines on the brainstem indicate where the slice preparation (Fig. 5 A) was cut. We collected samples from the ventral horn of the C4 spinal cord which contains the phrenic nucleus. Each sample of $10 \pm 1 \mu\text{L}$ was injected into the HPLC machine, and ACh and choline (Ch) were measured with an electrochemical (EC) detector. Panel (a): Chromatography traces of calibration: 100 fmol of ACh and Ch (10nM each in 10 μL of standard diluent). (b) Chromatography traces show ACh and Ch peaks in pre-drug control conditions. (c) Inhibition of AChEs by bath application of 20 μM neostigmine induced a substantial increase in ACh levels. Same scales apply to (a), (b) and (c). The Ch peaks in (b)

and (c) were clipped for better illustration of the ACh peaks. (d) ACh levels of a series samples collected before and during application of neostigmine at time points corresponding to the nerve activity trace. The time points were the midpoints of the 5-min sample collection periods. The sampling interval was 8 ± 1 min. Neostigmine also induced seizure-like activities in the ventral cervical nerve roots. The time scales of the nerve recording trace and the sample collection time are equivalent. Two insets show the nerve activities on an extended time scale. The time scales are equivalent for the two insets. (B) Changes in ACh levels in either left or right ventral horn as a function of time (mean $\pm$ SE, $n = 3$ to 6 for each column). Neostigmine application started at time 0. Asterisks “*” indicate significant differences ($p \leq 0.05$, repeated measures ANOVA) in ACh levels between the time points and the time prior to neostigmine application (4th column).

Fig. 4.

Differential ACh concentration distribution in the spinal cord of neonatal rats. (A) AChE activity staining with the modified copper thiocholine method (Ma et al., 2001) on transverse sections (60 μm thick) shows the anatomy of ventral motor columns that contain AChEs. The arrow indicates the middle motor column (phrenic nucleus) and the arrow head indicates the lateral motor column. The pipette tip for sample collections on the spinal cord at the C4 level (*en bloc* brainstem-spinal cord preparation as Fig. 3A) was positioned at: 1, left lateral (L lat) side; 2, left ventral (L vent) horn; 3, midline of ventral surface; 4, right ventral (R vent) horn; and 5, right lateral (R lat) side. (B) Summary of the ACh amounts (mean $\pm$ SE, $n = 4$ to 11 for each column) at the 5 loci before and during bath application of neostigmine (Neo, 10 – 20 μM). Asterisks “*” indicate significant differences ($p \leq 0.05$, repeated measures ANOVA) in ACh levels between the ventral horn (either left or right) and ipsilateral side at the same experimental conditions (either control or in the presence of Neo). Cross symbols “†” indicate significant differences in ACh levels between the ventral horn (either left or right) and the midline.

Fig. 5. Collecting fluid samples and electrode physiological recordings from the medullary slice. (A) The slice preparation was stabilized with a frame with nylon threads. Patch electrode was positioned to patch on neurons in the left preBötC and the left XIIIn was recorded with a suction electrode. The fluid collection pipette was positioned on the contralateral preBötC. (B) Application of neostigmine (10 – 20 μ M) elevated ACh levels separately collected from the preBötC and the hypoglossal nucleus (XII nu). The data represent mean $\pm$ SE ($n = 4$ to 5). Asterisk “*” indicates statistically significant difference ($p \leq 0.05$, repeated measures ANOVA) in ACh concentrations between control conditions and in the presence of neostigmine in XII nu. (C) Simultaneous recordings of a whole-cell patch-clamped preBötC inspiratory neuron (Im, voltage clamped at -65 mV) and the XIIIn respiratory-related rhythmic motor activity (XIIIn, integrated XIIIn activity) before and during bath application of neostigmine. Neostigmine excited the inspiratory neuron and increased the respiratory frequency, amplitude and induced tonic activities (indicated with arrows).