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Clarissa Muere
*Medical College of Wisconsin*

Suzanne Neumueller
*Medical College of Wisconsin*

Justin Robert Miller
*Medical College of Wisconsin*

Samantha Olesiak
*Medical College of Wisconsin*

Matthew Hodges
*Medical College of Wisconsin*

*See next page for additional authors*

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## Authors

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# Evidence for respiratory neuromodulator interdependence after cholinergic disruption in the ventral respiratory column

**Clarissa Muere**[a], **Suzanne Neumueller**[a], **Justin Miller**[a,1], **Samantha Olesiak**[a], **Matthew R. Hodges**[a], **Lawrence Pan**[b], and **Hubert V. Forster**[a,c,§]

[a]Physiology, Medical College of Wisconsin, Milwaukee, WI, 53226, USA

[b]Physical Therapy, Marquette University, Milwaukee, WI, 53201, USA

[c]Zablocki Veterans Affairs Medical Center, Milwaukee, WI, 53295, USA

## Abstract

Reverse dialysis of the muscarinic receptor antagonist, atropine (ATR, 50 mM), into the pre-Bötzinger Complex region of the ventral respiratory column (VRC) of awake and sleeping goats increases breathing frequency and serotonin (5-HT), substance P (SP), glycine, and GABA concentrations in the effluent dialysate. Herein, we report data from goats in which we reverse dialyzed 5 mM ATR or specific antagonists of M2 or M3 muscarinic receptors into the VRC. The effects on frequency of all three antagonists were not significantly different from time control studies. 5 mM ATR and the M3 antagonist increased SP seven-fold less than 50 mM ATR. The antagonists had no effect on 5-HT, glycine, and/or GABA, suggesting that the increases in glycine and GABA with 50 mM ATR were secondary to the larger increases in 5-HT and/or SP. These data are suggestive of neuromodulator interdependence, whereby attenuation of one neuromodulator is compensated for by local changes in other neuromodulators to stabilize breathing.

### Keywords

neuromodulator interdependence; muscarinic receptor; control of breathing

## 1. INTRODUCTION

The neuromodulator, acetylcholine (ACh), has been shown to play multiple roles in physiological function, including the neural control of respiration and sleep-state transitions (3, 6, 8, 11, 13, 23). There are five known G-protein-coupled muscarinic ACh receptor (mAChR) subtypes that are preferentially coupled to either excitatory $G_{q/11}$ (M1, M3, M5) or inhibitory $G_{i/o}$ (M2, M4) G-proteins (4, 12). These receptors differ in their CNS

[§]**Corresponding author:** Hubert V. Forster, Physiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI, 53226, USA (bforster@mcw.edu, **Phone:** 414-955-8533). cmuere@mcw.edu. sneumueller@mcw.edu. jumiller@mcw.edu. solesiak@mcw.edu. mhodges@mcw.edu. lawrence.pan@marquette.edu. bforster@mcw.edu.
[1]Present address: Zablocki Veterans Affairs Medical Center, Milwaukee, WI, 53295, USA

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expression patterns and synaptic distribution, but are known to be present in brainstem nuclei related to respiratory control in several animal models (1, 5, 18).

It has been found in reduced preparations that cholinergic receptor activation is excitatory to neurons within the pre-Bötzinger Complex (preBötC) (25), a site critical to normal respiratory rhythm and pattern generation (26, 28, 31). We, therefore, in a previous study on awake and sleeping goats, tested the hypothesis that reverse dialysis of the non-selective mAChR antagonist, atropine (ATR, 50 mM), into the preBötC region of the ventral respiratory column (VRC) would decrease breathing (22). We found no significant decrease in breathing, but rather that dialysis of 50 mM ATR significantly increased ventilation, driven primarily by an increase in breathing frequency. This effect was state-dependent, since frequency increased to a greater extent in the awake state compared to NREM sleep. We also found that during 50 mM ATR dialysis, the concentrations of serotonin (5-HT) and substance P (SP) in the effluent dialysate were markedly elevated. We concluded that the increases in 5-HT and SP served to offset or compensate for a presumed reduction in post-synaptic mAChR excitation of respiratory neurons. This conclusion is consistent with the recently formulated concept of “neuromodulator interdependence“, whereby attenuation of a single excitatory neuromodulator is rapidly compensated for by an increase in another excitatory neuromodulator to maintain a stable level of breathing (9, 10). Shown in **Figure 1** is a hypothesized mechanism for mediation of such rapid compensation. Interestingly, the increase in 5-HT and SP with 50 mM ATR appears to have been over-compensatory, as indicated by the sustained and significant increase in breathing frequency, rather than maintenance of a normal breathing frequency (22). Moreover, 50 mM ATR dialysis also significantly increased glycine (GLY) and *gamma*-aminobutyric acid (GABA) concentrations in the effluent dialysate, which may have been secondary compensatory responses to counter the increase in 5-HT and SP, and thus minimize the over-compensation and return breathing to normal levels. In line with this reasoning and the concept of neuromodulator interdependence, we hypothesize that dialysis of a lower concentration of ATR (5 mM) will slightly increase 5-HT and/or SP without changing GLY and GABA, such that breathing does not change during blockade of cholinergic transmission. One objective of the present study was to test this hypothesis.

A second objective of our current study was to gain insight into which mAChR subtypes were responsible for the effects of 50 mM ATR on breathing and neurochemical release. The M2 receptor has been reported to be the most abundant mAChR subtype in the brainstem and is known to be expressed pre-synaptically in cholinergic and non-cholinergic neurons at various locations in the CNS (14, 19, 32). These properties make the M2 receptor the ideal candidate for study, since its pre-synaptic localization would allow it to modulate the release of potentially multiple neurochemicals. On the other hand, the M3 receptor has been shown to play an excitatory role in controlling breathing frequency in brainstem slice preparations containing the preBötC (25). Since the M2 and M3 subtypes have also been shown to be the predominant mAChRs expressed in the preBötC (17), we chose to study the contributions to breathing and neurochemical modulation of these two subtypes by dialyzing specific antagonists to the M2 and M3 receptors in the VRC. Consistent with the mechanism depicted in **Figure 1**, we hypothesize that blockade of M2 mAChRs would recapitulate the

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effects of dialysis of 50 mM ATR in the VRC, and that blocking M3 mAChRs would depress breathing. Lastly, given that the effects of 50 mM ATR dialysis on breathing are state-dependent (22), we sought to test if dialysis of 5 mM ATR also had state-dependent effects on breathing. We hypothesize that 5 mM ATR would not change breathing in both the awake and NREM sleep states and have minimal effects on neurochemical concentrations in the effluent dialysate.

## 2. METHODS

### 2.1 Goats

Data were obtained from a total of 19 adult, non-pregnant female goats weighing 46.1 ± 2.1 kg. The goats were housed and studied in an environmental chamber with a fixed ambient temperature and 12-hour light/dark cycle (lights on 6AM). Goats were allowed *ad libitum* access to food and water, except during study periods and during a 24-hour fasting period before all surgeries. This study was approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee before studies were initiated.

### 2.2 Surgical procedures

Prior to surgery, goats were anesthetized with ketamine (5.0 cc, IV) for intubation, after which they were mechanically ventilated with 2% isoflurane in 100% oxygen. A non-steroidal anti-inflammatory analgesic, flunixin meglumine (Banamine, 1 mg/kg, IM) was given once preoperatively. Rectal body temperature ($T_r$), heart rate, respiratory rate, and blood oxygen saturation were monitored throughout and over the next 24 hours after each surgery. To reduce the risk of infection, ceftiofur sodium (Naxcel, 4 mg/kg, IM) was administered daily and all surgical sites treated with triple antibiotic for ≥7 days post-operatively. Buprenorphine hydrochloride (Buprenex, 0.005 mg/kg, IM) was administered twice daily 48 hours after surgery to minimize pain.

After a ≥3-day acclimatization period, two surgeries separated by two weeks were performed under sterile conditions. In an initial surgery, to monitor and score sleep state, electroencephalogram (EEG) and electro-oculogram (EOG) electrodes were implanted in the midline cranium and superior orbital ridge, respectively. The second surgery was later performed for chronic bilateral or unilateral implantation of stainless steel microtubules (MTs, 70 mm length, 1.27 mm outer diameter, 0.84 mm inner diameter) targeting the preBötC. This surgery required an occipital craniotomy, after which the dura mater was excised to expose the posterior cerebellum and dorsal medulla and for visualization of obex. The target site in the goat for implanting MTs was 2.5-3.5 mm rostral to obex, 4.0-5.0 mm lateral to midline, and 4.0-6.0 mm from the dorsal surface of the medulla (ventral to nucleus ambiguous). To avoid blood vessels on the dorsal medullary surface, the site of implantation had to be adjusted for some animals. The MTs were permanently secured with dental acrylic, the skin incisions sutured closed, and stainless steel stylets matching the length of the MTs were inserted into the MTs without penetrating the tissue. Stylets were removed only during study periods. Procedures for post-operative monitoring and medication regimes have been previously described.

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### 2.3 Physiological variables

During recovery from MT implantation, goats were trained to become accustomed to all testing procedures and equipment. To measure ventilation, a custom-made mask attached to a one-way breathing valve connected to inspiratory and expiratory tubing was secured to the goat's muzzle; breathing was continuously recorded using a Windaq data acquisition system for later offline analysis. Inspiratory flow (or minute ventilation, $V_I$, L/min), breathing frequency (breaths/min), and tidal volume ($V_T$, L/breath) were measured using a pneumotachograph attached in series to the inspiratory side of the breathing valve. $T_r$ was measured using a thermocouple inserted into the rectum. During night studies, data were collected in an identical manner, except EEG and EOG activity were additionally recorded for later scoring of sleep state.

### 2.4 In vivo *dialysis*

Dialysis probes (Harvard Apparatus, Holliston, MA) were 72 mm in total length (2 mm membrane tip, 0.5 mm membrane diameter, 20 kDA cut-off, 3 μL internal volume). The length of the MTs allowed for insertion of dialysis probes such that only the membrane tip penetrated the tissue. The dialysis perfusate was either mock cerebrospinal fluid (mCSF: 124 mM NaCl, 2.0 mM KCl, 2.0 mM $MgCl_2$, 1.3 mM $K_2PO_4$, 2.0 mM $CaCl_2$, 11 mM glucose, 26 mM $NaHCO^-$, and pH 7.32 in sterile distilled water) alone or containing one of the following: 1) the non-selective mAChR antagonist, ATR (5 mM, Sigma), 2) the M2 mAChR antagonist, methoctramine hydrate (MTC, 50 μM, Sigma), or 3) the M3 mAChR antagonist, 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 5 mM, Abcam). The concentration used for 4-DAMP was the highest we could achieve in mCSF and was chosen to maximize passage across the probe membrane. The concentration used for MTC was chosen to minimize inhibition of GTPase activity at mM concentrations (as reported by the supplier) and to maximize membrane passage. In a subset of goats, we also dialyzed MTC at concentrations of 2, 5, and 10 μM, but ventilatory results were less consistent at these lower concentrations and are not reported here. Prior to dialysis, solutions were warmed in a tonometer to 39°C and equilibrated with a nitrogen-balanced gas mixture of 6.4% carbon dioxide and 21% oxygen. For delivery of solutions to the probe, the syringe pump (Harvard Apparatus) used was placed outside of the animal chamber, requiring a 150 or 180 cm length of polypropylene tubing connecting the syringe to the probe. This length of tubing resulted in an approximate delay of 20 minutes between initiation of dialysis and when the perfusate reached the probe. Effluent dialysate was collected in modified cryotubes, aliquoted, and then frozen at −80°C for subsequent analyses by a core assay laboratory.

Control (pre-dialysis) data were obtained 30 minutes after probe insertion and collected for another 30 minutes. Three consecutive 1-hour dialysis periods (25 μL/min flow rate) followed in the order: 1) mCSF, 2) mCSF with mAChR antagonist, 3) mCSF. Effluent dialysate was collected for each hour in separate cryotubes.

Four groups of goats were studied differing only in the receptor antagonist dialyzed in Hour 2 or day/night condition. **Group 1** consisted of goats in which 5 mM ATR was dialyzed in the day; **Group 2** consisted of goats in which 50 μM MTC was dialyzed in the day; **Group 3** consisted of goats in which 5 mM 4-DAMP was dialyzed in the day; and **Group 4**

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consisted of goats in which 5 mM ATR was dialyzed at night. Daytime studies were completed between 9AM and 2PM and night studies were completed between 8PM and 2AM. A minimum of 36 hours separated consecutive studies within a single animal.

### 2.5 mCSF and glutamate receptor agonist injections

Since administration of glutamate receptor agonists to the preBötC elicits a distinct tachypnea, we injected a glutamate receptor agonist (N-methyl-D-aspartic acid, NMDA, 100 mM) as a physiological marker of MT placement in or near the preBötC region. Breathing was continuously measured over two hours during which either mCSF or NMDA was injected (500 nL) into the MTs at 30-minute intervals.

### 2.6 Neurochemical analysis

Glutamine (GLN), GLY, and GABA were measured via reverse-phase high-performance liquid chromatography, as previously described, with fluorescent detection using: Waters Resolve C18 column (150X3.9), a fluorescent detector with excitation at 229 nm and emission at 470 nm, β-alanine internal standard and O-phthaldialdehyde derivitization. 5-HT was measured with the same type of column with a potential setting of 0.6V vs. Ag/AgCl reference electrode and a N-methylserotonin internal standard. SP and thyrotropin-releasing hormone (TRH) were measured using commercially available assays (Assay Designs 900-018, range 9.76-10,000 pg/mL and MyBioSource MBS044339, range 0.625-20 μIU/mL, respectively) and a microplate reader at 405 nm.

### 2.7 Data and statistical analysis

For each study, the inspiratory flow signal was calibrated against known air flow rates and, in the case of night studies, EEG and EOG signals were used to score each breath as being in the awake or NREM sleep states. Data were analyzed on a breath-by-breath basis.

To determine if there were significant effects on breathing of daytime dialysis of 5 mM ATR, 4-DAMP, or MTC, we performed statistical comparisons of ventilatory parameters from these experiments to our previously reported time control studies (22), in which mCSF was dialyzed during Hours 1-3. These time control data were conducted under identical conditions and methodologies (22; **Fig. 3**). Importantly, we have re-used and re-analyzed these control data to: 1) minimize the number of animals used in our studies, since repetition of control experiments may violate policies regarding using the minimum number of animals required to address scientific questions; and 2) to make direct comparisons between control data and novel findings from our present studies, which we believe are critical for scientific interpretation by the audience (explained in further detail below).

Our previous studies (22) found that dialysis of 50 mM ATR elicited sufficiently large, sustained increases in $V_I$ and frequency, such that statistical analysis using 15-minute bins was feasible for comparing the effects of 50 mM ATR dialysis and time control studies. However, in the present study, since we hypothesize that dialysis of a lower dose of ATR (5 mM) would have no effect on breathing, we felt it necessary to compare these data to established control data and to analyze the data at a finer temporal resolution. We thus performed statistical analyses utilizing 15-minute bins, as per our previous report (22), and

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additional analyses using 1-minute bins to maximize detection of smaller changes in breathing. For both 1- and 15-minute bin comparisons, a two-way repeated measures (RM) ANOVA (one factor repetition, treatment and time as factors, Holm-Sidak *post-hoc*, when appropriate) was performed. The P-value of the interaction term provided the statistic of whether the effect of dialysis of an antagonist (5 mM ATR, 4-DAMP, or MTC) over time was significantly different from the effect of dialysis of mCSF over time in our time control studies. For 1-minute bin comparisons, to reduce noise, we limited the period of analysis to the last 15 minutes of Hour 1 (averaged and expressed as a single value) to the end of Hour 2, when either an antagonist or mCSF (in the case of time control studies) was dialyzed. One animal underwent both a time control study and daytime 5 mM ATR dialysis. For simplicity of analysis, this animal was coded as two different animals in statistical comparisons between 5 mM ATR and time control studies. The results of the 1- and 15-minute bin comparisons were consistent for the interaction term; thus, for clarity of presentation, all figures and data reported herein are in 15-minute bins. For night studies, a two-way RM ANOVA (two factor repetition, state and time as factors, Holm-Sidak *post-hoc*, when appropriate) was used to compare the effects of sleep state on breathing using 15-minute bins.

For each group, effluent dialysate concentrations of 5-HT, SP, GLN, GLY, and GABA were analyzed via one-way RM ANOVA to determine if there were significant effects on neurochemical concentration of mCSF or antagonist dialysis. For analysis of TRH, only the effluent dialysate from Hours 1 and 2 were analyzed, and a different subset of goats was used to measure the effect of daytime dialysis of 5 mM ATR. For each group, a paired t-test was used to compare TRH concentration between Hours 1 and 2. For all t-tests, two-tailed P-values are reported.

Ventilatory and neurochemical data from duplicate studies for a single animal were averaged. All statistical tests were performed using SigmaPlot software (version 12.0 or 12.5) and results with $P<0.05$ were considered statistically significant. All results are presented as mean ± S.E.M.

### 2.8 Histology

At the end of the protocol, goats were anesthetized with ketamine (2.3 cc, IV), the cranial circulation isolated, and the goat euthanized (B-euthanasia, 10 mL, IV). The head was perfused with phosphate buffered saline (PBS) via a carotid arterial catheter, followed by 4% paraformaldehyde in PBS; the brainstem was then harvested for processing and sectioning, as previously described. Nissl-stained 4000 DPI-scanned images of the entire MT tract were captured (Nikon Super Coolscan 9000), and Metamorph software used to measure MT placement (in mm, relative to midline and the ventral medullary surface) at the approximate middle of the rostral-caudal MT damage range.

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# 3. RESULTS

## 3.1 Histological analysis and NMDA responses

Previous studies in our laboratory demonstrate that the goat preBötC is located approximately 2.5-3.5 mm rostral to obex, 4.0-5.0 mm lateral from the midline, and 4.0-6.0 mm from the dorsal surface of the medulla (ventral to nucleus ambiguous) (22, 31). For the goats studied herein, histological analysis found that the center of the distal tip of the MTs were generally, but not always, within this range (**Fig. 2**). As in our past studies (21, 22), we utilized the breathing frequency response to glutamate agonist (NMDA) injections as a physiologic indicator of MT proximity to the preBötC region. Consistent with past studies, we found variation in this response to NMDA injections (inset in **Fig.2**). Accordingly, neither the histology nor the NMDA values provide absolute assurance that the MTs were within the preBötC, which is why we consistently state that the placements were generally within the VRC. While site-specificity of our effects is difficult to determine, dialysis within the right side of goats 9 (5 mM ATR in the day), 16 (4-DAMP), and 17 (MTC), whose MT placements were either too dorsal or rostral from the presumed preBötC, resulted in changes in SP concentration that were consistent with group results. Since we have historically found changes in SP to be the most consistent between goats (and thus, an indicator of an antagonist effect on neurochemical release), these goats were included in our analysis.

## 3.2 Effects on ventilation and local neuromodulator concentration of dialysis of 5 mM ATR during the day

Comparison of 5 mM ATR and time control studies found no significant interaction effects between treatment and time for any respiratory parameter ($P \geq 0.268$, **Fig.3**). 5 mM ATR had no significant effect on the effluent concentrations of 5-HT, TRH, GLY, and GABA ($P \geq 0.07$, **Fig. 4A,C,E,F**), but it significantly increased effluent SP levels ($P < 0.001$, **Fig. 4B**) nearly ten-fold. 5 mM ATR elicited a significant decrease in effluent GLN ($P < 0.05$, **Fig. 4D**).

## 3.3 Effects on ventilation and local neuromodulator concentration of dialysis of M2 and M3 mAChR antagonists during the day

Comparison of 50 μM MTC and time control studies found no significant interaction effects between treatment and time for any respiratory parameter ($P \geq 0.203$, data not shown). Likewise, comparison of 4-DAMP and time control studies found no significant interaction effects between treatment and time for any respiratory parameter ($P \geq 0.122$, data not shown). Dialysis of 4-DAMP significantly increased SP concentrations ($P < 0.001$, **Fig. 5B**), significantly decreased GABA concentration ($P = 0.002$, **Fig. 5F**), but had no effect on the effluent concentrations of 5-HT, TRH, GLN, or GLY ($P \geq 0.111$, **Fig. 5A,C-E**). Dialysis of MTC had no significant effect on the concentration of any neurochemical ($P \geq 0.192$, **Fig. 5A-C,E-F**), except significantly decreasing GLN ($P = 0.001$, **Fig. 5D**).

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### 3.4 Effects on ventilation and local neuromodulator concentration of dialysis of 5 mM ATR across sleep states at night

During dialysis of 5 mM ATR at night (data not shown), comparison of the awake state and NREM found significant main effects of state on $V_I$, frequency, and $V_T$ ($P \leq 0.01$), much of which may be due to normal differences in breathing between sleep states. There were significant main effects of time for frequency ($P < 0.001$) and $V_T$ ($P = 0.005$). There were no significant interaction effects between sleep state and time for $V_I$ and $V_T$ ($P \geq 0.213$); however, there was a significant interaction effect between sleep state and time for frequency ($P = 0.040$). Dialysis of 5 mM ATR at night had no significant effect on the effluent concentrations of 5-HT, GLN, or GLY ($P \geq 0.081$, data not shown). 5 mM ATR significantly increased effluent SP concentration ($P < 0.001$) and significantly decreased effluent GABA concentration ($P = 0.035$).

## 4. DISCUSSION

### 4.1 Summary of hypotheses and main findings

We hypothesized that dialysis of 5 mM ATR in the VRC would not change breathing (awake and NREM sleep), but would slightly increase 5-HT and/or SP without changing GLY and GABA. Our findings largely support these hypotheses, but do not support the hypotheses that blocking M2 receptors with MTC would affect breathing and neuromodulator concentration in a manner similar to 50 mM ATR, and that blocking M3 receptors with 4-DAMP would depress breathing.

### 4.2 Interdependence of neuromodulators controlling breathing

The concept of interdependence of neuromodulators implies that attenuation of a single excitatory neuromodulator results in changes in other neuromodulators that compensate for the attenuated neuromodulator. What then is the signal that triggers this compensation? In the present and the past studies (22), we did not observe a decrease in breathing which we predicted would occur with dialysis of ATR and the M3 blocker, 4-DAMP. If a change in breathing occurred, that change may have been within normal physiologic variation that occurs in freely behaving mammals. On the other hand, neuromodulator interdependence/compensation may not require or be dependent upon a global change in breathing or even a change in the activity of respiratory neurons as initiating factors. It is possible that events preceding changes in neuron activity and thus physiological behavior (e.g. a reduction in receptor activation) may trigger the release of neurochemicals. In other words, neuromodulator interdependence may occur at the local level and may not necessarily be reflex-mediated.

Indeed, we have several lines of evidence supporting the concept of local regulation of neuromodulators. First, as previously reported (22), when 50 mM ATR is dialyzed unilaterally into the VRC with mCSF simultaneously dialyzed into the contralateral VRC, 5-HT, SP, and GLY increase only on the side of ATR dialysis. This finding indicates that there are local, rather than reflex-mediated, compensatory changes when a single neuromodulator is altered. Second, while there is evidence that 5-HT, SP, and TRH are co-localized (15) in certain subsets of neurons, we found that 50 mM ATR markedly increased 5-HT and SP, yet

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had no effect or decreased TRH (previously unpublished, **Fig.6**). This dissociation in the changes in effluent 5-HT, SP, and TRH suggests that there are local mechanisms independently governing the release of these neuromodulators and/or that ATR may differentially affect these mechanisms. A third example of local regulation is that with dialysis of 50 mM ATR, the inhibitory neurotransmitters, GLY and GABA, increase coincident with increases in 5-HT and SP. In contrast, with 5 mM ATR, these inhibitory neurotransmitters tended to decrease with no change in 5-HT and a comparatively smaller change in SP. As we previously postulated (22), it is possible that the increase in GLY and GABA with 50 mM ATR may have resulted from the large increases in 5-HT and/or SP in an attempt by local control mechanisms to maintain a stable level of respiratory neuronal activity and breathing. These multiple, simultaneously-occurring local changes in neurochemicals seem inconsistent with a mechanism dependent upon a global change in breathing. Indeed, if ATR dialysis had caused a decrease in breathing, highly powerful chemoreceptors would have been activated, which likely would have caused global, rather than local, compensatory changes in excitatory neuromodulators.

### 4.3 Ventilatory and neurochemical effects of 5 mM ATR

We found that during 5 mM ATR dialysis (day and night, awake and NREM), frequency changed minimally or not at all. The only change in neurochemicals with 5 mM ATR dialysis was a small increase in SP and a decrease in GLN/GABA during ATR dialysis and during the recovery period. Consistent with the reasoning in the previous section, it is possible that the increase in SP may have offset a post-synaptic reduction in mAChR stimulation of breathing during ATR dialysis, which may be why we did not observe a reduction in breathing.

The small or absent change in breathing frequency, and the small changes in neurochemicals during dialysis of 5 mM ATR, are in sharp contrast to the large increases in breathing and in 5-HT, SP, GLY, and GABA during dialysis of 50 mM ATR. The difference in frequency resulting from dialysis of 5 mM and 50 mM ATR could be due to a combination of the comparatively smaller increase in SP, the lack of effect on 5-HT levels, and the lack of change (or reduction) in GLY and GABA with 5 mM ATR. Our current and past data do not permit definitive conclusions about the primary determinant driving the increase in breathing frequency with 50 mM ATR. However, the data do suggest that differences in neurochemical release between 5 mM and 50 mM ATR contribute to the difference in their ventilatory effects.

### 4.4 Ventilatory and neurochemical effects of M2 and M3 mAChR antagonists

As with 5 mM ATR, the effects of 4-DAMP and MTC on breathing were not different from time control studies. The small increase in SP with dialysis of 4-DAMP may have compensated for a presumed decrease in post-synaptic excitatory mAChR activity. Our data do not permit comment on the specific proportional contributions of each mAChR subtype to the effects of ATR. Despite this limitation, given evidence that the M2 receptor is the most abundant mAChR subtype in the brainstem (19, 32), it is conceivable that M2 mAChRs account for a larger proportion of the effects of ATR. We previously postulated that pre-synaptic inhibitory M2 mAChRs may mediate the 50 mM ATR-induced release of

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5-HT and SP (22). Since 5-HT, SP, and other measured neuromodulators did not increase with blockade of the M2 receptor, our current data do not support the hypothesis depicted in **Figure 1** for mediation of neuromodulator interdependence.

### 4.5 Effects of 5 mM ATR dialysis during sleep

Dialysis of 5 mM ATR at night during the awake state had a smaller excitatory effect on breathing frequency compared to 50 mM ATR, and there were significant differences (data not shown) between 5 mM and 50 mM ATR in their effects on 5-HT, SP, GLY, and GABA concentrations at night. The state-dependence of the respiratory effects of ATR may reflect different levels of cholinergic input to the VRC during wakefulness and NREM sleep (2). Alternatively, compensatory mechanisms to maintain breathing during sleep may be different from those in the awake state (24). Another possibility is that breathing during NREM is less dependent on ACh and therefore less vulnerable to the effects of ATR.

### 4.6 Caveats and limitations

As previously summarized (22), there are limitations to our studies which herein we briefly summarize. First, due to the importance of the preBötC in respiratory rhythm and pattern generation (26, 28, 31) and the known expression of mAChRs (17) at this site, we targeted the preBötC for mAChR blockade by dialysis of exogenous antagonists. However, the exact boundaries of the preBötC are unclear (see reference 16) and thus the precise placement of MTs for dialysis solely in this area was problematic. Additionally, the lack of an exact method for quantifying antagonist diffusion area is also a limitation. Indeed, differences in distance of diffusion may have contributed to the differences in effects of 5 mM versus 50 mM ATR, where a higher concentration of drug would diffuse over a larger area and affect a greater number of neurons.

Secondly, due to the length of inlet tubing required for delivery of antagonists, there was a delay between the initiation of dialysis of mAChR antagonists and when the antagonist actually reached the goat, which accounts for the delay in the ventilatory response. Third, because of the manual system for collection of effluent fluid, we were unable to separate neuromodulators according to the awake and NREM sleep states. Additionally, it has previously been reported (33) that sleep is associated with an increase in fractional interstitial space, facilitating exchange between the interstitial fluid and CSF. Since changes in extracellular space affect tissue concentration and diffusion of neurochemicals, potential state-associated changes in interstitial space are another caveat in our nighttime neurochemical values. There was also the potential for tissue damage from repeated insertion of the dialysis probe; thus, we were limited in the number of studies on each goat. Finally, sample size and variability between goats were also potential limitations to some aspects of the study. These limitations restrict the conclusions we can make from our data, but our conservative conclusions are warranted despite these and other potential limitations.

### 4.7 Significance of present findings

Over the last few decades, a variety of neuromodulators have been shown (primarily in reduced preparations) to be important in the control of breathing and in determining the excitability of respiratory neurons (25, 29, 30). The coordinated release of these

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neuromodulators is not well understood. Recent data in a reduced preparation suggest that there is interdependence among these neuromodulators, whereby “a modulator’s action is determined by the concurrent modulation and interaction with other neuromodulators” (10). Our unique data, under physiologic awake and sleeping conditions, support this concept. Moreover, we present several examples (summarized above) that this interdependence occurs locally within the environment of a neuron, rather than through a reflex mechanism.

Considering that the effects on breathing and neurochemical concentrations of 5 mM ATR were small compared to 50 mM, our data may represent two ends of a spectrum of compensatory changes in response to disruption of neural transmission. On one end, inactivation of a subset of mAChRs by 5 mM ATR may stimulate neurochemical release sufficient to maintain breathing within a normal range. The effects of the lower dose of ATR perhaps reflect homeostatic mechanisms within respiratory centers that allow them to respond to small fluctuations in neurochemical input. These fluctuations are compensated for without aberrant changes in physiological behavior. Such compensatory mechanisms may be important in maintaining function during normal changes in neural activity (e.g. during sleep state transitions), and may possibly be extended to control of non-respiratory functions. On the other end, a larger degree of mAChR inactivation by 50 mM ATR may elicit excessive or uncontrolled alterations in multiple neurochemicals, leading to abnormal changes in breathing (22). Accordingly, disruption of a single neuromodulator system can be adequately compensated for by local changes in other neurochemicals; however, if the perturbation is too great, perhaps as would be found in neurodegenerative diseases where there is failure of transmission of a neuromodulator (7), the compensatory capacity of the system may be disrupted, leading to over-compensatory changes in other neuromodulators. Such over-compensation may then require changes in inhibitory neurotransmitters (GLY and GABA) to maintain stable breathing. Whatever the sequence of events may be, the net effect of such large changes in neurochemical balance is a non-physiological level of breathing.

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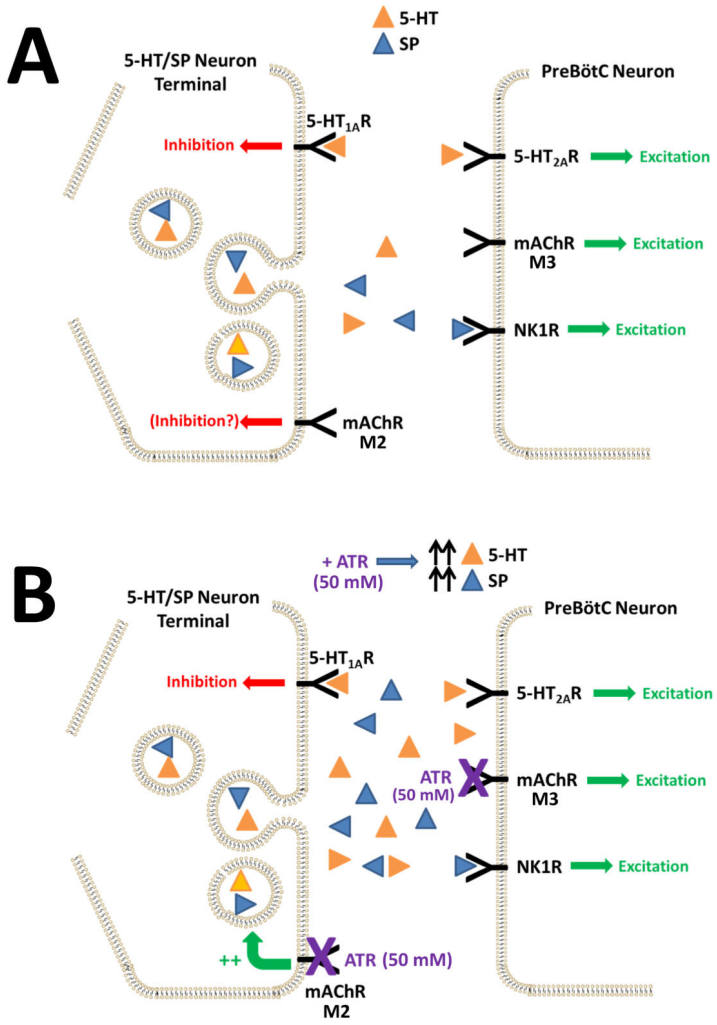


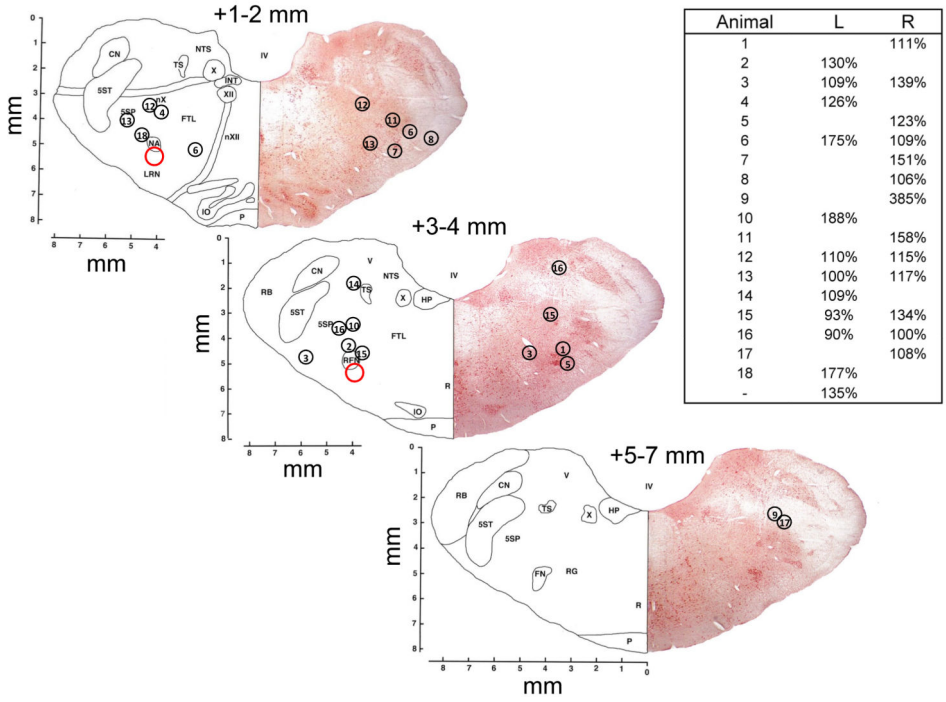

**Figure 1.**

Illustration of mechanism originally hypothesized to explain the increase in breathing frequency, 5-HT, and SP with dialysis of 50 mM ATR (22). Under normal conditions (**A**), 5-HT/SP-containing pre-synaptic terminals release 5-HT/SP into the extracellular space, where these neuromodulators act upon their respective receptors on a post-synaptic neuron, leading to excitation. Pre-synaptic terminals may express inhibitory mAChRs, the activation of which is thought to inhibit release of 5-HT/SP. Post-synaptic neurons may also express excitatory mAChRs, the activation of which would lead to post-synaptic excitation. For simplicity, ACh molecules have been omitted from the schematic. With dialysis of 50 mM ATR (**B**), post-synaptic excitation via mAChRs is dampened; however, this effect may be overridden by disinhibition of neurochemical release from pre-synaptic terminals due to blockade of inhibitory mAChRs. The net effect of ATR is thus an increase in the release of 5-HT/SP, leading to an increase in post-synaptic excitation and breathing frequency. 5-$HT_{1A}$R = 5-HT subtype 1A receptor; 5-$HT_{2A}$R = 5-HT subtype 2A receptor; mAChR = muscarinic ACh receptor; NK1R = neurokinin-1 receptor.

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| Animal | L | R |
|---|---|---|
| 1 | | 111% |
| 2 | 130% | |
| 3 | 109% | 139% |
| 4 | 126% | |
| 5 | | 123% |
| 6 | 175% | 109% |
| 7 | | 151% |
| 8 | | 106% |
| 9 | | 385% |
| 10 | 188% | |
| 11 | | 158% |
| 12 | 110% | 115% |
| 13 | 100% | 117% |
| 14 | 109% | |
| 15 | 93% | 134% |
| 16 | 90% | 100% |
| 17 | | 108% |
| 18 | 177% | |
| - | 135% | |

**Figure 2.**

Sections from the goat brain atlas illustrating approximate placement of MTs in which studies were done for animals coded according to the inset table (for one animal, post-mortem tissue was unusable for Nissl staining). The corresponding frequency responses of each animal after injection of NMDA into the MTs are also listed and expressed as percent of control. L = left MT; R = right MT. Distance rostral from obex is indicated above each section. Red circles indicate approximate location of the preBötC in the goat.

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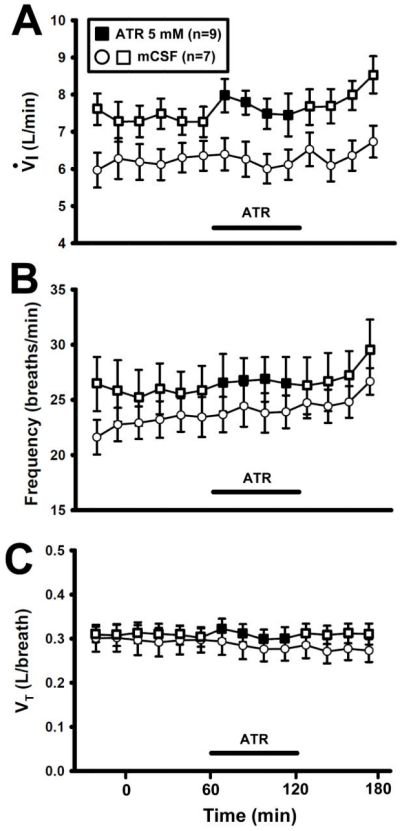



**Figure 3.**

The ventilatory effects over time of daytime dialysis of 5 mM ATR were not significantly different from the effects of mCSF dialysis in time control studies, as determined by lack of a significant interaction between treatment and time in two-way RM ANOVAs ($P \geq 0.268$). **A**, $V_I$; **B**, frequency; **C**, $V_T$. Note that while baseline ventilation and breathing frequencies were not the same between the two groups, the change in breathing over time, regardless of solution dialyzed, was the same between groups. x-axis is time (min) from start of dialysis; symbols left of 0 indicate pre-dialysis period. Open symbols indicate dialysis of mCSF; circles indicate time control studies (n=7); squares indicate studies with daytime dialysis of 5 mM ATR during Hour 2 (n=9). Black bar and closed symbols indicate period of ATR dialysis.

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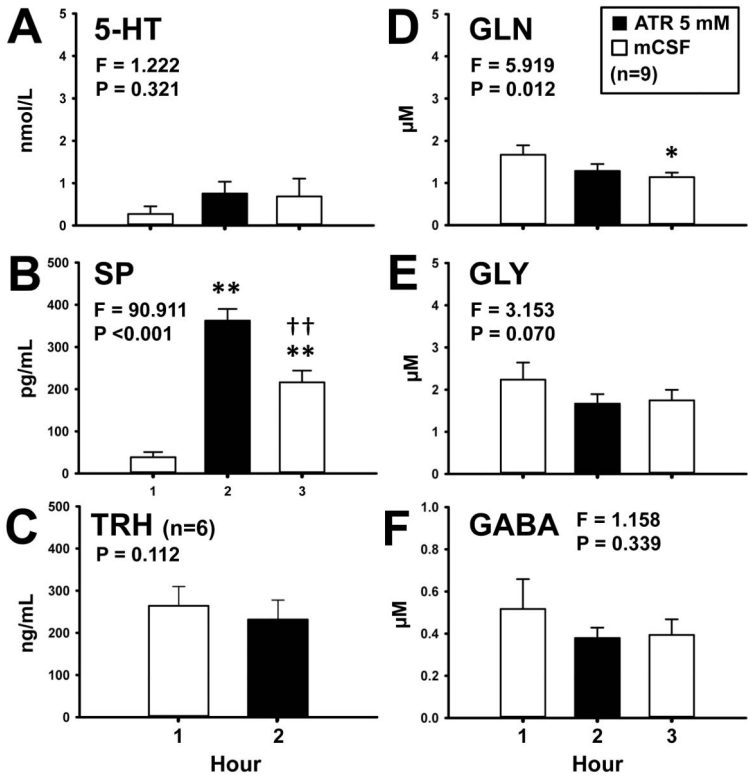



**Figure 4.**

Dialysis of 5 mM ATR in the day had no effect on the effluent concentrations of 5-HT (**A**), TRH (**C**), GLY (**E**), and GABA (**F**), but significantly increased SP (**B**) and significantly decreased GLN (**D**) concentrations. x-axis is hour of dialysis. White bars indicate dialysis of mCSF; black bars indicate dialysis of 5 mM ATR. F and P values from one-way RM ANOVAs (**A-B, D-F**, n=9) or paired t-test (**C**, n=6). *P<0.05, **P<0.001 vs. Hour 1, ††P<0.001 vs. Hour 2.

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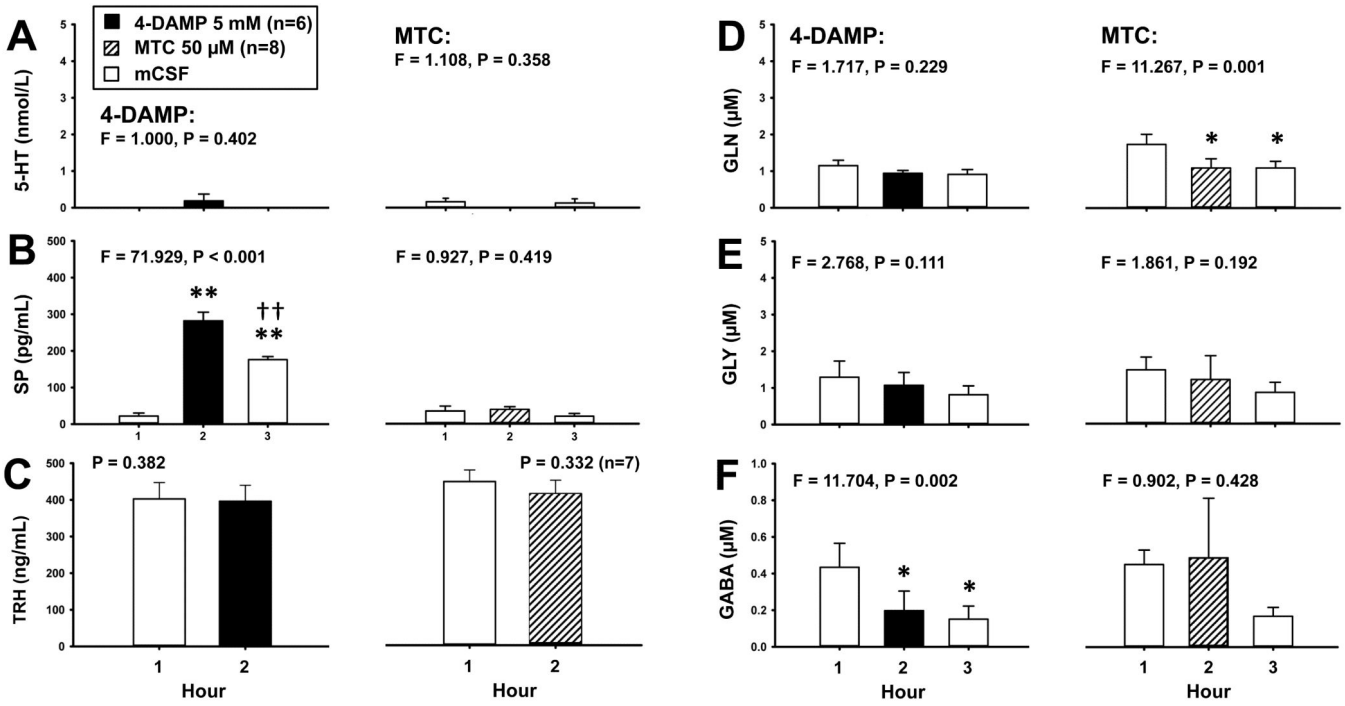



**Figure 5.**

Dialysis of either 50 µM MTC or 5 mM 4-DAMP had no effect on the effluent concentrations of 5-HT (**A**), TRH (**C**), and GLY (**E**); each compound either did not affect or significantly decreased GLN (**D**) and GABA (**F**) concentrations. While 4-DAMP significantly increased effluent SP concentration (**B**), MTC had no effect on SP levels. x-axis is hour of dialysis. White bars indicate dialysis of mCSF; black bars indicate dialysis of 4-DAMP; hatched bars indicate dialysis of MTC. F and P values from one-way RM ANOVAs (**A-B, D-F**, MTC n=8, 4-DAMP n=6) or paired t-test (**C**, MTC n=7, 4-DAMP n=6). *P<0.05, **P<0.001 vs. Hour 1, ††P<0.001 vs. Hour 2.

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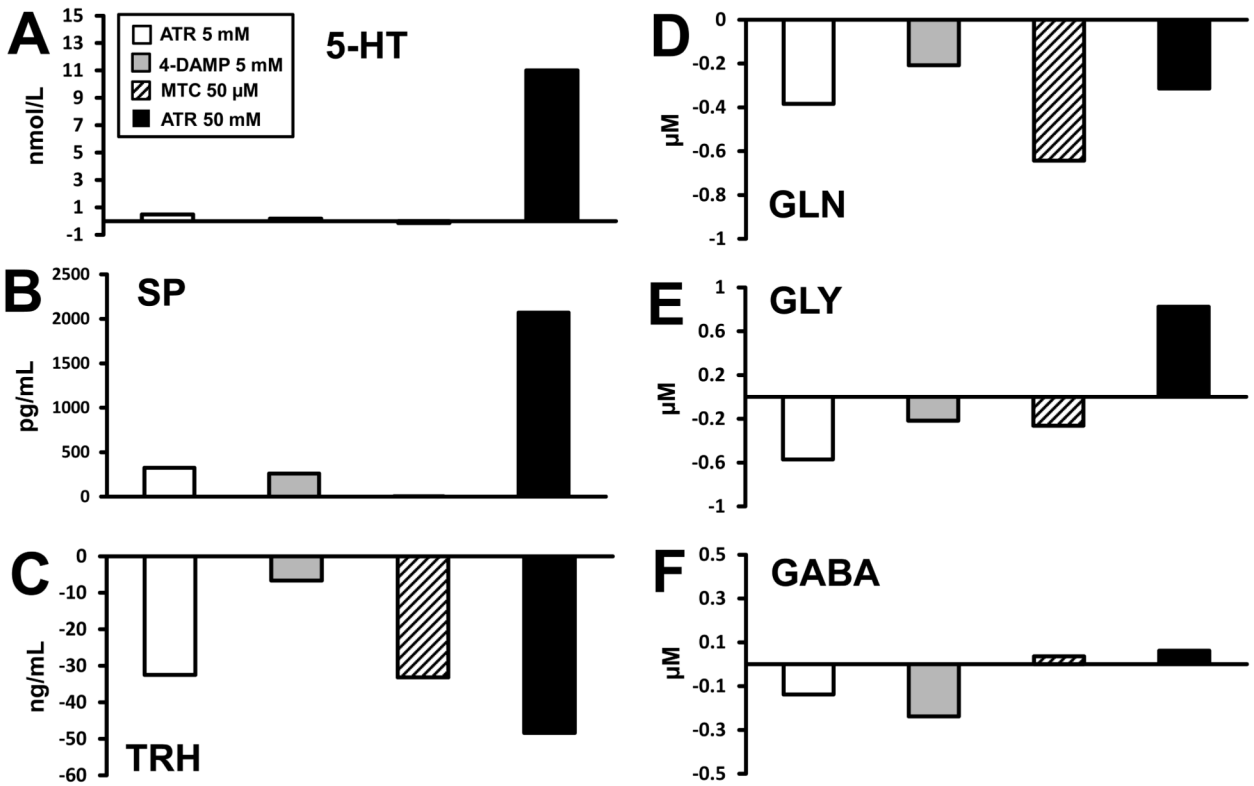



**Figure 6.**

Summary of changes in daytime neurochemical concentrations with dialysis of mAChR antagonists (see ***Sections 3.2, 3.3*** and **Figs. 4, 5** for results of statistical analyses). Each panel shows the change in effluent concentration of each neuromodulator between Hour 1 and 2 in response to dialysis of 5 mM ATR (white), 5 mM 4-DAMP (grey), 50 µM MTC (hatched), or 50 mM ATR (black) (22). The higher dose of ATR consistently elicited the largest absolute changes in neurochemical concentration, most notably in 5-HT, SP, GLY, and GABA. Inhibitory neurotransmitters increased with dialysis of 50 mM ATR, while they largely decreased with dialysis of 5 mM ATR or specific mAChR antagonists.