MEDIAL MEDULLA NETWORKS IN CULTURE:
A MULTICHANNEL ELECTROPHYSIOLOGIC
AND PHARMACOLOGICAL STUDY

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements
For the Degree of

Master of Science

By

Edward W. Keefer, B.S.
Denton, Texas
August, 1998

Spontaneously active primary cultures obtained from dissociated embryonic medial medulla tissue were grown on microelectrode arrays for investigating burst patterns and pharmacological responses of respiratory-related neurons. Multichannel burst rates and spike production were used as primary variables for analysis. Pacemaker-like neurons were identified by continued spiking under low Ca\(^{++}\)/high Mg\(^{++}\) conditions. The number of pacemakers increased with time under synaptic blocking medium. Sensitivity to CO\(_2\) levels was found in some neurons. Acetylcholine changed activity in a complex fashion. Curare, atropine and gallamine modified ACh effects. Eserine alone was ineffective, but potentiated ACh-induced responses. Norepinephrine caused channel-specific increases or decreases, whereas dopamine and serotonin had little effect at 30 µM. GABA and glycine stopped most spiking at 70 µM. Developmental changes in glycine sensitivity (increasing with age) were also observed. It is concluded that pacemaker and chemosensitive neurons develop in medial medulla cultures, and that these cultures are pharmacologically histiotypic.
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CHAPTER 1

INTRODUCTION

1.1 Overview

Recent evidence indicates that structures necessary and sufficient for the central generation of basic respiratory rhythms are located within a limited region of the medulla (Smith et al., 1991). Lesioning experiments in intact animals abolish respiration when sectioned at the level of the obex (Dawes, G.S., 1984). The rat and murine brainstems with attached cervical spinal cords maintain respiratory-like drive on the ventral spinal roots for several hours (Suzue, T., 1984, DiPasquale et al., 1992). This brainstem-spinal cord preparation, by eliminating all peripheral input, greatly facilitated study of the central mechanism of respiratory rhythmogenesis. Several groups utilized it to demonstrate that neurons contained within the rostral ventrolateral medulla (RVLM) fire bursts of action potentials correlated with the phrenic discharge (Arata et al., 1990; Murakoshi et al., 1985; Murakoshi and Otsuka, 1985; Onimaru et al., 1987; Onimaru and Homma, 1987).

Six classes of respiratory-related neurons have been characterized, based upon their phase relationship to respiratory activity (Onimaru, H., 1995). One of these, termed the pre-inspiratory by Onimaru et al., 1989, continues to fire
action potentials under conditions of synaptic blockade. Neurons possessing this quality are commonly termed pacemakers (Johnson et al., 1994). Models of central respiratory pattern generation often incorporate pacemaker cells in order to provide the fundamental rhythm (Ogilvie et al., 1992; Ramirez and Richter, 1996; Richter et al., 1992; Smith et al., 1990). This unique class of neuron has received considerable research interest, but their functional contribution to the respiratory pattern generator as a whole remains undefined (Onimaru et al., 1987; Riggato et al., 1992; DiPasquale et al., 1993; Johnson et al., 1994).

Slice preparations of the RVLM maintain a respiratory-like rhythm on rootlets of the Xth and XIIth cranial nerve (Ramirez et al., 1996; Ramirez et al., 1997). A limited region of the RVLM, the pre-Botzinger complex, has been proposed as the cytoarchitectonic feature containing the kernel of respiratory rhythm generation (Smith et al., 1991). Utilizing transverse slice preparations, several investigators have demonstrated that a sub-population of pre-Botzinger neurons exhibit pacemaker properties (Johnson et al., 1994). The presence of pacemaker cells in the neuronal population essential for rhythm generation is consistent with the idea that they participate in generation of the fundamental respiratory rhythm, but is by no means conclusive.

Application of various neurotransmitters and neuro-modulatory substances to the brainstem-spinal cord and slice preparations has provided a large amount of data on the receptor populations expressed in the respiratory-related
structures (Bianchi et al., 1995). Glutamate, glycine, GABA, ACh, norepinephrine, dopamine, serotonin, ATP, and various peptides have been shown to effect respiratory rate and/or the respiratory-related neurons themselves (Fukuda and Loeschke, 1979; Murakoshi et al., 1985; Erridichi et al., 1990; DiPasquale et al., 1992; Shao and Feldman 1997; Seller et al., 1997).

The ventral medulla surface has been shown to contain neurons that respond to altered CO₂ and H⁺ concentrations (chemosensors) with alterations in firing frequency and increased transcription (Rentero et al., 1992; Rittuci et al., 1997; Teppema et al., 1997). Other structures in the medulla contain chemosensitive cells including the nucleus tractus solitarius (NTS) and the medullary raphe (MR) (Dean et al., 1989; Richerson, G. B., 1995).

While numerous groups have utilized acute slice and brain-stem spinal cord preparations to study respiratory-related and other neurons of the medulla, experiments utilizing cultured and long-term explanted neuronal networks have been limited (Riggato et al., 1992; Neubauer et al., 1991). The use of neuronal networks grown directly on multi-channel recording arrays has been demonstrated to provide excellent signal-to-noise ratios and to offer the ability to record continuously for many days without deterioration of the experimental preparation (Gross, 1994). Preparations utilized by other researchers require extensive manipulation of the temperature, O₂ levels, extra-cellular potassium concentrations and glucose levels in order to maintain stable activity (Onimaru et
al., 1987; Smith and Feldman, 1987; Hilaire et al., 1990). For these reasons, it was felt that the non-invasive multi-channel environment in which this study was conducted provided unique opportunities to study neurons obtained from this physiologically essential region of the murine brainstem. In complex systems, only multi-channel exploration provides the necessary real-time monitoring of neuronal network components to allow quantitative studies of the dynamics of specific networks.

The primary goal of this study was to explore the neuronal components of the respiratory-related structures found in the RVLM. However, neurons from the NTS, the nucleus ambiguus, the MR, the hypoglossal nuclei, and the medullary reticular formation were included in the cultures used. For this reason, the neuronal population studied, while greatly enriched in respiratory cells, was by no means purely respiratory-related. The production of cultures with exclusively respiratory-related neurons is certainly possible, but would require refinements in the dissection and cell culture procedures.

1.2 Objective of Study

1) Establish neuronal networks cultured from dissociated tissue dissected from the embryonic murine medial medulla on multi-microelectrode recording arrays.
2) Test the responses of these cultured networks to pertinent pharmacological compounds.
3) Compare and contrast the pharmacological responses to those seen in slices,
brainstem-spinal cord, and *in vivo* preparations.

4) Demonstrate that pacemaker cells develop in medial medulla cultures.

5) Test the pharmacological responses of pacemaker cells under two conditions;  
   a) with synaptic interactions intact, and b) during inhibition of synaptic  
   transmission.
CHAPTER 2

MATERIALS AND METHODS

2.1 Fabrication of Electrode Arrays

All fabrication occurred in-house by the Center for Network Neuroscience (CNNS) staff. The techniques used to fabricate and prepare multimicroelectrode plates (MMEPs) have been described elsewhere (Gross, 1979; Gross and Lucas, 1982; Gross et al., 1985; Gross and Kowalski, 1991). Briefly, the MMEPs consisted of an array of photo-etched electrodes onto which monolayer neuronal networks were grown. The plates (5 cm x 5 cm) were prepared from 1.2 mm thick indium-tin oxide (ITO) sputtered barrier glass (soda lime glass with a 100 nm quartz layer, Applied Films Corp., Boulder, Co.). The electrode conductor pattern consisted of a central 0.8 mm$^2$ recording matrix of 64 microelectrodes (4 rows, 16 columns). Photo-etching was performed with standard procedures. The plates were spin-insulated with a 2 - 4 μm thick layer polysiloxane resin yielding a shunt impedance of approximately 15 MOhms. The matrix microelectrode sites were de-insulated with single laser pulses (Gross, 1979). This resulted in a 20 μm diameter crater at the end of each 10 μm wide ITO conductor. Low recording crater impedances of 1- 2 MOhms were achieved by electroplating a thin layer of gold on each exposed ITO tip (Gross et al., 1985).
2.2 Tissue Culture

Dissociated tissue cultures were prepared according to the basic method established by Ransom et al. (1977). Embryos were obtained from timed pregnancy Hsd:ICR mice at 14.5 to 18 days gestational age. The dissection of the tissue used to prepare the medial medulla cultures followed roughly that of Fitzgerald et al., 1992. Modifications to ensure the dissected tissue contained the respiratory-related structures and to accommodate the limited equipment available were necessary (Figure 1).

After delivery of the fetus from the amnion, the fetus was placed in chilled HEPES-buffered, low Ca physiologic saline (D15GH) and decapitated with forceps at the cervical level. Proceeding under microscopic control, the cranium was then entered through the foramen magnum with fine forceps and the skin of the head and the skull dissected away. The brainstem was separated from the rest of the brain at the pontine-collicular junction. The brainstem was then held dorsal side up with forceps, while the caudal medulla and attached spinal cord was cut away at the obex utilizing a micro-scalpel. A second transverse cut was made 3-500 μm rostral to the first, and the resulting medullary slice was freed from remaining meninges. The slices from 8-20 fetuses were collected and given to tissue culture personnel. Dissection time for each individual slice was approximately 4 minutes.
Figure 1: Area dissected from the medulla containing the elements necessary for the production of the respiratory rhythm. The brainstem is sectioned transversely at the level of the obex. A second cut is made just caudal to the most caudal rootlet of cranial nerve X (panel A). As seen in panel B, the resulting slice contains the preBotzinger complex, a region demonstrated to be necessary for respiration. Other cytoarchitectonic elements contained in the slice are shown in panel C.
The tissue was dissociated enzymatically and mechanically, seeded onto MMEPs, and maintained under Minimal Essential Medium (MEM) supplemented with 10% horse serum and 10% fetal bovine serum (MEM 10/10) for the first 7 days. Thereafter, the use of fetal bovine serum was discontinued and the cultures were maintained in MEM supplemented with 10% horse serum (MEM 10). Seeding concentrations utilized in this study ranged from 250 to 600 thousand cells per milliliter. Each MMEP was seeded with .5 milliliters MEM 10/10 containing the dispersed cells. The applied MEM 10/10 was confined to a 4 cm² area by a silicone gasket.

Before seeding, the MMEPs were prepared to allow for maximum cell adhesion. Butane flaming (1 sec) oxidized the methyl groups of the polysiloxane to hydroxyl groups and formed a hydrophilic surface conducive to cell adhesion. Two adhesion areas, a centrally located recording island (typically 2 - 6 mm in diameter) and a separate, off-center “medium conditioning area” measuring approximately 1 cm x 2 cm always were formed before seeding of cells. Polylysine and laminin adhered only to the flamed regions and were used to enhance cell attachment and growth.

The cultures were incubated at 37°C in a 10% CO₂ atmosphere until ready for use. The age of medulla cultures used in this study ranged from 8-43 days. Cells were “fed” twice a week with MEM containing 10% horse serum. Further descriptions of procedures for cell culture and maintenance can be found in
previous publications (Gross and Lucas, 1982; Gross et al., 1985; Gross and Kowalski, 1991).

Figure 2: The experimental chamber on the stage of an inverted microscope. 32 preamplifiers are shown attached to the right side of the MMEP. The plastic cap utilized to maintain a 90% air/10% CO$_2$ atmosphere is not shown.
The central island of the MMEP culture, which overlaid the recording electrode matrix, typically developed a confluent glial carpet. Intermixed and atop this carpet, a monolayer neural network formed. Among the network, neuronal somata generally were found on top of the carpet, and axonal processes were found both below and at the surface of the glial layer. Previous unpublished work suggested that basic network morphology is established by 15 days \textit{in vitro} (D.I.V.) and that, on MMEPs, networks can remain viable, stable, spontaneously active, and pharmacologically responsive for more than six months (Gross, 1994). MMEP fabrication and tissue culture facilities represent a standard support infrastructure for research.

2.3 Data Acquisition and Analysis

The following arrangement allowed for simultaneous recording of spontaneous extracellular spike activity. The MMEP was placed on a base plate and pressed in place by a silicone 0-ring attached to a stainless steel chamber (Figure 2). The culture was maintained at 37°C by heating the base plate and at a pH of approximately 7.4 under a humidified 10% CO₂ atmosphere. A plastic cap covered the chamber and was equipped with lines carrying 10% CO₂ and a heating unit to prevent condensation on the cap. This allowed for maintenance of the pH, osmolarity control, and prevention of culture contamination. pH was monitored by a phenol red indicator in the medium. A peach color signified a pH around 7.4, which is optimal for culture survival and maintaining stable activity.
Chamber components were sterilized by autoclaving and UV light before each experiment. Once the culture was stable, a full medium change using 1 ml of wash medium was performed. All subsequent medium applications originated from the same stock solution.

2.3.1 Workstation 1

Analyses of subtle aspects of network dynamics incorporating spike separation and spike pattern statistics was performed on a third generation version of the hardware available at Station 2 (Plexon Inc.). The statistics on pacemaker activity were generated with a data analysis program package with the hardware (Nex). The ability to isolate and monitor single units, as opposed to the multi-unit analysis available at Station 2 was essential to identification and characterization of these neurons.

2.3.2 Workstation 2

Figure 3 shows a schematic of the experimental arrangement available at Workstation 2. Activity was recorded by a custom multi-amplifier system (Spectrum Scientific) consisting of 2 sets of 32 preamplifiers connected to 64 second-stage amplifiers. Total system gain was 10K. Signal output from the amplifiers was distributed to 1) a 32-channel patch panel which are then integrated (analog RC circuits), 12 of the 32 integrated signals can be assigned to a 12 pen strip chart recorder, 2) 14 raw data channels can be assigned to a
Racal Store 14DS tape recorder, 3) 14 raw data channels can be assigned to a Masscomp 5700 computer for off-line burst analysis, 4) 16 channels can be assigned to speaker amplifiers for auditory feedback. Channels for recording were selected based on the best signal-to-noise ratios.

Spike integration was used as a method of major feature extraction. Integration generated slow voltage changes that were proportional to the spike frequencies recorded and were easily graphed by chart recorders. Integration allowed extraction of bursts, which are generally more easily recognized and measured than spikes. In addition, burst patterns represent a simplified level of activity that often reveal major states or modes of the network activity without massive statistical calculations on very large spike data sets (Gross et al., 1994). If the channel reports a single unit, then the integrated amplitude indicates the instantaneous spike frequency, and the integrated area under the curve represents the total spike production during the burst. However, most channels report more than one unit. Therefore, the integrated burst amplitude is influenced by the number of units, each instantaneous spike frequency, and the action potential size of the various units. Nevertheless, integration provides a useful and informative method of data extraction, especially since there is a strong tendency for units to exhibit approximate synchronization.
Figure 3: Depiction of the experimental apparatus available at Workstation 2. The assembled chamber shown in Figure 2 is connected to the 64 preamplifiers in a Faraday cage. The spike trains are sent to 64 2nd stage amplifiers (MNAP system). Channels selected for recording are assigned to 1) 4 oscilloscope traces, 2) a 32 location patch panel, 3) 14 tape recorder channels, 4) 14 computer channels (Masscomp 5700). 12 channels of integrated data can be recorded on a strip chart. The 14 raw data channels assigned to the tape recorder can also be monitored with 16 speakers.
2.4 Experimental Paradigm

Medial medulla cultures exhibiting phase-bright neuronal profiles with extensive process development and fasciculation were routinely produced with the cell culture techniques described above. Such cultures were termed "healthy", and were the ones included in the List of Experiments. These neuronal cells appeared singly and in clumps of 3-20 cells. The cells were typically 10-20 μm in diameter, with occasional larger neurons up to 40 μm. Neuronal cells lay on top of a non-phase-bright glial carpet, with processes running both above and below the glial layer. Cultures with smooth neuronal somata without cytoplasmic granularity typically produced bursting and spiking behavior that remained consistent over time if experimental environmental parameters such as temperature and osmolarity were maintained.

A typical experiment began by transferring the culture from the tissue culture incubator to the experimental chamber shown in Figure 2. The experimental chamber, forceps used to make the transfer, and the retaining screws used to attach the experimental chamber to the base plate were sterilized by autoclaving. The cap used to maintain constant atmospheric conditions above the experimental medium was sterilized with alcohol. All experiments were assembled under a laminar flow hood. The efforts to maintain aseptic conditions routinely resulted in cultures that remained active at or near baseline values for 4-5 days.
The recording medium used for most experiments consisted of conditioned flask media, drawn from a tissue culture flask containing actively growing neurons and glia, and an equal volume of MEM. Enough of the recording medium was made prior to the beginning of the experiment to ensure that the culture was bathed in the same solution throughout the recording session. The experiments where this solution was not used were the ones exploring pacemaker activity, where a modified low Ca/high Mg Ringer's solution was employed. The composition of this solution is detailed in Protocol 2 of the results section.

Once the culture was mounted on the inverted microscope stage with the pre-amplifier assemblies shown in Figure 2 attached, active channels were assigned to the various recording devices shown in Figure 3. The recording medium was adjusted to a known volume, usually 1000 μl, and once the temperature of the experimental chamber had stabilized at 37°C, recording of baseline activity begun. Typically, 30-60 minutes of spontaneous culture activity was recorded and used as a reference for measuring the effects of the subsequent experimental manipulations. Pharmacological applications were made with Eppendorf pipet and disposable tips, with stock solution concentrations of the drugs figured to ensure that total experimental volume was varied less than 5%. All drugs were dissolved in distilled water. All drugs were made fresh on the morning of the experiment except for bicuculline and strychnine.
Application intervals varied with the experimental protocol, but no interval was less than 20 minutes. Reversibility of drug effects was tested by washing the drug from the experimental medium. Washes consisted of removing all but 100 μl of the medium from the experimental chamber, and replacing with equal volumes of fresh medium. Typically, 2 washes were done separated by 20-30 minutes. The activity of the culture was allowed to stabilize for at least one hour before beginning another experimental manipulation. If the activity of the culture did not return to near baseline values, no further work was done with that culture and the effects of the prior drug application were considered irreversible. Strychnine and bicuculline induced an altered burst pattern in medulla cultures that was irreversible by washing if the drugs were left on the cultures for period exceeding 120 minutes. However, some further drug applications to the bicuculline or strychnine treated cultures were done.

In the experiments where chemosensitivity was tested, alterations in the CO₂ levels supplied to the culture were effected by changing the settings on the Aalborg gas controller. The resulting pH changes were monitored with an Accumet pH meter utilizing a combination electrode. The pH shifts induced by raising the ambient CO₂ levels from 10 to 15% ranged from -0.2 to -0.3 pH units, and occurred within 4 minutes of changing the gas mixture. Reversal of the pH changes occurred on a similar time course when the CO₂ levels were readjusted to 10%. A cell was classified as chemosensitive if the firing rate was
raised by raising the CO$_2$ levels. Only preliminary work was done investigating
the occurrence of chemosensitive neurons in the medial medulla cultures.
CHAPTER 3

RESULTS

Medulla neurons when cultured on MMEPs formed spontaneously active networks with complex patterns of spike and burst production. The age of cultures recorded from ranged from 8 to 43 days in vitro (Table 1).

<table>
<thead>
<tr>
<th>Experiment Name</th>
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<th># Active Channels</th>
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<td>17</td>
<td>22</td>
<td>300K</td>
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<td>500K</td>
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<td>EK 08</td>
<td>12/22/97</td>
<td>23</td>
<td>14</td>
<td>500K</td>
</tr>
<tr>
<td>EK 09</td>
<td>12/24/97</td>
<td>10</td>
<td>13</td>
<td>500K</td>
</tr>
<tr>
<td>EK 10</td>
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<td>14</td>
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<td>MED 6</td>
<td>6/8/98</td>
<td>10</td>
<td>16</td>
<td>500K</td>
</tr>
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**TABLE 1:** The list of experiments included in the study
The seeding concentrations used ranged from 250 to 600 thousand cells per ml. With the technique utilized to locate the suspended cells over the recording matrix of the MMEPS, this was equivalent to 477 to 1136 cells per mm². The cultures tested that were seeded at less than 500 cells per mm² (n=4) averaged 25 active channels, for an electrode yield of 40%. Conversely, at the high density of 1136 cells per mm² (n=4), electrode yield was only 21%. The high-density cultures had massive cell growth for the first 7-10 days, followed by an apparent wasting period resulting in an accumulation of debris over the surviving cells. The SNR of the high-density cultures was invariably low, averaging 1.9 for all cultures. The data for the high-density cultures is not shown in Table 1 as no results from these cultures were included in this study.

The cultures tested with intermediate seeding densities of 645-950 cells/mm² (n=14) averaged 28 active channels (45% electrode yield) with a 3.7 mean SNR. When appropriate measures were used to maintain sterility and osmolarity, these intermediate-density cultures produced stable activity for periods of 4-5 days. In addition to the 4 high-density culture mentioned above, several culture dates with 4-10 MMEPs seeded per date yielded no usable cultures.

Figure 4 shows the waveforms of two medulla culture units exhibiting spontaneous activity on channel 5. The sort window is 1400 μs wide, and the vertical divisions are 500 μv. The larger amplitude unit has a negative going
component of time of 1500 μv, with a positive deflection of 1000 μv. The total time baseline to baseline is approximately 1ms. The smaller unit has a negative component of approximately 750 μv, followed by a positive deflection of 250 μv. The width of the action potential is approximately 800 μs. The two units displayed in Figure 4 had mean frequencies of 6.2 Hz (large unit) and 2.7 Hz. (small unit) for the 34 minute baseline period of the recording session. The signal-to-noise ratios seen here of approximately 6 and 12:1 were typical for the medulla cultures. A signal-to-noise ratio of 1.5:1 was used as a cutoff for determining active channels.

Figure 5 is a digitized stripchart recording showing 16 channels of spontaneous activity from a medulla culture. The approximately 30-second interval represented in Figure 5 provides some idea of the complicated network dynamics seen in these cultures. Burst and spike patterns are seen to display transient synchronization across channels. Transitions between bursting and spiking modalities could be induced with pharmacological applications. Spike frequencies of discriminated units ranged from 0.05 to 400 Hz, with the peak frequencies usually occurring during burst-onset. Burst durations ranged from 7-8 ms to 7-8 seconds. Often, burst initiation on one channel coincided with burst shutoff on another. A pattern also seen repeatedly was the initiation of a burst on one channel followed within 1-2 ms by burst initiation on another. The multichannel environment allows the monitoring of complex network dynamics.
on a real-time basis.

Figure 4: Example of spike separation of multiple units on one channel. Spikes were separated by passing through two boxes (shown). When the spike amplitudes differed sufficiently, multiple units (maximum of 5 per channel) could be discriminated with 100% accuracy.
Figure 5: 16-channel display of spontaneous activity of a medulla culture. The time interval shown is approximately 30 seconds. Numerous patterns are evident. Some channels display continuous spiking, while others have their activity confined nearly exclusively to bursts. The arrow indicates a temporary synchronization of bursts occurring on 7 channels.
3.1 Pharmacology of Medulla Cultures

3.1.1 Responses to Cholinergic Compounds

Concentrations of ACh as low as 2 μM reliably produced responses in all medulla cultures (n=6). Figure 4 shows the response of a medulla culture to titrating ACh from 1-125 μM. ACh was seen to be inhibitory to burst rate with an IC$_{50}$ of 60 μM. Eserine, a cholinesterase inhibitor, produced no discernible effect on medulla cultures when applied alone (n=4). However, when eserine was applied in combination with ACh (n=4), it potentiated the effect seen from ACh applied singly. It required less ACh to elicit a given response in the presence of eserine and the response lasted for a longer time (Figure 6). A reasonable interpretation of these results is that acetylcholinesterase was expressed in medulla cultures, but ACh was not released endogenously.

The variety of cholinergic receptors expressed in medulla cultures was assayed with atropine, a non-selective muscarinic antagonist, and tubocurarine, an antagonist at several nicotinic receptor sub-types. Neither curare nor atropine, when applied alone or in combination elicited a discernible reaction (n=4). Atropine, when applied subsequent to ACh application, caused a further reduction in burst rate among the units depressed by the ACh (Figures 6 and 9). In order to identify the type of muscarinic receptor involved in this response, applications of gallamine (M$_2$-selective) and pirenzepine (M$_1$-selective) were made.
Figure 6: Decline in burst rate as a function of time demonstrating a characteristic decrease in activity with increased [ACh]. The bath concentrations of compounds added are given on the vertical lines. Atropine is ineffective in altering the burst rate decline induced by 116 μM ACh, but 5 μM curare reverses the effects with a latency of 8 minutes. Curare is an antagonist at the neuro-muscular junction and a non-subtype selective antagonist at central nicotinic receptors. The curare response seen here indicates that at least part of the inhibitory effect produced by ACh is mediated by nicotinic receptors.
Figure 7: Burst rate of medulla cultures, while sensitive to applications of ACh in the low micromolar range as shown in Figure 4, did not respond to eserine, an acetylcholinesterase inhibitor, without addition of exogenous ACh. The response induced by eserine was a potentiation of the effect produced by ACh when applied singly. This potentiation can be seen by comparing the 75% decrease in burst rate seen here caused by 10 µM of ACh when eserine is present, to that shown in Figure 4. It required 11 times the concentration of ACh to produce the same percentage inhibition of burst rate. Eserine also prolonged the response induced by ACh (data not shown). The data indicate that acetylcholinesterase is expressed in the medulla cultures, but that little if any endogenous release of ACh takes place.
Figure 8: Gallamine triethiodide, when applied to medulla cultures in the presence of ACh, produced an immediate response (n=2). The response opposed that produced by curare. Gallamine is a selective antagonist at the muscarinic m₂ receptor, and the effect on medulla cultures indicates that m₂ receptors are expressed and functional.
Figure 9: Curare reversed the decline in burst rate caused by ACh application. Titrating ACh from 0.5 to 50 μM induced an 80% decline in the mean burst rate. Concentrations as low as 0.5 μM produced a transient reduction in rate followed by a partial recovery to a level lower than that seen with the previous concentrations. 10 μM atropine reduced the burst rate even further, similar to the effect seen in Figure 6 with gallamine. 5 μM curare restore the burst rate to baseline levels within 20 minutes.
Only gallamine (10 μM) was successful in reversing the ACh effect (Figure 8), indicating the presence of functional M₂ receptors. This result is in accord with that obtained by Kubo et al., 1997 in intact male rats and by Fukuda and Loeschke, 1979 in ventral medullary slices.

Curare (10 μM), applied after concentrations of ACh sufficient to inhibit burst rate, totally reversed the ACh effect in medulla cultures (Figure 9). Histiochemical studies indicate the presence of cholinergic neurons within the cranial nerve nuclei, and the presence of nicotinic receptors in the nucleus tractus solitarius, the cranial motor nuclei, and the RVLM (Bianchi et al., 1995). Ueno et al., 1993 showed the predominate response to ACh application to acutely dissociated neurons from the NTS was the elicitation of a transient inward current (K₀=84 μM), an effect blocked by curare.

At first glance, the experimental results reported for medulla cultures indicated the presence of functional muscarinic receptors (predominately M₂). These receptors functioned to maintain burst rate in the presence of exogenously applied cholinergic agonists. Curare sensitive nicotinic receptors mediate a burst rate decrease in the presence of the same agonists. The total picture is much more complex however, as demonstrated in Figure 10. ACh, while decreasing burst rate on 1 channel, simultaneously suppresses spike production on another channel. Figure 11 shows the effect of ACh (1-50 μM) on the burst rate and the spike rate of one channel. Although the burst rate is inhibited over 70% by 50
μM ACh, the spike rate is unchanged. The spike rate remains essentially
unchanged throughout the experiment, declining only in response to 10 μM
atropine, which also has the effect of suppressing the burst rate even further.
Reversal of all effects, with a transient rebound acceleration of spike production
above baseline values is elicited by 10 μM curare.

Considering the above results, it would seem that the cholinergic
mechanisms expressed in the medulla cultures are almost as complex as those
seen in preparations where the neuronal circuitry remains intact. Muscarinic
receptors appear to mediate one effect of exogenous ACh, while the nicotinic
receptor population apparently recruited inhibitory mechanisms that
preferentially effected burst production. Murakoshi et al., 1985 reported that ACh
applied to the brainstem-spinal cord preparation increased respiratory rate, an
effect 80% antagonized by atropine and completely by atropine and curare.
ACh Produces Varied Effects on Medulla

![Graph showing varied effects of ACh on medulla culture activity](image)

Figure 10: ACh suppresses burst rate on Channel 7 concomitant with spike rate suppression on Channel 2 and spike rate increase on Channel 4. The mixture of opposing effects was common to all medulla cultures tested with ACh (n=6). ACh reliably changed medulla culture activity at concentrations as low as 0.5 μM, but the exact effect varied between channels and even between neurons on the same channel. The reversal of ACh effects with application of curare was also seen in all cultures. The use of an antagonist to reverse effects indicates the effects induced by ACh are receptor mediated.
Figure 11: ACh applied to medulla cultures preferentially suppressed burst rate. Spike rate remains essentially unchanged while the burst rate declines by approximately 45%, induced by 50 µM ACh. The decline in burst rate was accompanied by a corresponding increase in burst duration (not shown). The opposing effects induced by ACh seem at first glance to mirror those induced by NA (Figure 12). However, NA effected both burst and spike production simultaneously.
Eserine sulfate, an acetylcholinesterase inhibitor, increased the respiratory frequency in 50% of the preparations they tested it on. Application of cholinergic agonists to the dorsal medulla depresses respiratory rate as do systemic organophosphates which indirectly increase levels of ACh. The same agonists applied to the ventral medulla increase respiratory rate (Bianchi et al., 1995). The ventral medulla contains neurons that respond to increased H$^+$ and CO$_2$ by increasing their firing frequency (Fukuda and Loeschke, 1979). Muscarinic antagonists block this chemosensory response (Bianchi et al., 1995).

In conclusion, the cholinergic responses seen in medulla cultures mimic some of those found in more intact in vitro preparations as well as in vivo studies. The presence of both muscarinic and nicotinic receptors was established. However, no endogenous release of ACh was detectable in the cultures as determined from the lack of response to inhibitors of acetylcholinesterase.

3.1.2 Responses to Norepinephrine

Norepinephrine (1-30 μM), when applied to medulla cultures, induced increased activity on some channels, concomitant with reduced activity on other channels. This is similar to the activity-shift induced by ACh. In the brainstem-spinal cord preparation, it has been demonstrated that the respiratory center receives tonic input from the noradrenergic A5 area, located in the caudal pons (Erridichi et al., 1990; Hiliare et al., 1989). The result is an inhibition of respiratory rate that is alleviated by removal of the pons (Erridichi et al., 1994).
Recent work with knockout mice lacking a homeobox gene, Krox-20, which controls the development of the caudal pons, indicates that another previously unrecognized noradrenergic region in the pons tonically inhibits the A5 area (Borday et al., 1997; Jaquin et al., 1996; Schneider-Maunoury et al., 1993).

Murakoshi et al., 1985 showed that norepinephrine (10-30μM) increased respiratory rate in the brainstem-spinal cord. Hilaire et al., 1989, utilizing the same preparation, showed that α2-antagonists applied to the bathing medium increased respiratory rate. In pons-attached preparations, norepinephrine applied focally to the pons increased the respiratory rate while application to the medulla decreased the rate (Erridichi et al., 1994). Al-Zubaidy et al., 1996 reported bath application of 100 μM NA caused a 40% reduction in XII discharge rate in rhythmically active slices. The above evidence indicates that endogenous NA released from the pons has an inhibitory role in respiratory rate, mediated via α2 receptors.

Hayer et al., 1997 tested norepinephrine and other adrenergic agonists on pacemaker-like neurons in coronal brainstem slices, as well as tissue punches containing only the RVLM. 1-100 μM NA applied to the bath depolarized or increased the discharge rate of all putative pacemaker cells in a dose-dependent manner. They also found that 40% of the RVLM cells examined exhibited strychnine sensitive post-synaptic potentials with a frequency virtually identical to the discharge frequency of the pacemaker-like units. They hypothesize that the
pacemaker cells are inhibitory interneurons stimulated by norepinephrine released from the A5 area of the pons. This stimulation induces an increased release of glycine, which in turn inhibits the activity of other RVLM units, resulting in the reduced respiratory frequency described above.

Figure 12 shows the effect of NA (10-30 μM) when bath-applied to a medulla culture under low Ca/high Mg synaptic blocking media. Instead of the mixed effect seen with similar concentrations of NA in normal media, where some units were excited and other units concomitantly inhibited, under synaptic blockade all units that continued to fire action potentials were excited by the NA. These results would seem to support the above hypothesis of Hayer et al., 1997. In medulla cultures with intact synaptic interactions, the excitation of glycine-releasing neurons would induce a depression of activity in the post-synaptic cells, producing the pattern of activity seen in Figure 13. In the medulla cultures with synaptic interactions disrupted by ionic manipulations, the excitation of the cells with adrenergic receptors occurs without subsequent glycine release. The pacemaker-like responses to NA seen in the medulla cultures are remarkably similar to those reported by Hayer et al., 1997 considering the disparate experimental preparations and protocols utilized to identify pacemaker cells (Table 2).
Figure 12: Norepinephrine, when applied to medulla cultures produced opposing changes in spike production on different channels. Contrast this with the effect seen in Figure 13. With intact synaptic interactions, NA altered activity on different channels in a reciprocal manner as seen here for example on dsp 3a and dsp 4a. When synaptic connections were disrupted as in Figure 13, NA was almost uniformly excitatory. Possible explanations for this are discussed in the text. This figure shows individual unit activity plotted on separate axes. Spike rate (spikes per minute) is shown on the y-axis and the time (in seconds) is shown on the horizontal.
Figure 13: Application of NA to a medulla culture with synaptic interactions disrupted with low Ca/high Mg medium. This manipulation removes network interactions allowing reactions of single neurons to be visualized. 3 μM NA produced increases in spike rates in all 23 units shown averaging 212%. Raising the concentration to 30 μM generated more complex responses, with spike rate increases in 18 units, decreases in 2, and no discernible response in the remaining 3 neurons.
Figure 14: Response of a medulla culture to application of serotonin. 10 µM serotonin produced a transient increase in spike production on 22 of the 25 units depicted in the graph. The overall effect at 30 µM was a slight increase in spike rate in the majority of the units shown. The 4 cultures where serotonin was tested reacted consistently in this way, increasing the activity of 77% of the channels (n=4).
3.1.3 Responses to Serotonin

Figure 14 shows the response of a medulla culture to bath application of 10-30 \( \mu \text{M} \) serotonin (5-HT). The predominate effect was to increase firing rate (spike production) in approximately 85% of the units monitored during this experiment. The drug elicited no discernible response in the other units shown. The average increase was 27% 15 minutes after the concentration was raised to 30 \( \mu \text{M} \). 5-HT consistently affected the medulla cultures in this fashion \((n=4)\).

Murakoshi et al., 1985 reported that 5-HT had a biphasic effect on respiratory rate with an initial increase followed by persistent rate decrease in the continued presence of 5-HT. DiPasquale et al., 1992, observed a 70% increase in respiratory rate induced by 30 \( \mu \text{M} \) serotonin in the brainstem-spinal cord preparation when the pons was attached. However, with the pons removed, the respiratory rate increase was abolished. Focal application of 5-HT showed that the structures responding to the drug during the rate increase were located in the medulla. They hypothesize that the adrenergic-serotonergic systems interact to modulate respiratory rhythm. The medullary raphe contains serotonergic neurons that project to the respiratory structures. These cells participate in the cardiorespiratory and emetic responses (Bianchi et al., 1995). In summary, serotonin apparently participates indirectly in central respiratory control, modulating the rhythm during transient events such as nausea.
3.1.4 Responses to Dopamine

Dopamine was tested on a total of 3 medulla cultures. Figure 15 depicts the response of a medulla culture to bath application of dopamine (10-30 μM). The response was similar to that seen with comparable concentrations of serotonin. 38% of the units shown responded to dopamine with an increase in spike production averaging 11%. The remaining units appeared unaffected by the drug. Haloperidol (10 μM), antagonized the increase in spike rate induced by 30 μM dopamine (not shown).

In the brainstem-spinal cord, Murakoshi et al., 1985 reported similar results with dopamine on the respiratory frequency. They also were successful in antagonizing the dopamine effect with haloperidol. It is suggested that dopamine participates in respiratory control in the intact animal by acting peripherally at the carotid bodies to stimulate ventilation and possibly participating in the central response to hypoxia (Bianchi et al., 1995).

3.1.5 Responses to Inhibitory Amino Acids

Figure 16 depicts the response of a 30-day-old medulla culture to application of glycine and gamma-amino butyric acid (GABA). Both compounds are seen to be reversibly inhibitory to spike production in the majority of the units depicted. Glycine had an IC<sub>50</sub> of 40 μM in cultures older than 18 days (n=2), while GABA had an average IC<sub>50</sub> of 60 μM in the 2 cultures in which it was tested.
Figure 15: Response to application of dopamine to a medulla culture. The most common effect seen with dopamine was a slight increase in spike production. In the experiment shown, 8 units increase spiking, 1 unit decreases and the remaining 12 were essentially unchanged by 30 μM DA. The three experiments where DA was used had activity increases averaging 14% on an average of 42% of the channels.
Figure 16: The response of a medulla culture to application of the inhibitory amino acids GABA and glycine. The graph depicts the mean spike rate (spikes per minute) of 31 discriminated units. Glycine reversibly inhibits the culture shown with an EC$_{50}$ of 60 µM. GABA has an EC$_{50}$ of 50 µM and is also reversible with washing. The culture shown was 30 days old at the time of the experiment. Compare this graph with Figure 18, showing the response of a 10-day-old culture to glycine.
The GABA<sub>A</sub> antagonist, bicuculline (1-100 μM), when applied to medulla cultures, elicited a profound alteration in the bursting behavior. The burst patterns of individual channels were merged into a network-wide pattern of synchronized bursting. The effect of bicuculline on burst rate was not as predictable as its effect on synchronization. The burst rate was increased in 2 cultures by bicuculline and decreased in 2 others. A common effect seen in 75% of the bicuculline treated cultures was the appearance of newly active channels after 10-20 minutes of bicuculline induced bursting. The synchronization of activity was not 100% reversible even with repeated washings and delays of up to 12 hours if the bicuculline was left in the bath for periods of more than 1 hour. Spike production was invariably increased by bicuculline concentrations in excess of 10 μM.

The glycine receptor antagonist, strychnine (.25-10 μM), produced similar effects to those seen with bicuculline in medulla cultures older than 15 days. The burst pattern elicited by strychnine was somewhat different, with shorter average burst durations and decreased average burst amplitudes. The inhibitory effect of exogenously applied ACh on the burst rate of certain units of medulla cultures was prevented by prior application of 1μM strychnine (Figure 17), but not by 30 μM bicuculline. The observation noted above, that activation of nicotinic receptors are necessary to decrease the burst rate, when combined with the finding that nicotinic receptors are involved in the release of other
neurotransmitters (Bianchi et al. 1995), may at least partially explain this result. If the nicotinic receptors were located on glycinergic cells, then ACh-induced release of glycine from these cells would still occur, but glycine's effect at the post-synaptic cell would be antagonized by the strychnine blockade of the glycine receptor.

Several studies have examined the contribution to respiratory rhythm made by inhibitory neurotransmission (Murakoshi and Otsuka, 1985; Onimaru et al., 1990; Paton et al., 1994; Paton and Richter, 1995; Ramirez et al., 1996; Shao and Feldman, 1997). The dependence of the respiratory-related rhythm both in vivo and in tilted-sagittal slices of mice upon glycine-mediated neurotransmission was found to differ profoundly between neonates and adults (Paton et al., 1994; Paton and Richter, 1995). Specifically, concentrations of strychnine (0.2-50 μM) that disrupted the respiratory rhythm in adults were ineffective in neonates. Shao and Feldman, 1997 found that although reciprocal inhibition circuitry within the pre-Botzinger complex is glycinergic and mediated by a glycine-activated Cl⁻ channel, that channel is not required for respiratory-related rhythm generation in transverse slices from neonatal rats.

Tapia and Aguayo, 1998 reported that the functional properties of glycine receptors differed significantly between age groups of spinal cord neuronal cultures. In cultures aged 3-12 days, glycine produced a depolarizing response, whereas in cultures 17-24 days old glycine was hyperpolarizing. They attribute
this difference to a change in the reversal potential of the glycine current from -27 mV in young cultures to -52 mV in mature cultures. The resting potential of the neurons changed concomitant with the reversal potential, from -41 mV in the young cultures to -51 mV in the mature ones. It has been known for over 100 years that neonatal rodents are resistant to strychnine poisoning. This resistance is possibly due to a neonatal glycine receptor subunit that exhibits low strychnine sensitivity when expressed in *Xenopus* oocytes and a negative correlation between mRNA abundance and age (Kuhse et al., 1990).

Possible developmental changes in glycine receptor function were explored in 4 medulla cultures (2 < 14 days, 2 > 14 days). Figure 18 shows the effect of glycine (20-120 μM) when applied to a 10-day-old medulla culture. The spike rate of 11 of the 14 units shown was essentially unchanged by the glycine application; 1 unit increased spike production and 1 unit was partially inhibited. Only 1 of the 14 units was completely silenced by glycine concentrations above 40 μM. Compare this result to Figure 16 showing a 30 day old medulla culture, where 28 of 29 units are totally silenced by 90 μM glycine. It appeared that an age-dependent alteration in glycine sensitivity was present in the medulla cultures.
Figure 17: Pretreatment of a medulla culture with 1 μM strychnine, an antagonist at the glycine receptor, blocked effects of subsequent applications of ACh, eserine, and atropine. The lack of response to these cholinergic compounds indicates that some of the cholinergic-induced effects seen in medulla cultures are possibly mediated by glycinergic neurons.
Figure 18: A 10-day-old medulla culture shows little overall change in spike rate with increasing concentrations of glycine from 20 to 100 μM. Contrast with the near total suppression of activity seen in a 30-day-old culture shown in Figure 14 at a glycine concentration of 90 μM.
3.2 Occurrence of Pacemakers in Medulla Cultures

There are no specific criteria for what constitutes a pacemaker cell. Neurons which burst rhythmically during application of depolarizing current were termed pacemakers in murine spinal cord (Legendre et al., 1985). Cells that continued to fire after bath-application of kynurenic acid to block excitatory transmission in slices containing respiratory-related neurons from rats were termed pacemakers by Sun et al., 1988. Smith et al., 1997 applied bicuculline, strychnine, and CNQX to thin transverse slices of murine medulla containing the pre–Botzinger complex in order to study pacemaker behavior. Hayer et al., 1997 identified neurons as pacemakers based upon their location in the RVL and the firing of action potentials at 7-8 Hz, either spontaneously or after a small current injection.

However, blocking synaptic transmission with manipulation of the ionic environment appears to be the most common method used for identification of pacemakers. Low calcium high magnesium solutions have been applied by Johnson et al., 1994 (.2mM CaCl₂, 1mM MgSO₄, 4mM MgCl₂), Dean et al., 1990 (.2mM CaCl₂, 11.4mM MgSO₄), and G. B. Richerson 1995 (.5mM CaCl₂, 20mM MgCl₂). Riggatto et al., 1992 added .5μM TTX in addition to 3mM MgCl₂, while Kawai et al., 1996 used .2mM CdCl₂, 1μM TTX, .2 mM CaCl₂ and 5mM MgCl₂. (Table 2).
<table>
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<tr>
<th>Experimenter</th>
<th>Year</th>
<th>Protocols Utilized to Identify Pacemaker Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Legendre et al.</td>
<td>1985</td>
<td>Rhythmic bursting induced by depolarizing current injection</td>
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<tr>
<td>Sun et al.</td>
<td>1988</td>
<td>Fire action potentials with kynurenate in the bath</td>
</tr>
<tr>
<td>Smith et al.</td>
<td>1997</td>
<td>Maintain activity with bicuculline, strychnine, and CNQX</td>
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<tr>
<td>Hayer et al.</td>
<td>1997</td>
<td>Location and characteristic frequency</td>
</tr>
<tr>
<td>Dean et al.</td>
<td>1990</td>
<td>Maintain activity in .2mM CaCl$_2$, 11.4mM MgSO$_4$</td>
</tr>
<tr>
<td>Riggato et al.</td>
<td>1992</td>
<td>Maintain activity with .5μM TTX, 3mM MgCl$_2$</td>
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<tr>
<td>Johnson et al.</td>
<td>1994</td>
<td>Maintain activity with (.2mM CaCl$_2$, 1mM MgSO$_4$, 4mM MgCl$_2$)</td>
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<tr>
<td>G. B. Richerson</td>
<td>1995</td>
<td>Maintain activity with .5mM CaCl$_2$, 20mM MgCl$_2$</td>
</tr>
<tr>
<td>Kawai et al.</td>
<td>1997</td>
<td>Maintain activity with .2mM CdCl$_2$, 1μM TTX, .2 mM CaCl$_2$ and 5mM MgCl$_2$</td>
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**TABLE 2: Summary of the protocols used to identify pacemaker-like neurons**

Two different protocols were used to study pacemaker type neurons in cultures obtained from murine medial medulla. Protocol 1 involved recording spontaneous activity under control media (.9 mM MgCl$_2$, 2mM CaCl$_6$) and then titrating MgCl$_6$ to 10-12 mM in 2mM increments (n=3). The 64 electrodes were then checked for activity. If units continued to fire on channels that were not...
originally selected for data recording, the channels were then reassigned to reflect the units active under the synaptic blockade. Protocol 2 differed in that the recording media was a low Ca, high Mg Ringer's solution (119 mM NaCl, SmM KCl, 26 mM NaHCO₃, 2 mM CaCl₂, 1.24 mM KH₂PO₄, 11.4 mM MgSO₄, 10 mM glucose). This duplicated the conditions used by Dean et al., 1992 and has been shown to inhibit synaptic response to Schaffer collateral stimulation (Dean and Boulant, 1989). After an equilibration time of 15-20 minutes, the channels were scanned for any remaining activity and then reassigned (n=6).

Protocol 1: Network activity ceased in medulla cultures for 3-7 minutes when Mg levels reached 5 mM, and for periods in excess of 4 hours when levels reached 7 mM. Single unit activity (predominately spiking) remained on 10-15% of the electrodes. Neurons that remained active after synaptic blockade are generally low SNR (1.5-3) units. The firing frequency ranged from .25 to 5 Hz (Table 3). Recording of pacemaker cells under these conditions was performed for durations of up to 4.5 hours. The activity pattern was stable for approximately 2 hours, after which time the majority of units exhibited an increase in spike frequency with an occasional transition to infrequent bursting. Some pacemaker units responded to changes in CO₂ concentration with altered activity, indicating that chemosensitivity was an intrinsic property possessed by a certain percentage of pacemaker cells, and was not dependent upon network interaction. Figure 19 demonstrates the response of two units, which maintained
Figure 19: Pacemaker-like units shown under low Ca/high Mg synaptic blocking medium during alterations in the CO₂ levels of the atmosphere supplied to the experimental chamber. Note the tripling of spike activity of neuron 3 after raising the CO₂ level from 10 to 15%. A reversal of the effect is seen when the atmosphere is readjusted to a baseline mixture. The application of carbachol, a persistent cholinergic agonist is shown at 84 minutes. Neuron 1 responds with an increase in spike rate averaging 94% which continues until the end of the experiment 30 minutes later. The demonstration of chemosensitivity in one pacemaker cell, concomitant with a cholinergic response in another, reveals both the diversity of the pacemaker properties existing in the medulla cultures and the power of the multi-channel environment.
activity in the presence of 12 mM MgCl₂, to an increase in the CO₂ content of the atmosphere supplied to the culture from 10 to 15%. Unit 3 increased firing within 3 minutes of the CO₂ adjustment, coincident with a decline in pH from 7.3 to 7.1. This increased firing rate was maintained for 40 minutes, and returned to previous levels within 6 minutes of restoration of control conditions. The pH of the culture chamber media returned to 7.3 over the same interval. This was consistent with the results obtained with other preparations (Dean et al., 1990; G. B. Richerson, 1995; Kawai et al., 1997, Riggato et al., 1992). Figure 19 also shows the response of the same five neurons to application of the persistent cholinergic agonist carbachol (30 μM). Unit 1, which had maintained a spike rate of 40 per minute in excess of 90 minutes, increased its spike production by over 100% within 45 seconds of the carbachol application. The spike production remained elevated until termination of the recording session, 20 minutes later.

Protocol 2: Application of the low Ca/high Mg medium to the medulla cultures caused a nearly immediate cessation of bursting and spiking on the majority of electrodes. The average number of units that initially maintained activity under these conditions was 6 per culture upon initial exposure to the medium. Some idea of the actual percentage of neurons exhibiting pacemaker properties may be gained by considering that the average number of electrodes active under control conditions was 45 and most electrodes had at least 2 units. The figure arrived at, 7% (6/90), compared fairly closely with that given by
several groups utilizing brainstem-spinal cord and transverse slice preparations; (5/33) or 15%, Johnson et al., 1994, and (7/68) or 10%, Di Pasquale et al., 1993. The figures demonstrate that a percentage of neurons sampled from the medial medulla exhibited activity under synaptic blockade irrespective of the preparation used to study them (brainstem-spinal cord, transverse slice, or cultured network).

Two experiments allowed the examination of the activity of the pacemaker cells from the beginning of the experiment. The units that remained active when synaptic blockade was initiated were among the ones selected for data recording initially. The other three experiments conducted with Protocol 2 detected the pacemaker units only after the application of low Ca/high Mg solution. Several parameters were examined in an attempt to quantify the activity of the pacemaker cells. Table 3 lists the values for the mean firing frequency of the pacemaker-like units during the exposure to synaptic blocking medium. Figure 18 shows interspike interval histograms for one experiment, chosen as representative of the pacemaker population surviving in the medulla cultures.

The pacemaker cells exhibited exclusively spiking for the initial period of exposure to synaptic blockade. Some of these cells changed to bursting behavior upon prolonged exposure to low Ca/high Mg solution (> 4 hours). Correlation of the activity with that reported in the literature was complicated by the diverse experimental conditions used by investigators.
<table>
<thead>
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<th>Experiment</th>
<th># Initially Active</th>
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**TABLE 3:**
Figure 20: Interspike-interval histograms showing the characteristic firing patterns of 11 pacemaker units. The histograms summarize the intervals for a 7200-second period. The pacemaker units had been exposed to low Ca/high Mg medium for 6 hours at the beginning of the recording session. The y-axis shows the total number of interspike-intervals accumulated in each bin for the 7200-sec experiment. The x-axis shows the interval each bin represents.
Several studies reported units that continued to fire bursts of action potentials during synaptic blockade. However, all studies in which pacemaker-like cells had bursting activity were conducted with glucose levels at 30 mM (Johnson et al., 1994; DiPasquale et al., 1994; Kawai et al., 1996).

Figure 21 shows the activity of 4 pacemaker neurons immediately after changing to low Ca/high Mg solution. Obviously, the medium change dramatically lowers the spike rate of all units. This decreased activity level continued for the duration of the exposure to low Ca/high Mg solution. The spike rate of all neurons increased immediately upon return to norm-ionic media, although the baseline rate was not restored. A total of 11 units maintained a spike frequency of .05 Hz or greater for the duration of synaptic blockade.

Figure 22 shows the same culture 18 hours later under control medium as the experiment is repeated. What is immediately notable is the fact that both the spike rate and the number of pacemaker units are increased when compared to Figure 21). The majority of units altered their spike rate minimally when the culture was again exposed to low Ca/high Mg solution. There were 4 active units after the initial exposure to synaptic blockade, and only 1 of those maintained a spike rate of 1 Hz. 9 units were active during the repeat application and 6 of those had a spike rate of 1 Hz or greater (maximum of 4.2).

These two figures demonstrate a very interesting result. There was a positive correlation between the number of neurons possessing the pacemaker-
like property of continuing to fire action potentials while functionally dissociated, and the duration and the frequency of exposure to ionic conditions shown to cause synaptic blockade. This same effect was seen when Protocol 1 was used. The continued exposure to high Mg solution resulted in spontaneous increases in the spike frequency and the number of neurons active. It seemed an adaptation to altered ionic conditions occurred in medulla cultures when the time course of the exposure is prolonged.
Figure 21: Initial exposure of a medulla culture to low Ca/high Mg medium reveals the presence of pacemaker-like neurons. Prior to application of the blocking medium, there were 35 active channels. The activity of all units was suppressed by the solution. However, 4 units continued to fire action potentials with a mean frequency ranging from .27 to .92 Hz. Compare this graph to Figure 22. The number of units continuing to fire under synaptic blocking conditions is increased by 225%.
Figure 22: Repeated exposure of the same medulla culture to low Ca/high Mg medium results in an increase both in the number of neurons (from 4 to 9) that continue to fire action potentials and in the average frequency of the units that maintained activity during each exposure. The range of mean firing frequencies also increases. This adaptation to alterations in the ionic environment also occurred spontaneously when the exposure was prolonged beyond 4 hours in both cultures tested in this manner.
CHAPTER 4

DISCUSSION

The origin of the mammalian respiratory rhythm that begins in utero and continues for the lifetime of the animal is not completely understood. Invertebrate pattern generating networks have been used as the basis for models for motor pattern generating networks in higher organisms. Although it is composed of only twelve neurons, the swimming pattern generator of Tritonia has at least 79 of the possible 132 monosynaptic connections (Bianchi et al., 1995). The mammalian respiratory CPG is obviously much more complicated, both in the number of neurons comprising it and in the complexity of motor outputs it produces. An important development in the study of the respiratory CPG was that of Suzue, 1984 who utilized the brainstem-spinal cord en bloc preparation to study the respiratory network and the motor neurons it controls. Additional insights have been provided by the use of brain stem slices in which the respiratory rhythm is preserved on cranial nerve rootlets (Ramirez et al., 1996; Paton et al., 1994; Johnson et al., 1994).

The data accumulated utilizing these preparations indicates that a specific area of the mammalian brainstem, the pre-Botzinger complex, is both necessary and sufficient for the generation of respiratory rhythm (Smith et al., 1991;
Ramirez et al., 1996; Greer et al., 1996). However, the pre-Botzinger complex may not explain the respiratory network function in intact animals. Respiratory-related neurons are found throughout the brainstem (Dick et al., 1995). The relation between neurons in these other areas and the pre-Botzinger complex is undefined.

Based upon the depolarization pattern of individual neurons in relation to the pattern of respiratory output measured at the phrenic nerve roots or the rootlets of the hypoglossal nerve, 6 different classes of respiratory neurons have been characterized both in vivo and in vitro (Ramirez and Richter, 1996; Onimaru, H., 1995). All six of these classes have been found in the pre-Botzinger complex. Pacemaker neurons, which possess intrinsic cellular properties allowing them to fire action potentials while functionally isolated from other neurons, have been identified with one of these neuronal types, the pre-inspiratory (Onimaru et al., 1995; Johnson et al., 1994). These neurons fire immediately prior to the initiation of inspiration, and have been hypothesized to be important for respiratory rhythmogenesis in the in vitro preparations (Smith et al., 1990; Onimaru et al., 1989). The functional role of these cells in a network with intact synaptic connections is as yet unproven beyond the demonstration that they excite another class of respiratory neuron, the inspiratory-throughout (Onimaru et al., 1992).

Dissociated cells obtained from the murine RVLM formed spontaneously
active networks composed of neurons and glia when cultured on MMEPs. The networks responded to applications of pharmacological compounds in a histiotypic manner. Comparisons of the responses seen with the same substances in various other preparations reveal many similarities. Acetylcholine produced a variety of effects. Many of the cholinergic responses were apparently induced by the simultaneous activation of both nicotinic and muscarinic receptors. The m2-muscarinic receptor sub-type contributed to the responses.

The medulla cultures transiently responded to ACh concentrations as low as 1 μM. The acetylcholinesterase inhibitor, eserine, potentiated the effect induced by ACh application. This potentiation worked to both magnify and prolong the ACh responses. However when applied singly, eserine elicited no discernible responses, requiring exogenous ACh to produce an effect. Apparently, functional acetylcholinesterase enzyme is expressed in medulla cultures. If endogenous ACh release was occurring however, application of eserine alone would seemingly have changed culture activity.

The effects induced by norepinephrine were also diverse. When applied to medulla cultures with intact synaptic interactions, NE increased the activity of approximately 70% of the units, and decreased activity of the remaining units. Interestingly, NE was nearly exclusively excitatory to all pacemaker units at concentrations as low as 3 μM. This observation is remarkably similar to that made by Hayer et al., 1997 in the slice preparation. The respiratory rhythm is
tonically suppressed by a noradrenergic feed from pontine regions in the intact animal and in the brainstem-spinal cord preparation with pons attached. The observation that NE is nearly 100% excitatory to pacemaker-like units in both medulla culture and slice preparations possibly indicates that the NE induced respiratory rhythm inhibition arises from activation of these pacemaker units, which could then modulate rhythm via release of inhibitory substances.

Alterations in the sensitivity of medulla culture glycine receptors to glycine occurred in an age-related fashion. This developmental change in glycine sensitivity has been observed in other preparations (Paton and Richter, 1995) and in cultured neuronal networks grown from spinal cord tissue (Tapia and Aguayo, 1998). The molecular mechanisms behind this alteration have been explored by several groups (Kuhse et al., 1990; Takahashi et al., 1992). The timing of this glycine sensitivity alteration coincides with a reorganization of the respiratory rhythm generation mechanisms in mice and rats occurring about post-natal day 15 (Paton et al., 1994). What contribution the alterations in inhibitory mechanisms make to the maturation of rhythm generation is unclear.

Another special class of neuron, thought to mediate the CNS response to alterations in CO₂ and H⁺ necessary for maintenance of homeostasis in vivo, the chemosensitive cell, developed in the medulla cultures. These cells respond to decreased pH with an increased activity level. The presence of chemosensitive cells has been documented in the ventral medulla (Fukuda and Loescheke, 1979).
the medullary raphe (Richerson, G. B., 1995) the NTS (Dean et al., 1990) and the caudal hypothalamus (Dillon and Waldrop, 1992). Some of the pacemaker neurons found in the medulla cultures also demonstrated the ability to increase their firing rate when CO₂ levels were raised.

The observation that pacemaker cells responded to various neuroactive compounds while functionally dissociated, and that the response differed between units provides new information regarding the heterogeneity of the pacemaker population. Repeated application of low Ca/high Mg medium to medulla cultures revealed a previously unreported phenomenon; the number of cells that continue to fire action potentials while under conditions of synaptic blockade increased with the frequency and the duration of the exposure. There was also a change in the quality of the pacemaker activity with continued synaptic blockade. The mean firing frequency of the majority of pacemaker neurons (17 of 24) increased spontaneously when exposure to altered ionic conditions persisted for periods greater than 4 hours. A possible explanation for this seeming adaptation might invoke homeostatic-like mechanisms functioning on a single-neuron level to maintain activity.

LeMasson et al., 1993 proposed a model in which maximal conductances of ionic currents depend upon intracellular Ca++ concentrations. The Ca++ levels are an indirect indicator of the activity level of the neuron. Intrinsic neuronal properties such as the active current conductances are dynamically regulated
through protein phosphorylation and altered gene expression. When a neuron is silent or slowly firing, the intracellular Ca\(^{++}\) concentrations are low. If this condition persists, a decrease in the potassium conductance through downregulation of channel numbers or decreases in the Ca\(^{++}\) dependent K\(^{+}\) current could result in a shift in the equilibrium potential for K\(^{+}\) and a subsequent increase in the firing rate. The increase in the number of pacemaker cells with continued exposure to synaptic blocking conditions could be an example of such activity-dependent dynamic regulation.
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