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Anatomical Localization of Membrane Progesterone Receptors in Brainstem Respiratory Areas

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Résumé

La progestérone est un stimulant respiratoire, mais l'implication des différents types de récepteurs de progesterone dans ces effets n'est pas connue. La progesterone possède 2 types de récepteurs: des récepteurs nucléaires (nPR) et des récepteurs membranaires (mPR). nPR est exprimé dans le noyau du tractus solitatrius (NTS), un des noyaux du tronc cérébral impliquées dans le contrôle respiratoire. En revanche on ne sait pas si les mPR sont exprimés dans ce noyaux. Nous avons effectués des marquages immunohistochimiques sur des coupes de tronc cérébral de souris adultes (mâles). Une coloration dense a été trouvé pour les récepteurs mPRα et mPRβ dans les NTS caudal et rostral, le noyau vague et le noyau hypoglosse. mPRα est exprimé dans les corps cellulaires, tandis que mPRβ se trouve dans les fibres neuronales. Des experiences de double marquage en immunofluorescence montrent une co-localisation de mPRα et mPRβ dans des neurones exprimant la tyrosine hydroxylase, enzyme de synthèse des catécholamines dans le NTS. De plus, la 3β-hydroxystéroïde déshydrogénase, impliquée dans la synthèse de progestérone, est également exprimé dans le NTS. Ces résultats suggèrent que mPRα et mPRβ peuvent jouer un rôle dans le contrôle respiratoire et que la synthèse de progestérone locale peut moduler la fonction respiratoire.

Abstract

Progesterone is a potent respiratory stimulant, but the implication of progesterone receptor subtypes on this effect are not known. Progesterone has two main types of receptors, the "classical" nuclear receptor, and the recently identified membrane progesterone receptors. While it has been shown that the nuclear progesterone receptor is expressed in the nucleus tractus solitatrius, a brainstem nuclei involved in respiratory control, much less is known relatively to the expression of membrane progesterone receptors in this area. Accordingly, we used immunohistochemistry to determine the localization of membrane progesterone receptors (mPR) in respiratory-related areas in the brainstem of adult male mice. Serial slices were incubated with antibodies against alpha and beta mPR (mPRα and mPRβ). A prominent staining for mPRα, and mPRβ appeared in caudal and rostral parts of the nucleus tractus solitarius (NTS), X and XII nuclei, but while mPRα stained cell bodies, mPRβ stained fibers. With double fluorescence labeling and confocal microscopy we showed that mPRα is co-localized in catecholaminergic neurons (TH+) in NTS. mPRβ is expressed in TH+ fibers in these regions. Furthermore, 3β-hydroxysteroid dehydrogenase (3β-HSD), which is involved in progesterone synthesis, was also densely expressed in these regions. These results suggest that mPRα and mPRβ may play a role in respiratory control, and that local progesterone synthesis may modulate respiratory function.

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Introduction

The main function of the respiratory system is to ensure adequate exchange of arterial gases through the lungs. Alveolar Oxygen (O_2) is diffuses from the alveoli to the blood, while carbon dioxide (CO_2) diffuses from the blood to the alveoli, and is exhaled during expiration. Therefore, the main function of pulmonary ventilation is to keep partial pressures of oxygen and carbon dioxide in arterial blood at their optimal levels. Oxygen serves as the final electron receptor in the mitochondrial respiratory chain, which produces energy in the form of Adenosine Tri-Phosphate (ATP), a molecule with high-energy covalent links. CO_2 is a waste product of oxidative processes in the cells and needs to be continuously released from the body in order to avoid toxic hypercapnic (high CO_2) conditions.

The regular contractions of the diaphragm and the resulting movements of the thoracic cage ensure the lung movements necessary for convection of air and proper exchange between alveolar and exterior gases. Generation of the respiratory pattern depends on the activity of a neural network localized in the pre-Bötzinger complex (PBC) in the ventrolateral medulla of the brainstem. The respiratory pattern produced by these neurons is sent to the spinal cord for relay to motoneurons, which then innervate respiratory muscles, such as the diaphragm and intercostal muscles (Pena 2006). This motor system receives afferent fibers from several elements of the peripheral and central nervous system (Feldman 2003) and its activity is modulated by numerous elements. In particular, sex and sex-hormones are two major determinant of the activity of the respiratory system (Bayliss 1992).

1   Sex Steroids and Respiratory Control

The current evidence suggests that hormones have a relevant role in the regulation of breathing via several mechanisms. They may stimulate breathing at the level of the central nervous system or at peripheral chemoreceptors (i.e progesterone) or by altering the basic metabolic rate, also indirectly affecting breathing (i.e thyroid hormones). The multiple locations of progesterone, estrogen, androgen, prolactin, and human chorionic gonadotropin/luteinizing hormone receptors suggests that these hormones may act in various tissues, including the trachea, lungs, brain, and brainstem, and thereby have possibly involvement in breathing. Progesterone is believed to be a

pivotal role player in the regulation of respiration (Saaresranta 2002). If this is indeed the case, then how is progesterone implicated in key respiratory illnesses concerning humans, namely apneas?

The occurrence of apneas among humans is higher in males than females (Bixler E. et al., 2001), perhaps due to the effects of ovarian hormones such as progesterone. Previous research has effectively demonstrated that progesterone combined with estrogen treatment reduces apnea frequency in post-menopausal women from 15 to 3 episodes per hour (Pickett C. et al., 1989). The necessary question to then ask is: if progesterone does play a role in the regulation of respiration, through which mechanisms is it acting?

Ovarian steroids have been shown to act directly on peripheral chemoreceptors in adult mammals and increase carotid sinus nerve activity, resting minute ventilation, and hypoxic chemo-responsiveness (Bayliss D. et al., 1990; Bayliss D. et al., 1987). The carotid sinus nerve activity has been shown to increase following chronic elevation of progesterone levels in cats (Bayliss D. and Millhorn D. 1992). Progesterone has been found to stimulate resting minute ventilation by acting on the following central respiratory regions: NTS, medulla oblongata, and hypothalamic nuclei structures (Behan M. and Kinkead R. 2012; Hannhart et al., 1990; Tatsumi K. et al., 1997).

In a study of healthy men, administration of medroxyprogesterone increased minute ventilation as well as the hypoxic and hypercapnic ventilatory responses (Skatrud J. et al., 1978). In rats, progesterone stimulation of resting minute ventilation has been shown to involve a mechanism that reduces the inhibitory role of D2 dopamine receptors on breathing in the carotid bodies (Josephn 2002). Progesterone may be playing a role in respiratory regulation through selective expression of progesterone receptors in the elements of the central and peripheral nervous system involved in respiratory regulation. While PRs have been localized in the brainstem, hypothalamus and carotid bodies, in-vivo results obtained in mice (see Fig. 1) indicate that these receptors only partially contribute to the sex-specific effect on respiratory control (Joseph 2012). Since not all effects of progesterone are explained by the classical receptor, this steroid hormone may be exerting its effects on respiration via a signaling pathways (i.e - mPR) at the membrane level (Zhu 2003, Brinton 2008).

1.1 Importance of Sex Hormones in Respiratory Control

In addition to being at lower risk for the development of sleep apneas, it is well known that women have decreased alveolar and arterial pressures of CO_2 during pregnancy (a period which exhibits high circulating levels of sex hormones; Behan M. and Wenninger J. 2008). Progesterone plays a pivotal role during pregnancy: by stimulating the maternal respiratory drive it ensures adequate O_2 furniture to the fetus and washout of fetal CO_2 through the maternal circulation, (Soliz J. and Joseph, V. 2005). Cyclic fluctuations in ventilation also take place during the normal menstrual cycle. For example premenstrual asthma has been strongly linked to progesterone levels at the end of the menstrual cycle. Reduced progesterone levels are a great risk factor for premenstrual or menstrual asthma whereas increased levels of progesterone during pregnancy lead to an alleviation of symptoms in asthma patients. Progesterone's may be rendering its effects through the potentiation of catecholamines on bronchial smooth muscles (Mirdal G. et al., 1998). Thus there is abundant indication as to the importance of sex hormones as key modulators of the activity of the respiratory system (Behan M. and Wenninger J. 2008). Hyperventilation during pregnancy has long been linked to heightened progesterone levels. Hyperventilation and decreased arterial pressure of CO_2 are seen in the luteal phase of the menstrual cycle when progesterone levels peak. Treatments with synthetic progesterone were shown to increase minute ventilation and the ventilatory response evoked by decreased arterial pressure of oxygen in healthy men. Over the past decades evidences have accumulated suggesting that progesterone acts at several levels of the respiratory control system, including the peripheral chemoreceptors and areas of the central nervous system that contribute to respiratory regulation. Estrogen also has some effects on respiration and decrease sleep disorders in women on estrogen monotherapy after menopause. These effects are believed to be a consequence of estrogens upregulation of the expression of progesterone receptors (Behan M. and Wenninger J. 2008).

In another study conducted by Lefter R. et al., (2007 & 2008), it was also demonstrated that postnatal treatment with progesterone, and progesterone + estradiol enhanced the hypoxic ventilatory response in newborn male rats, showing that progesterone is also effective in newborn mammals.

1.2   Peripheral chemoreceptors

The peripheral chemoreceptors have the job of sensing changes in the partial pressures of O_2 and CO_2 pressures of the arterial blood. The chemoreceptors send information via afferent neurons in the carotid sinus nerve, a branch of the glossopharyngeal nerve, to the brainstem. The inputs from peripheral chemoreceptors are mainly localized on the dorsal portion of the brainstem, in the nucleus tractus solitaries (Finlay J. and Katz D., 1992). This information is integrated by a specific neural network, which modulates the activity of the respiratory network. The end result is regulation of lung movement patterns via motoneurons (effectors) and phrenic nerve activity.

1.3   Carotid and aortic bodies

In 1868, Pflüger discovered that hypoxia increased ventilation, thus began the search for an O_2 sensitive receptor in the periphery and brain (Pflüger E. 1868). Heymans observed that ventilation increased when there was a reduction in O_2 content passing through the carotid artery bifurcation, for which he was awarded the Nobel Prize in 1938 (Heymans C. et al., 1931). He proceeded to identify the localization and physiological significance of chemosensitive receptors known as the carotid bodies, found in the carotid sinus regions. The search for the O_2 chemosensor was believed to be finalized until it was observed that carotid body denervation followed by hypoxemia still produced an increase in respiration. This increase in respiration was lost once the vagus nerve, which the aortic body nerve afferents are linked to, was cut. This was indicative of an extra-carotid chemoreceptor for Heymans. The precise physiological location of the aortic bodies however, remained unknown (Lopez-Barneo J. et al., 2009).

Comroe who was initially recognized for demonstrating that the carotid bodies responded to drops in O_2 pressures (rather than O_2 concentrations), was also intrigued by the fact that a residual ventilatory response to hypoxia was still present when the carotid body was no longer intact. To address this curiosity, he designed an experiment to show that hypoxia excited respiration in a physiological setting without influence from carotid body chemoreceptors and this excitation could be induced by stimulating cell bodies along the aortic arch. His intimate understanding of local anatomy of afferent nerves and blood supply to cell bodies, thanks to his clinical knowledge as a surgeon, was necessary for the strategic experimental design which led

to the discovery of the precise neural and circulatory anatomy of the aortic bodies, as well as their physiological response to hypoxia (Comroe J. 1939).

Hypoxia is the main natural stimulus of the carotid bodies. When O_2 partial pressures fall below baseline levels (100 mm Hg at sea level), afferent chemoreceptor discharge and ventilation rise in an exponential manner. This response is rapid, given a constant stimuli from hypoxia (Kumar P. 2009). The peripheral chemoreceptors are able to sense low levels of arterial pressure of O_2 (PaO_2) through a subset of specialized cells named "glomic cells". When PaO_2 decreases the glomic membrane K+ channels are closed, resulting in membrane depolarization, followed by calcium influx, a release of neurotransmitters at the synapse from presynaptic vesicles and excitation of afferent carotid sinus nerve (López-Barneo 2008). So far the identity of the molecular components of oxygen sensing system able to close K+ channels remains controversial, but mechanisms involving heme-oxygenase 2, Adenosine-Mono-Phosphate Kinase, and H_2S have been evocated (Prabhakar 2006, Ortega-Sáenz 2007, Olson 2011).

Hypoxia was shown to increase extracellular glutamate in the caudal nucleus of the NTS when Ve rose in unanesthetized rats. This was eliminated once the carotid body was denervated. It was also demonstrated that injecting glutamate to the NTS increased Ve. These three facts indicate that carotid sinus nerve terminals mainly release glutamate within the nucleus tractus solitarius (Burton 2000). This results in a stimulation of breathing, which increases PaO_2 and reduces $PaCO_2$. For these reasons, the NTS is considered as the central relay for the hypoxic ventilatory response.

1.4   Impact of steroid hormones on peripheral chemoreceptors

To test the hypothesis that progesterone enhances carotid body responsiveness during hypoxia, castrated male adult cats were studied for their hypoxic ventilatory responses and carotid body neural output (CBNO) after treatment with progesterone (Hannhart 1990). Estrogen treatment was used in combination with progesterone treatment in order to induce expression of the progesterone receptor.

Combined chronic (1 week) treatment of estrogen and progesterone raised alveolar ventilation as well as the hypoxic ventilatory response but when the treatments were given separately (Fig. S1),

minimal effects were observed (Hannhart B 1990). CBNO responsiveness to hypoxia was greater in the two groups receiving progesterone treatment than the groups simply receiving estrogen or placebo. Combined treatment of progesterone with estrogen did not increase CBNO responsiveness to hypoxia compared to treatment with just progesterone. What was observed however was that cats treated with estrogen and progesterone had greater ventilation than those treated with progesterone alone during progressive hypoxia (Hannhart 1990, Tatsumi 1991). Since combined treatment of estrogen and progesterone didn't increase CBNO relative to progesterone treatment alone, it was postulated that estrogen could be working in the CNS to modulate peripheral sites in order to raise ventilation as well as HVR (Hannhart 1990). Since estrogen treatment resulted in enhanced CNS transduction and progesterone raised CBNO responsiveness during hypoxia, it can be said that combined, these hormones act at peripheral and central sites in order to stimulate HVR (Hannhart 1990). In addition when ovarian function was eliminated by ovariectomy in female cats, the ventilatory and carotid sinus nerve responsiveness to hypoxia declined (Tatsumi 1997). It has also been demonstrated that, in addition to estradiol, progesterone reduces dopamine synthesis in the carotid body (Joseph 2002). Since dopamine is mainly considered as an inhibitory transmitter in the carotid body, this further support the hypothesis that progesterone acts on peripheral chemoreceptor to stimulate breathing.

In addition to these effects on peripheral chemoreceptors, progesterone also acts on the central nervous system to stimulate breathing.

1.5   Impact of steroid hormones on central areas of the respiratory control system.

To investigate the matter of progesterone's involvement at the central nervous system level, researchers examined CNS chemosensitivity by looking at neuronal response to progesterone during hypoxia on brainstem slices from adult male rats (Pascual 2002). Hypoxia caused a decline in spontaneous activity in about 2/3 of neurons recorded in the NTS. In the remaining neurons, hypoxia increased spontaneous activity. Applying progesterone at physiologically relevant concentrations (1 µM) reversed these effects of hypoxia on spontaneous activity of all recorded neurons. Due to the speed of progesterone's action, which requires between 2 and 3 minutes for effects to occur, the authors suggested that progesterone was using a non-genomic mechanism of action to interact with the cellular signaling component of hypoxia (Pascual 2002).

1.6   Molecular mechanisms by which progesterone stimulates breathing

Progesterone is a steroid hormone that plays a key role in feminine physiology. Progesterone works to prepare and maintain the gestation phase by acting on reproductive tissues and the brain. Progesterone is synthesized in the gonads, adrenal glands and placenta (during gestation). It is also produced in females and in males in the central and peripheral nervous system by neurons and glial cells, which makes it a neurosteroid. Progesterone also has multiple non-reproductive function in the CNS that include the regulation of: cognition, mood, inflammation, mitochondrial function, neurogenesis, protection and recovery from traumatic brain injury. Progesterone's neural responses are mediated by an array of receptors which include the classical progesterone receptor (PR), the membrane progesterone receptor (mPR) and progesterone receptor membrane component 1 (PGRMC1 also known as 25 Dx; Brinton 2008). The classical progesterone receptor is a member of the super-family of the steroid receptors, which upon activation by its ligand, is translocated to the nucleus, binds DNA, to enhance or repress transcription of its target genes. Membrane receptors also bind progesterone, but they act as modulators of intracellular second messenger pathways, and might allow rapid responses to hormone exposure (Hammes 2007). Ultimately, second messenger pathways may activate specific transcription factors and also modulate genomic expression. While the localization of the classical progesterone receptors in the brain is well described, little is known about membrane receptors. In particular, the localization of these receptors in areas involved in respiratory control is not known.

1.7   Classical Progesterone Receptors

Mechanisms of action for progesterone can take place through specific intracellular progesterone receptors (PR) which belongs to the classical nuclear receptor family. The classic PR was characterized in the 1970's and has since been localized in the hippocampus, cortex, hypothalamus and cerebellum (Dressing G.E. et al., 2011). Two types of classical receptors exist for progesterone: PR-A and PR-B, which are 80 and 120 kDa long respectively. PR-A has a truncated N-Terminal. Once PR binds progesterone, the receptor-ligand-complex enters the nucleus to bind to its transcription element on DNA (Brinton R. et al., 2008).

The nuclear receptor functions as a transcription factor, acting to activate or repress expression of target genes (Brinton R. et al., 2008). Progesterone's effects on these two receptors are regarded as classical genomic mechanisms (Behan M. and Wenninger J. 2008). However, many effects can't be explained by classic mechanisms of steroid action, which are relatively slow and involve transcription and translation. For this reason, we look to non-genomic mechanisms of action, which may be able to explain rapid progesterone action (Delville Y. 1991). Non-genomic mechanisms of action involve the expression of specific membrane progesterone receptors as well as modification to the allosteric configuration of $GABA_A$ receptors and effects on: ion transport, neurotransmitter release, protein kinase activity, nuclear translocation (Lambert J. et al., 2009; Dressing G.E. et al., 2011; Thomas P. 2008).

1.8   Membrane Progesterone Receptors

In 1996, a putative membrane receptor of progesterone called 25 Dx different from PR was discovered with 194 amino acid sequence and a single membrane-spanning domain isolated and cloned from porcine liver (Guennoun R. et al., 2008). In 2003, a new mPR, unrelated to PR or 25 Dx, was cloned from fish oocytes by Zhu et al (2003). This receptor met the criteria of true membrane receptors including the presence of a trans-membrane domain, expression in steroid targeted tissues, selective steroid binding, regulation of intracellular signaling pathways and regulation by hormones (Dressing G. et al., 2011). The mPR is a protein with seven trans-membrane domains, a characteristic of G-protein coupled receptors. The mPR belong to a large and ubiquitous family of proteins in prokaryotes and eukaryotes called progestin and adiponectin receptors (PAQR). Distinct mPR, encoded by specific genes have been described to date as mPRα, mPRβ, mPRɣ, mPRδ, and mPRε (Fernandes 2008, Pang 2012).

1.9   Progesterone Biosynthesis & PR Expression

The brain was first observed to synthesize neurosteroids de novo when pregnenolone, the immediate progesterone precursor, was found in the brains of gonadectomized and adrenalectomized rats by Corpechot (1981). Steroid hormones are derived from cholesterol. The biosynthesis of progesterone is regulated by the expression of enzymes such as, cytochrome

P450 side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD; Micevych 2008).

In peripheral tissues such as the ovaries progesterone synthesis is regulated by follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH activate G-protein coupled receptors and their corresponding intracellular pathways that result in the genetic transcription of enzymes, which regulate progesterone synthesis. Progesterone synthesis can be regulated rapidly (non-genomic) and non-rapidly (genomic). Genomic regulation requires genetic transcription and translation and thus requires more time. We know little about the rapid regulation of progesterone synthesis but researchers have speculated that it involves the membrane receptor (Micevych 2008).

The rate limiting step in progesterone synthesis is cholesterol transport across the inner mitochondrial membrane by steroid acute regulatory protein (StAR). Upon entering the inner mitochondrial membrane, cholesterol is enzymatically cleaved by P450scc into pregnenolone (Micevych 2008).

Steroidogenesis varies from cell to cell in the CNS. For example, astrocytes have been shown to be the most productive in terms of steroid synthesis. They express P450scc, 3β-HSD, StAR, and are able to synthesize pregnenolone, progesterone, estrogen and testosterone. Oligodendrocytes on the other hand express P450scc, 3β-HSD and can synthesize pregnenolone, while neurons express all the different types of steroid enzymes. However, the primary steroids produced by astrocytes and oligodendrocytes are progesterone and pregnenolone respectively (Fig. S2; Micevych 2008).

1.10 Hypothesis and objectives

From a clinical perspective, progesterone is interesting because it decreases the occurrence of apneas, one of the leading causes of respiratory morbidity throughout life (Bixler E. et al., 2001). The effect of progesterone on respiratory control is thought to explain the fact that adult woman have a lower occurrence of sleep apneas than men (Behan M. and Wenninger J. 2008; Bixler E. et al., 2001; Soliz J. and Joseph V. 2005).

The mechanisms underlying progesterone's effects are poorly known. In particular, the implication of different progesterone receptor subtypes has not been directly addressed. A preliminary study in our laboratory has demonstrated that in adult male and female PR knock-out mice (PRKO), minute ventilation is reduced compared to their wild type counterparts both under normoxia and in response to acute hypoxia (Fig. S3). However, wild-type female mice had higher minute ventilation than males and interestingly enough, this sex specific difference was also observed in PRKO mice. Thus PR contributes to mediating some of the stimulatory effects of progesterone on ventilation, but it does not explain the sex specific differences (Joseph 2012). mPR which we discussed earlier has been described and firmly identified as of 2003. It is plausible to think that the gender specific effects in respiration not accounted for by PR could be due to progesterone activity at the mPR (Brinton R. et al., 2008).

Given these findings, the hypothesis of my master research is that **mPRs are expressed in respiratory related structures of the brainstem.** Thus my primary objective was to search for the presence of membrane progesterone receptors in the brainstem. Presence of mPR's in the brainstem could account for the molecular mechanism through which progesterone is acting to modulate respiration not accounted for by the PR.

2   Materials and Methods

2.1   Tissue preparation for Immunohistochemistry

Experimental procedures used were approved by the ethics committee for animal use and care at Laval University. We used a total of 4 mice between the ages of 3-6 months for immunohistochemistry and 4 for immunofluorescence examination Animals were anesthetized intra-peritonally with ketamine before the chest cavity was opened and the heart was perfused with ice-cold PBS solution. After perfusion, the brains were removed and placed on dry ice immediately and stored at -80 ˚C. Using a cryostat, the brainstem was cut coronally at 15 µm and sections were collected on Superfrost slides. Slides were then stored at -80 ˚C. Prior to immunostaining, tissues were post-fixed in 4% para-formaldehyde in PBS solution for 15 minutes with gentle agitation.

2.2   Immunohistochemistry

The antibodies used have been well recognized for binding specificity (see Table 1). All steps performed in immunohistochemistry were accompanied with gentle agitation. Tissues were washed 3 x 5 minutes in PBS after post fixation and between every consecutive step. Endogenous peroxidase activity was blocked for 30 minutes with 0.3% H_2O_2 diluted in PBS. Non-specific binding was blocked for 4 hours in blocking solution (2% non-fat skim milk and 0.3% Triton diluted in PBS). Tissues were then incubated 18 or 36 hours at 4 ˚C with the primary antibody in blocking solution. Secondary biotinylated antibody incubation (Table 1) with blocking solution was performed for 2 hours before 30 minutes incubation using the Vectastain Elite avidin-biotinylated enzyme complex (ABC) method. This antigen-antibody complex is then visualized using diaminiobenzidine peroxidase (DAB – Vector Laboratories). Sections were then dehydrated using alcohol and cleared with xylene before coverslipping. Similar caudal and rostral sections were observed for all animals used. Although staining intensity varied between protocols, this did not interfere with the analysis as length of the staining procedure was recorded and shown to correlate with staining intensity. Intensity scores in Tables 2 and 3 were given based on comparatively consistent degrees of staining throughout conducted experiments (NCCLS 1999; Mukai K. and Rosai J. 1981).

Antigen	Primary Antibody	Secondary Antibody Immunohistochemistry	Secondary Antibody Immunofluorescence
mPRα – consists of an extracellular N-terminus, intracellular C-terminus and seven transmembrane domains. G protein-coupled receptor.	Affinity purified goat polycolonal antibody for peptide mapping derived from an internal region of mPRα of human origin (1/200) -- Santa Cruz Biotechnology INC.	Biotinylated horse anti-goat (1/200) – Vector Laboratories	Donkey anti-goat TexasRed (1/200) – GeneTex
mPRβ – 354 amino acid G protein-coupled receptor expressed in brain and spinal cord. Has an extracellular N-terminus, intracellular C-terminus and seven transmembrane domains.	Affinity purified goat polycolonal antibody for peptide mapping derived from the N-terminus of mPRb of human origin (1/200) -- Santa Cruz Biotechnology INC.	Biotinylated horse anti-goat (1/200) – Vector Laboratories	Donkey anti-goat TexasRed (1/200) – GeneTex
TH – Recombinant protein, C-terminal portion of mouse sequence .Rate-limiting enzyme in catecholamine synthesis, involved in converting tyrosine to DOPA.	Mouse monoclonal antibody. Cross-reactive with mouse, human and rat (1/200) -- GeneTex	Not Applicable	Rabbit anti-mouseFluorescein (1/200) – Jackson ImmunoResearch Laboratories

Table 1 - List of antibodies and their antigens used to perform immunohistochemistry of mPRα, mPRβ and TH analysis.

2.3   Double Immunofluorescence Staining

The double fluorescence staining protocol deviates from the Immunohistochemistry protocol after the primary antibody incubation. Since tissues are visualized using fluorescence from a confocal microscope under ultra-violet light, the need for the ABC method, DAB coloring and endogenous peroxide blocking is unnecessary. The tissues were washed and then incubated for 2 hours with a fluorescent secondary antibody against the primary animal host. After washing, tissues were cover slipped after 2 drops of 4',6-diamidino-2-phenylindole (DAPI) were applied. We used antibodies against the membrane PR (α and β), a tyrosine hydroxylase (TH) antibody (a marker of noradrenergic neurons, used as anatomical reference for NTS), as well as a nucleic acid staining agent which targets A-T rich regions of DNA, DAPI (SantaCruz). These primary antibodies were made visible using fluorescent secondary antibodies FITC green (488nm) for

TH, DAPI blue (550nm) and Texas red (594nm). The visualization of the nucleus (DAPI) and cytoplasm of noradrenergic neurons (TH) was used to provide a context for anatomical localization of the progesterone receptors presence by staining catecholaminergic neurons.

3   Results

3.1   mPRα

mPRα immunoreactivity was observed to be strongest in the medial solitary tract (SolM), dorsolateral solitary tract (SolDL), intermediate solitary tract (SolIM), solitary tract (sol), area postrema (AP) and dorsal motor nuclei of the vagus cranial nerve (10N) of the adult mouse caudal brainstem using immunohistochemical analysis (Fig. 1). Light to medium immunoreactivity for mPRα was seen in the raphe obscurus (Rob), commissural solitary tract (SolC), ventral solitary tract (SolV), ventrolateral solitary tract (SolVL), lateral solitary tract (SolL), SolI and sub postrema (SubP).

mPRα showed consistent immunoreactivity in regions resembling the cell body of the neuron. The black arrows in Figure 1c and 1e point to the typical features observed in mPRα immunoreactive sites.

Figures 6 and 7 show immunoreactivity for mPRα and TH using the double immunofluorescence technique. Colocalization of the enzyme TH and mPRα is seen as hybrid of the two separate fluorescent dyes (Texas Red and FITC) resulting in an orange colour. Colocalization is indicated by the white arrows in the panels of 6a and 6b in the caudal region of the adult mouse brainstem. Neuronal nuclei were visualized blue by DAPI and are seen as the blue spotted structures present throughout merged panels. Colocalization occurred in the SolM, and 10N. TH staining seemed to thoroughly cover cytoplasms.   mPRα. Figure 7 shows a detailed view of the colocalization at 2400x. TH staining is strong and seen throughout the cell while mPRα staining is in the form of dotted clusters (bottom panel Fig. 7). Negative controls in 6c show what appears to be minor autofluorescence but otherwise no positive immunoreactivity (Neumann M. and Gabel D. 2002).

3.2   mPRβ

Structures bearing the greatest mPRβ immunoreactivity were found in the adult mouse caudal brainstem and included SolC, SolVL, SubP, sol, AP and 10N during immunohistochemical analysis (Fig. 3, Fig. 4). Light to medium immunoreactivity for mPRβ was seen in SolM, SolV,

interstitial solitary tract (SolI) and SolDL. Negative controls were void of immunoreactivity for mPRα (Fig. 1d and 1f) and mPRβ (Fig. 3d, 3f and 4d).

mPRβ immunoreacted mainly with fibers, projection axons and in the cell body region at 18 hours (Fig. 3) and 36 hours (Fig. 4) incubation with the secondary antibody. The black arrows in Figure 3c and 3e are indicative of long projection fibers immunoreactive for mPRβ. The dashed black arrow shows sites immunoreactive for mPRβ resembling cell bodies or synaptic boutons (Fig. 3c). The white arrow points to the highly noteworthy SolC staining by mPRβ which displayed extensive immunoreactivity throughout in the form of a column (Fig. 3c). This column appears to contain a network of nerve fibers. The red arrow in Fig. 4b points to what resembles a synaptic bouton/large cell body linked to several strongly strained mPRβ axons and fibers.

Figures 8 and 9 show immunoreactivity for mPRβ and TH, as indicated with the white arrows. TH was colocalized with mPRβ in caudal region of the adult mouse brainstem which represented the AP, SolC, SolM and 10N. mPRβ demonstrated a particularly interesting staining pattern in addition to colocalization, by staining interneural axons positive for TH (white arrows in enlarged panels of Fig. 8a, 9a and 9d). The negative control in 8c displays no immunoreactivity for mPRβ or TH. A key finding from immunofluorescence analysis is the great degree of immunoreactivity for mPRα and mPRβ in neurons not expressing TH. This form of staining was the most commonly observed.

3.3   Neuron Types

Neurons were categorized by type in each structure in order to gain a functional understanding from their morphological characteristics. Neurons with small cell bodies and extensive axon collaterals were considered Type I neurons as seen with the white arrow in Fig. 3e. Neurons with large cell bodies and a long projection axons were identified as Type II and III neurons as seen by the black arrow in Fig. 1c (Kawai Y. and Senba E. 1999). Based on this system of categorization, the AP, SolC, sol, SolL and SubP all had type I neurons. Type II and III neurons were observed in the following structures: 10N, 12N, SolIM and SolVL. SolM, SolV and SolDL were the only regions where both types of morphological features were observed.

3.4   3β-HSD

3β-HSD, the enzyme which converts pregnenolone into progesterone was found in the same structures expressing the mPR except for SolIM and SolL (Fig. 4c). Greatest immunoreactivity for the enzyme was in SolC, SolM, SolV, SolVL, SolDL and sol. A white arrow indicates the typical staining for 3β-HSD in Fig. 4c.

3.5   Rostral NTS

The rostral NTS was also examined for mPRα (Fig. 2) and mPRβ (Fig. 5) immunoreactivity. Positive staining for mPRα was observed in 12N, and can be seen with the black arrow in Fig. 2c and 2e. SolM had strong immunoreactivity for mPRα and this is indicated via the dashed black arrow in Fig. 2c. 12N also displayed mPRβ positive staining which is seen in Fig. 3c and 3e (black arrow). Negative controls were essentially void of immunoreactivity (Fig. 5d and 5f) except for a faint staining in large cel bodies (Fig. 2d and 2f)

3.6   Summary of Staining

Table 2 and 3 summarize the localization and intensity of mPRα, mPRβ, 3β-HSD and TH expression as well as the types of neurons identified in the respiratory related structures of the caudal brainstem. Figure 10 provides a visual representation of mPRα and mPRβ immunoreactivity in respiratory related structures of the caudal brainstem based on varying intensity. The most intense staining was for mPRβ in SolC and the AP. Figure 11 provides a visual representation of where colocalization of TH and mPRα/mPRβ was observed.

Figure 12 demonstrates the similarity in staining morphology of mPRα seen using two separate techniques, immunohistochemistry and immunofluorescence. As we can see, mPRα is distributed throughout the cell body. Figure 13 demonstrates the similarity in staining morphology for mPRβ using the two techniques, which both indicate that mPRβ is primarily found on fibers and axonal projections and to a lesser degree on cell bodies. These observations are indicative mPRβ having greater cellular distribution than mPRα.

1a mPRα 100x

1b
Cu · cu · SolDL · SubP · cu · C
Cu · SolI · AP · sol
x · SolL · SolM
SolVL · 10N · SolC
SolV · CeCv · CC · CeCv · IR
SolIM · 12N · Ro
IRt · Ro · mlf · IR
mb · MdV · ROb · MdV

1c mPRα 200x
SolIM · SolM
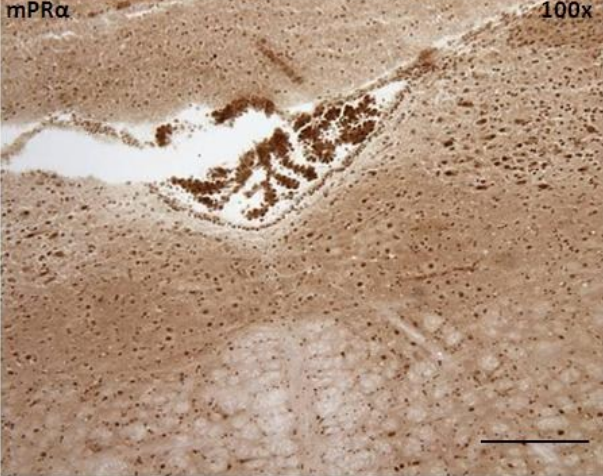10N

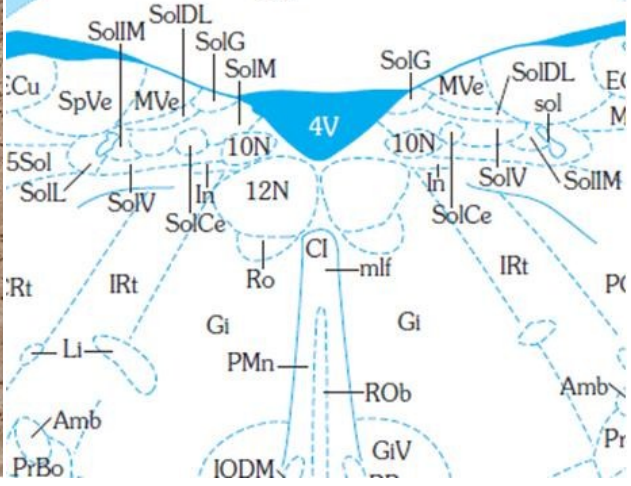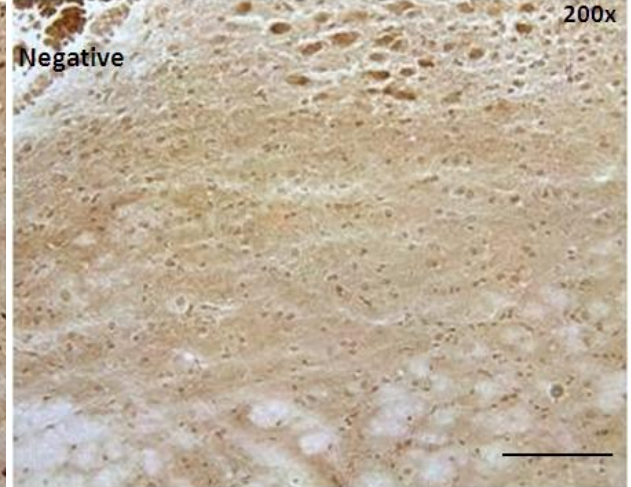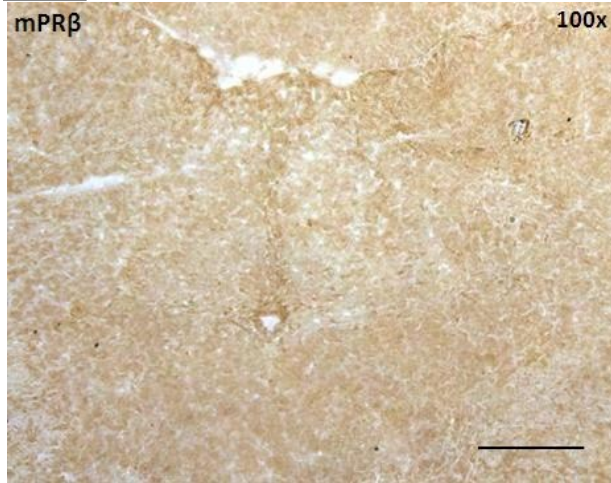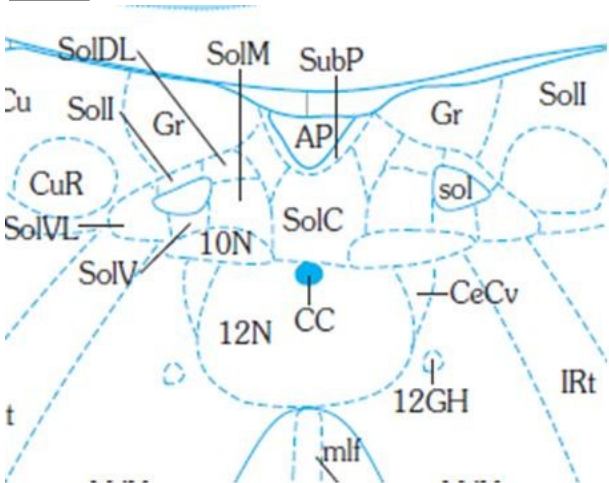**1d** Negative 200x

1e mPRα 200x
12N

1f Negative 200x
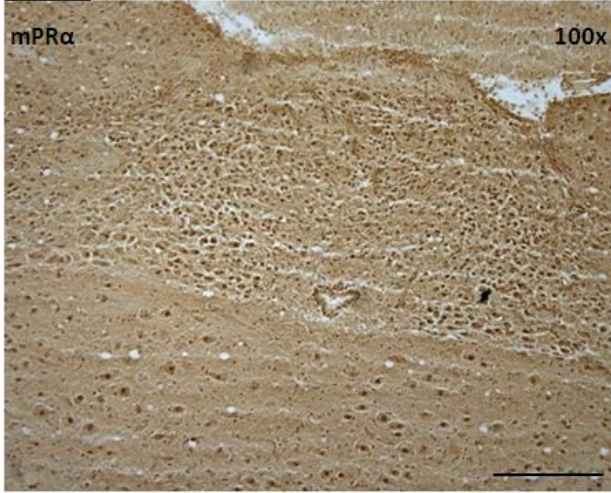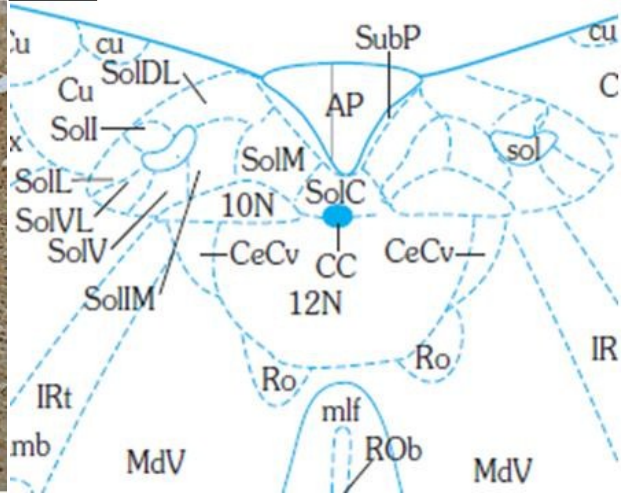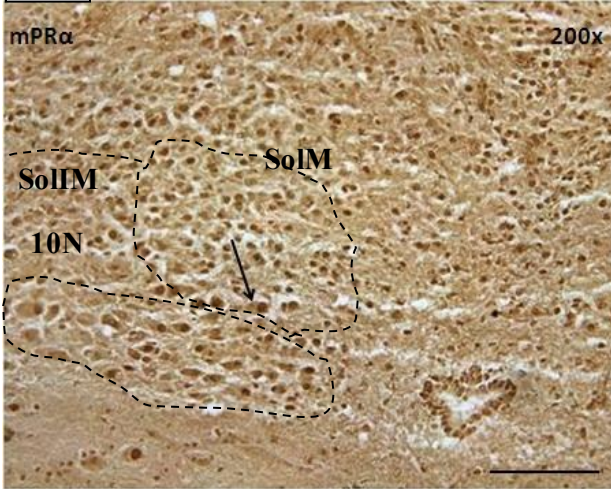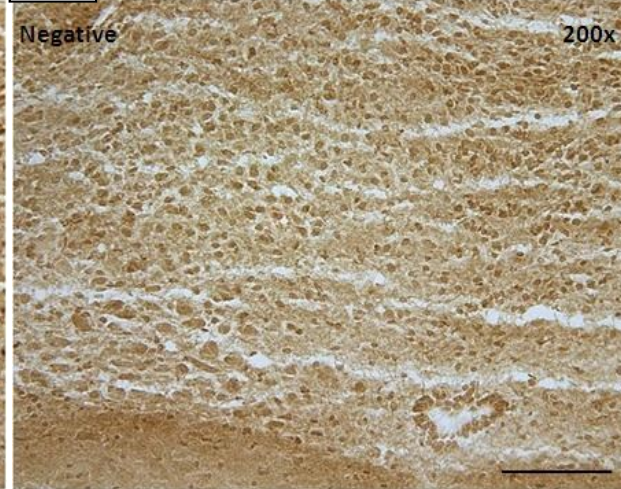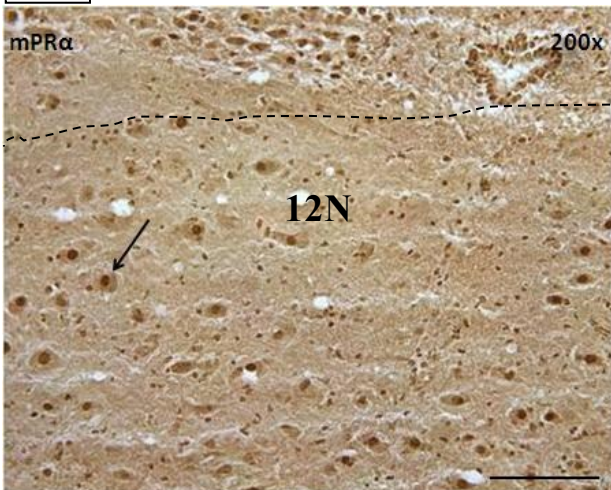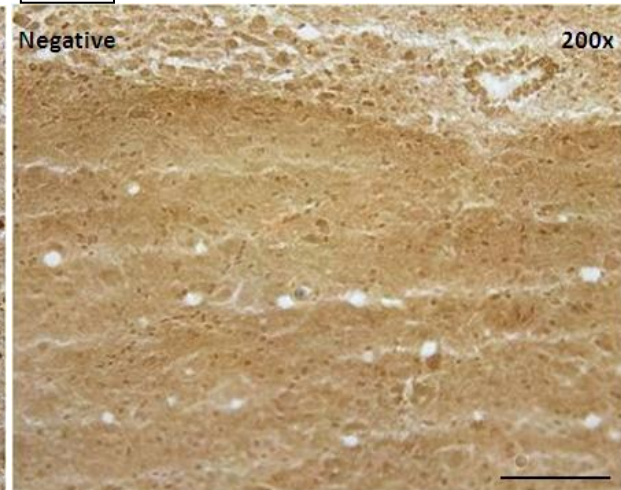
Figure 1 – **Immunohistochemistry for mPRα in the caudal region of the adult mouse brainstem.** It is estimated that this region coincides approximately with bregma – 7.56 mm. a) mPRα at 100x. b) Anatomy of adult mouse brainstem at bregma – 7.76 mm (Adapted from The Mouse Brain in Stereotaxic Coordinates, Third Edition). c) Dorsal view of mPRα at 200x. d) Dorsal view of negative at 200x. e) Ventral view of mPRα at 200x. f) Ventral view of negative at 200x. Scale bar at 100x = 100 μm and at 200x = 50 μm.

2a mPRα 100x
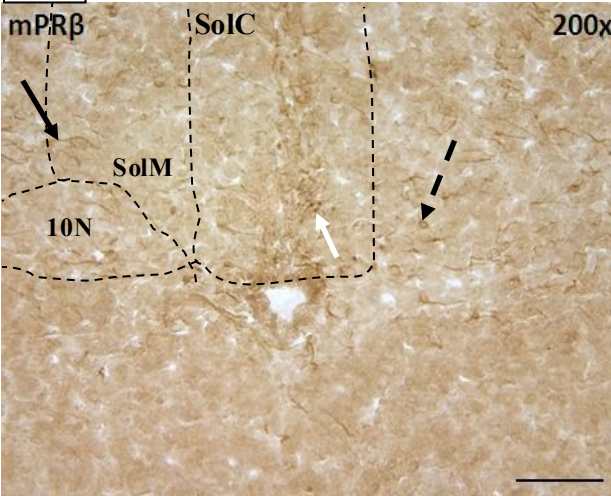
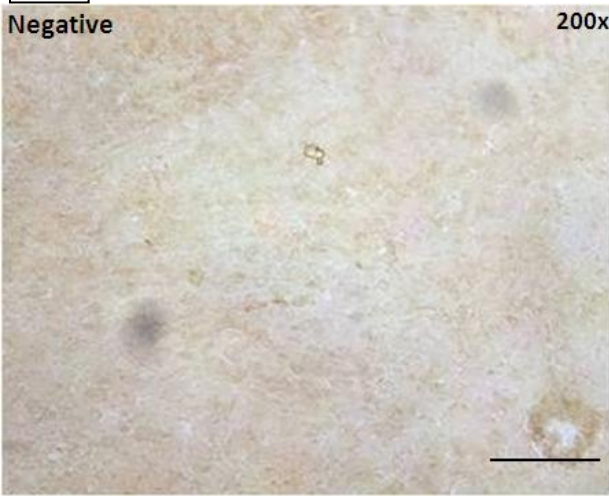**2b**
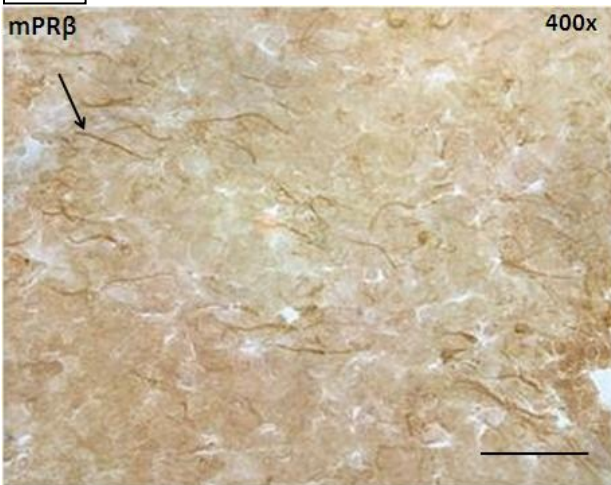
SolIM SolDL SolG SolG SolDL
ECu SpVe MVe SolM MVe sol M
5Sol 10N 10N
SolL SolV In 12N In SolV SolIM
SolCe SolCe
CRt IRt Ro Cl mlf IRt P(
Gi Gi
Li PMn ROb Amb Pr
Amb GiV
PrBo IODM
4V

2c mPRα 200x
SolM
12N
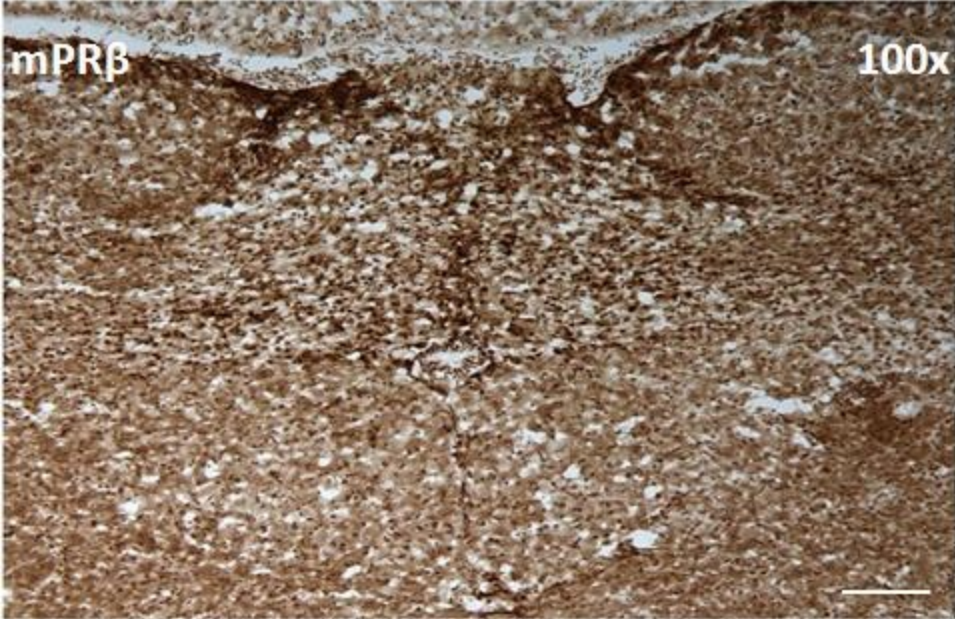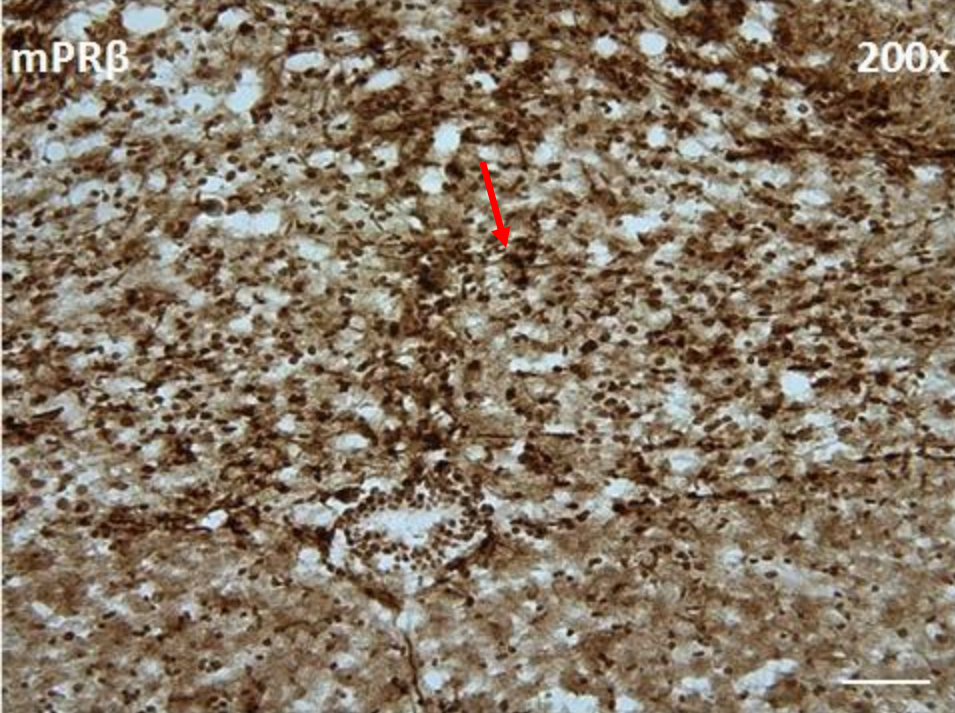
2d Negative 200x

2e mPRα 200x

2f Negative 200x
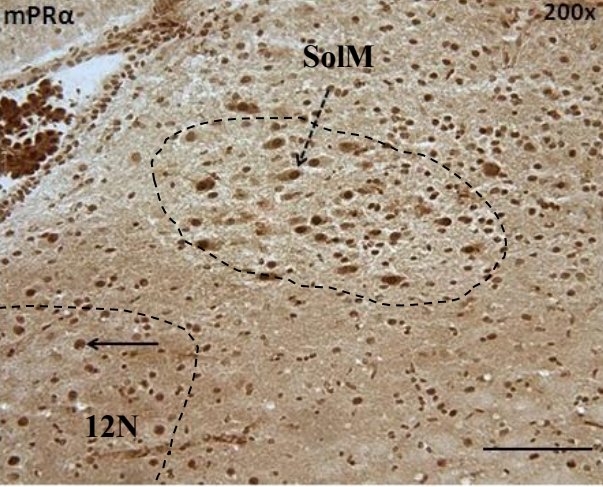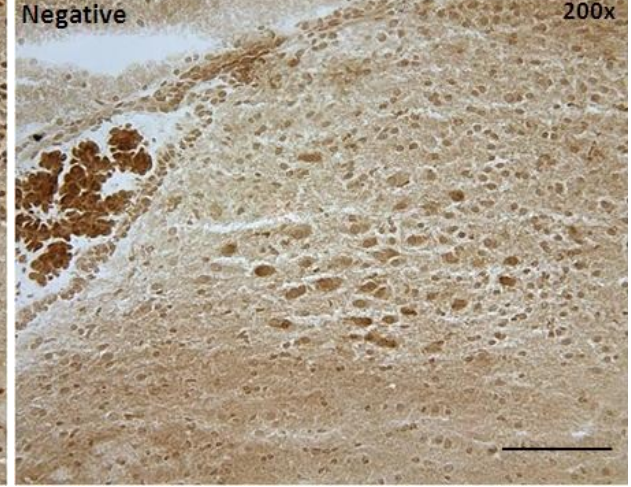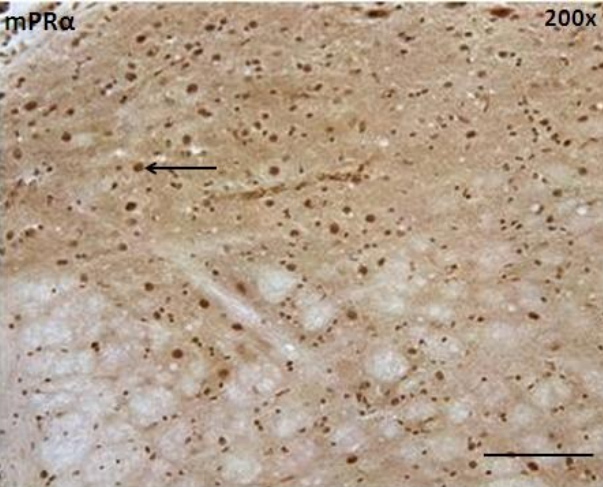
Figure 2 – **Immunohistochemistry for mPRα in the rostral region of the adult mouse brainstem. It is estimated that this region coincides approximately with bregma – 7.08 mm.** a) mPRα at 100x. b) Anatomy of adult mouse brainstem adapted from The Mouse Brain in Stereotaxic Coordinates, Third Edition. c) Dorsal view of mPRα at 200x. d) Dorsal view of negative at 200x. e) Ventral view of mPRα at 200x. f) Ventral view of negative at 200x. Scale bar at 100x = 100 μm and at 200x = 50μm.

3a

mPRβ   100x

3b

SolDL   SolM   SubP

Cu   SolI   Gr   AP   Gr   SolI

CuR   SolC   sol

SolVL   10N

SolV   CeCv

12N   CC

12GH   IRt

mlf

3c

mPRβ   SolC   200x

SolM

10N

3d

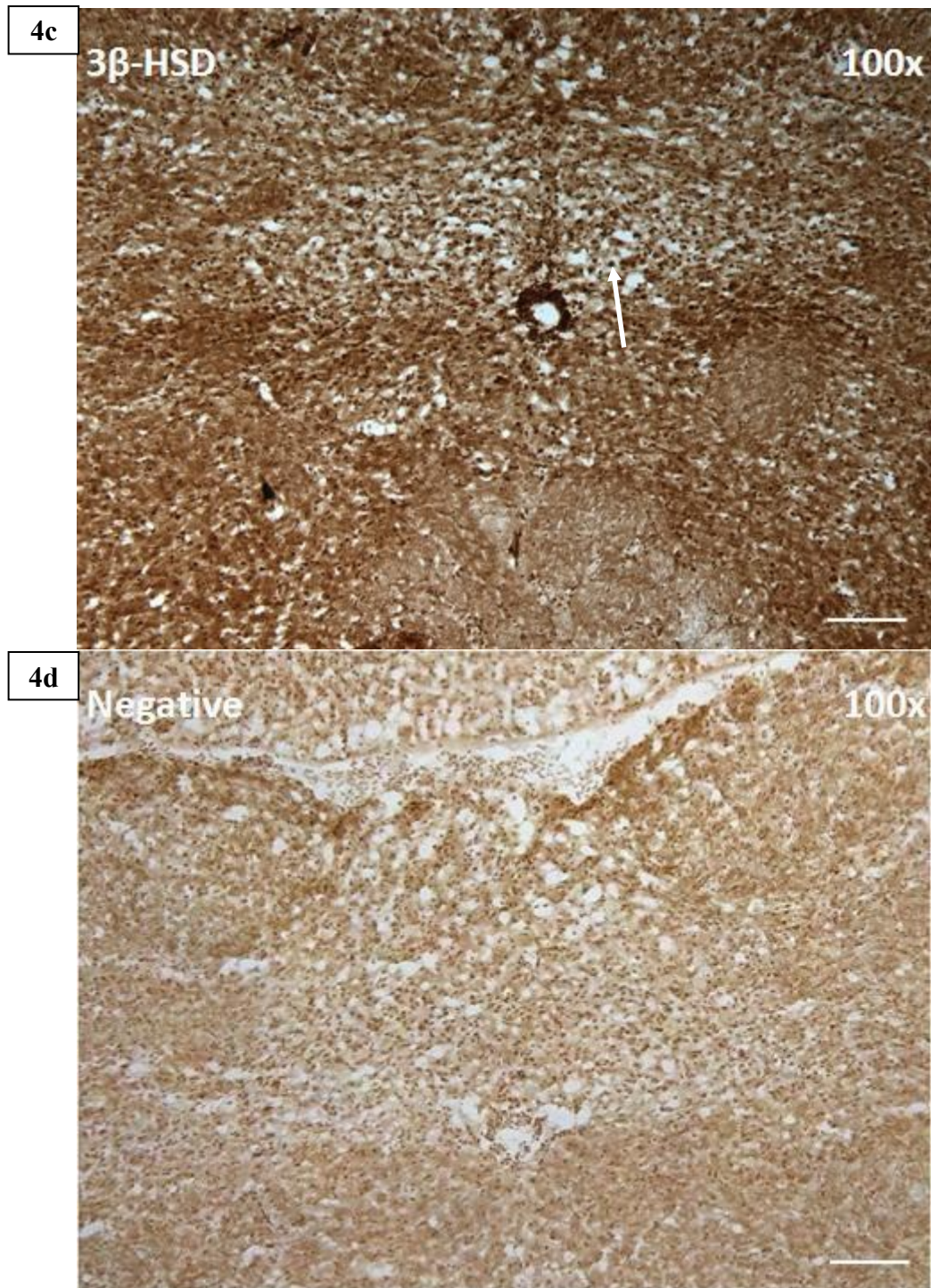Negative   200x

3e

mPRβ   400x

3f

Negative   400x
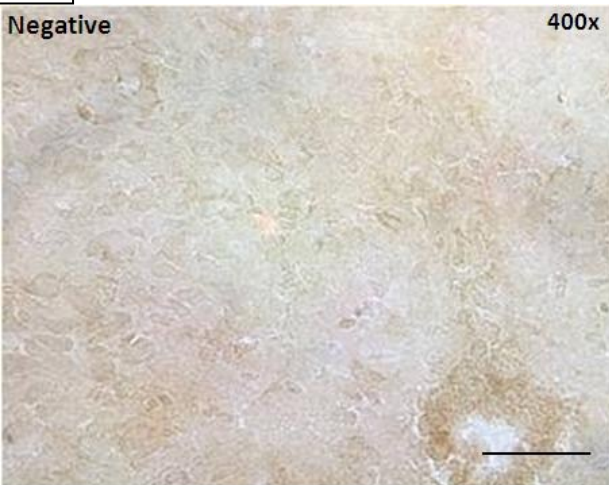
Figure 3 – **Immunohistochemistry for mPRβ in the caudal region of the adult mouse brainstem. It is estimated that this region coincides approximately with bregma – 7.76 mm after 18 hours incubation with secondary antibody.** a) mPRβ at 100x. b) Anatomy of adult mouse brainstem adapted from The Mouse Brain in Stereotaxic Coordinates, Third Edition. c) Dorsal view of mPRβ at 200x. d) Dorsal view of negative at 200x. e) Dorsal view of mPRβ at 400x. f) Dorsal view of negative at 400x. Scale bar at 100x = 100 μm, at 200x = 50μm and at 400x = 25μm.
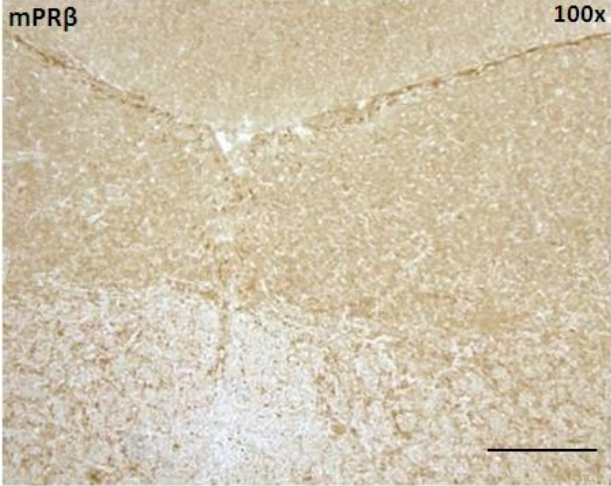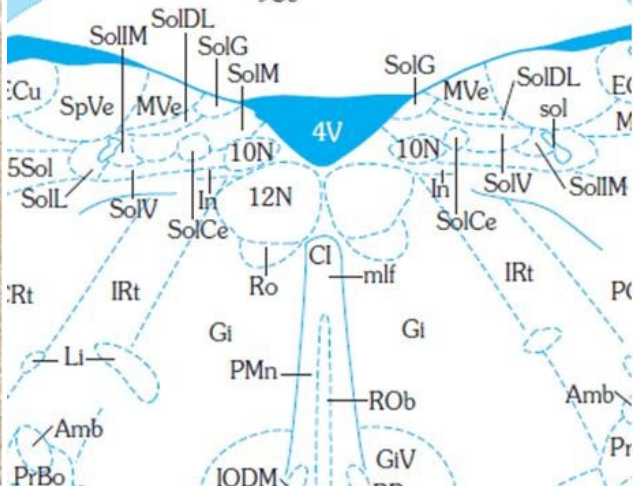
Figure 4 – Immunohistochemistry of mPRβ and 3β-HSD in the caudal region of the brainstem approximately coinciding with bregma -7.76mm after 36 hours incubation with secondary antibody. a) mPRβ at 100x. b) mPRβ at 200x. c) 3β-HSD at 100x. d) View of Negative 100x. Scale bar at 100x = 100μm.

5a mPRβ 100x

5b
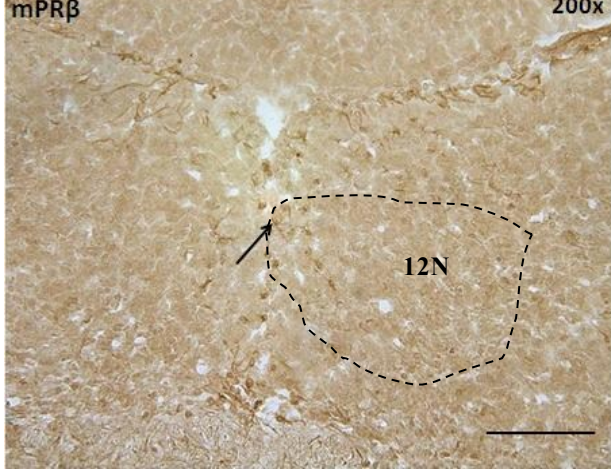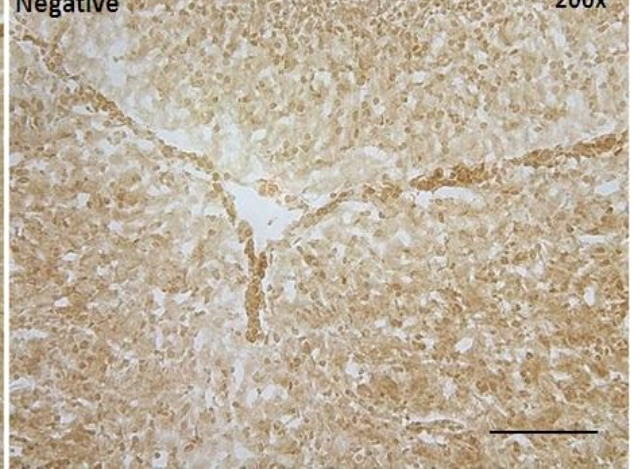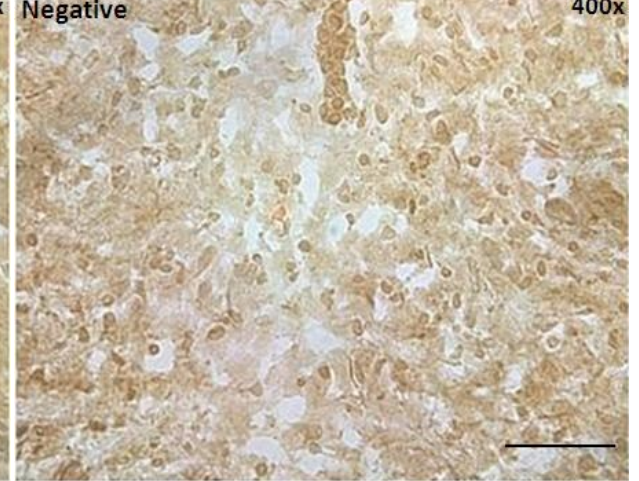SolIM SolDL SolG SolG MVe SolDL
ECu SpVe MVe SolM sol
5Sol 4V 10N 10N In SolV SolIM
SolL SolV In 12N SolCe
SolCe Ro CI mlf IRt
ERt IRt Gi Gi
Li PMn ROb Amb
Amb GiV Pr
PrBo IODM

5c mPRβ 200x 12N

5d Negative 200x

5e mPRβ 400x

5f Negative 400x
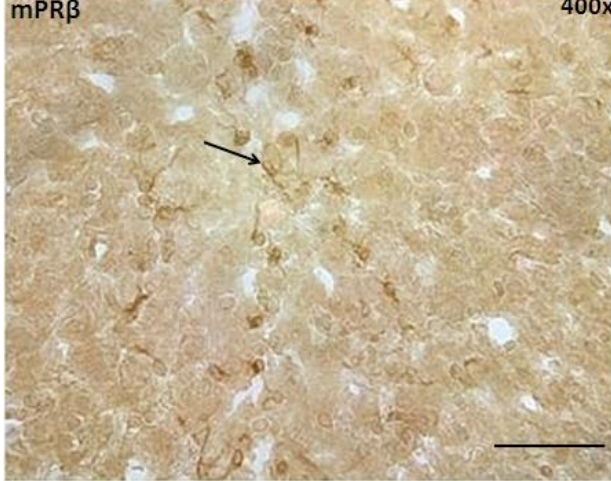
Figure 5 – **Immunohistochemistry for mPRβ in the rostral region of the adult mouse brainstem. It is estimated that this region coincides approximately with bregma – 7.08 mm.** a) mPRβ at 100x. b) Anatomy of adult mouse brainstem adapted from The Mouse Brain in Stereotaxic Coordinates, Third Edition. c) Dorsal view of mPRβ at 200x. d) Dorsal view of negative at 200x. e) Ventral view of mPRβ at 400x. f) Ventral view of negative at 400x. Scale bar at 100x = 100 μm, at 200x = 50μm and at 400x = 25μm.

a) SolM 1200x   b) SolM 1200x   c) Negative 1200x
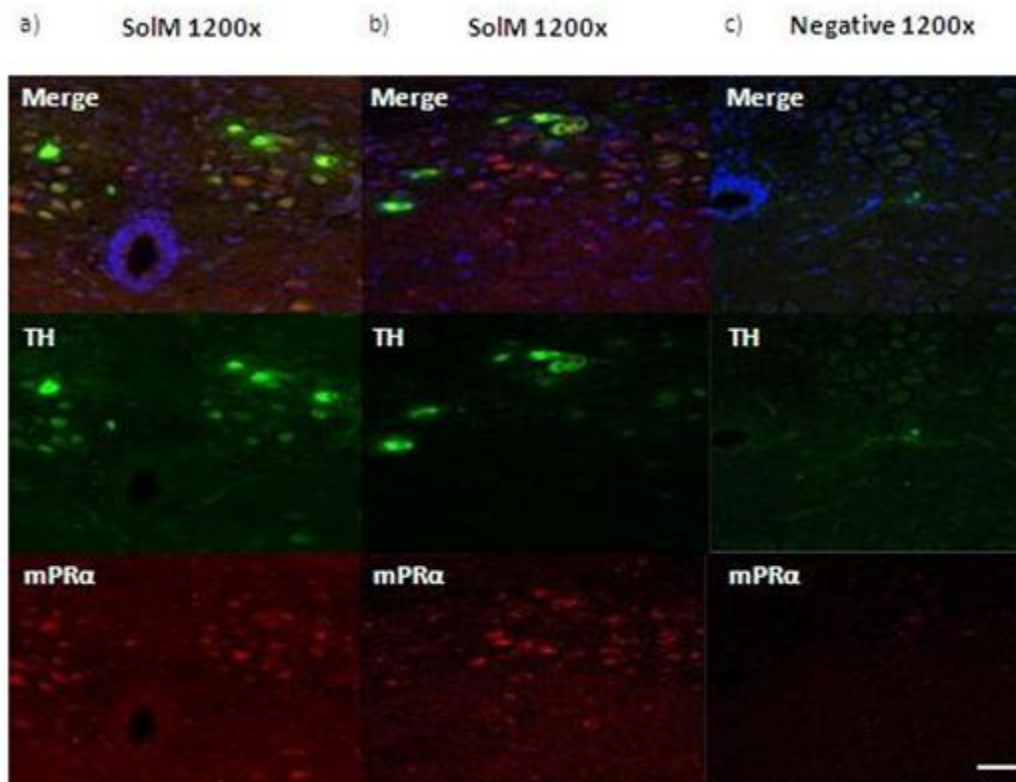
Figure 6 – **Double Immunofluorescence for mPRα and TH in the caudal region of the adult mouse brainstem. It is estimated that this region coincides approximately with bregma – 7.56 mm.** a) and b) mPRα, TH and DAPI are merged at 1200x in a region corresponding to SolC. Structures were identified based on anatomical schematic seen in Fig. 3b. c) Negative for mPRα, TH and the merge at 1200x. Scale bar at 1200x = 50 μm. Below is a magnified view of individual images. cc- central canal.
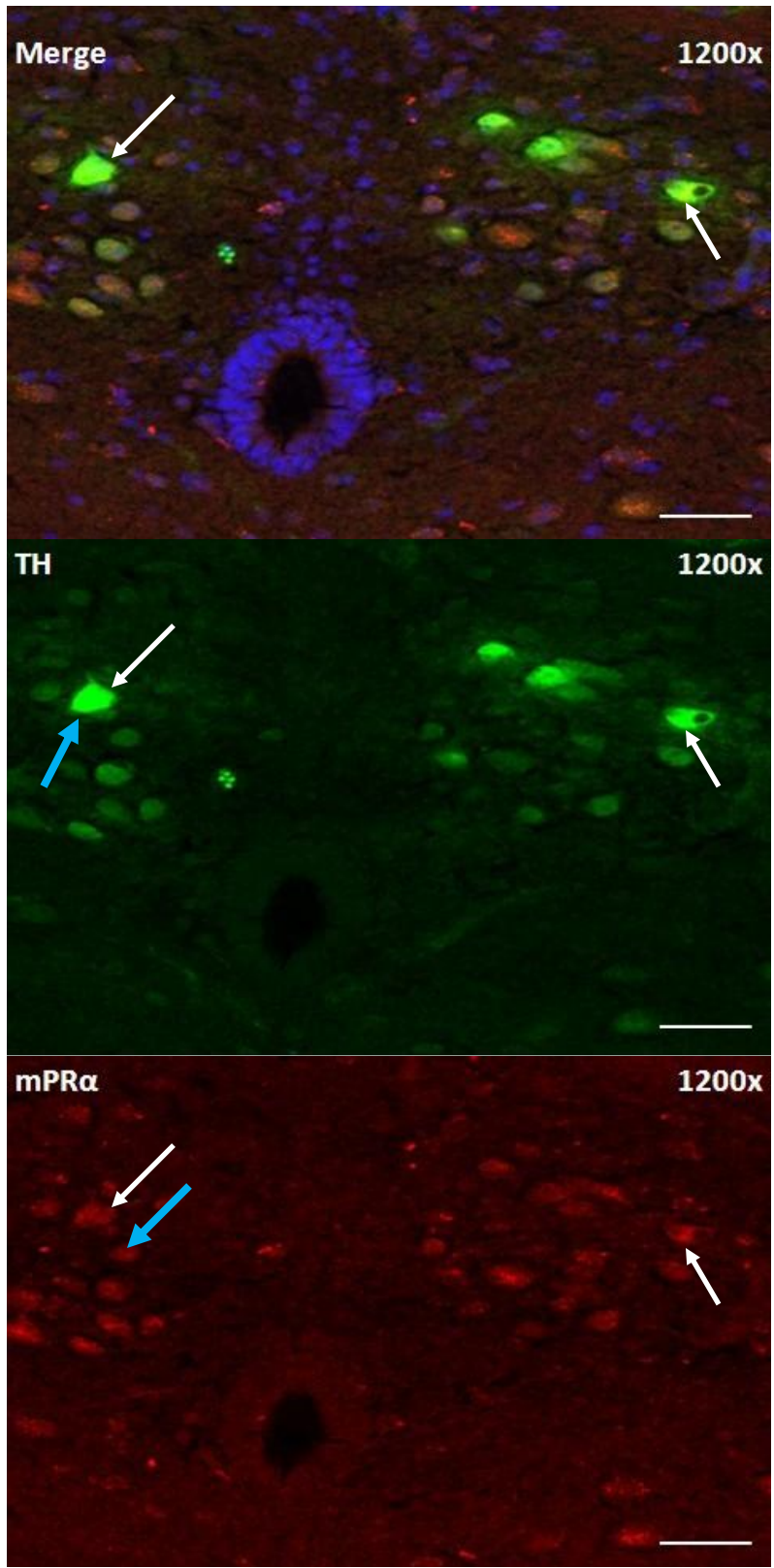
6a
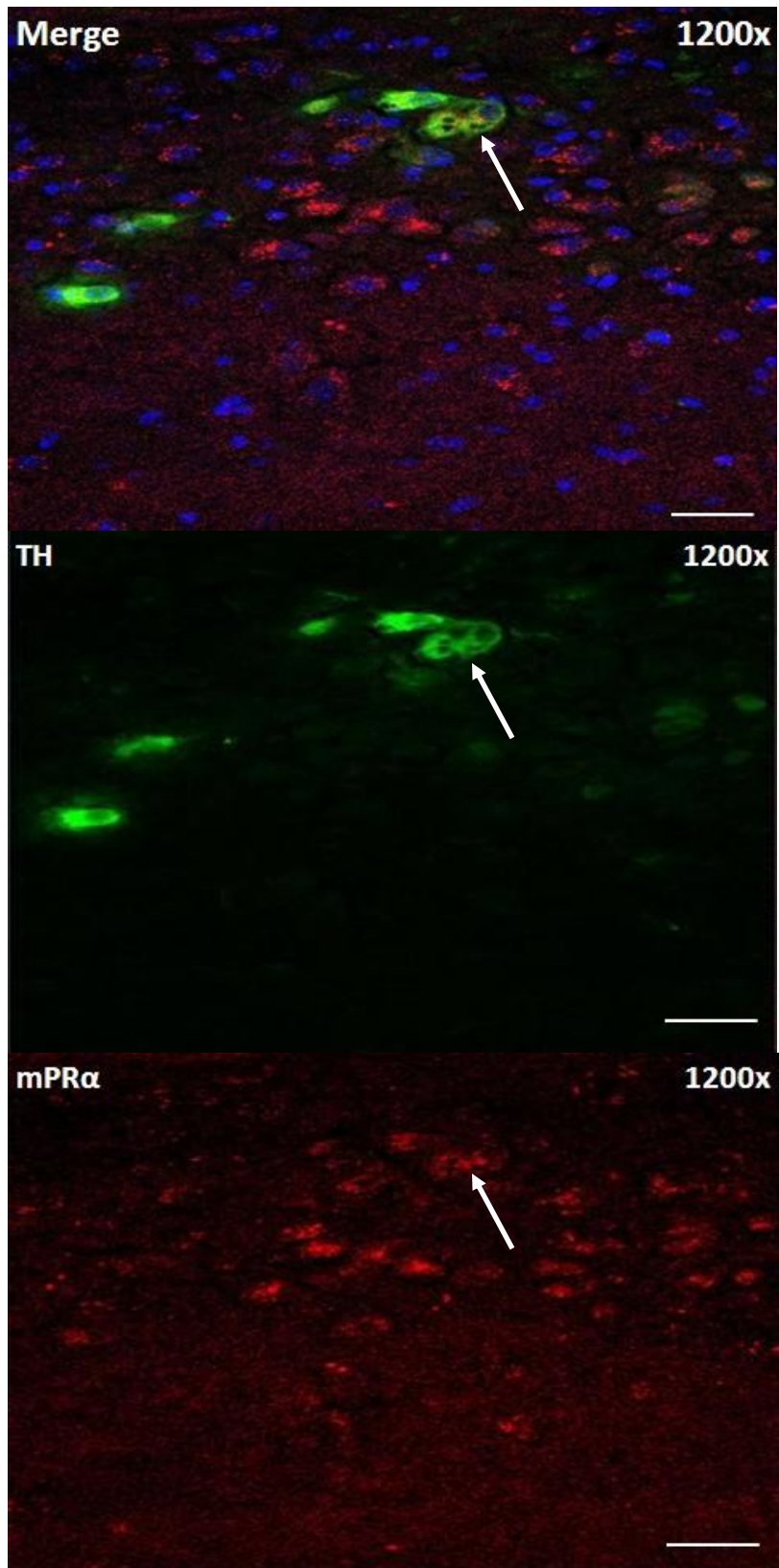
6b

6c

Figure 7 − Double Immunofluorescence for mPRα and TH in the caudal region of the adult mouse brainstem. mPRα, TH and DAPI are merged at 2400x in the NTS. TH can also be seen alone in the middle panel at 2400x as well as mPRα alone in the bottom panel. The region seen corresponds to SolM based on the anatomical schematic seen in Fig. 3b and is approximated to be between bregma -7.56 mm to 7.76 mm. Scale bar at 2400x = 25 μm.
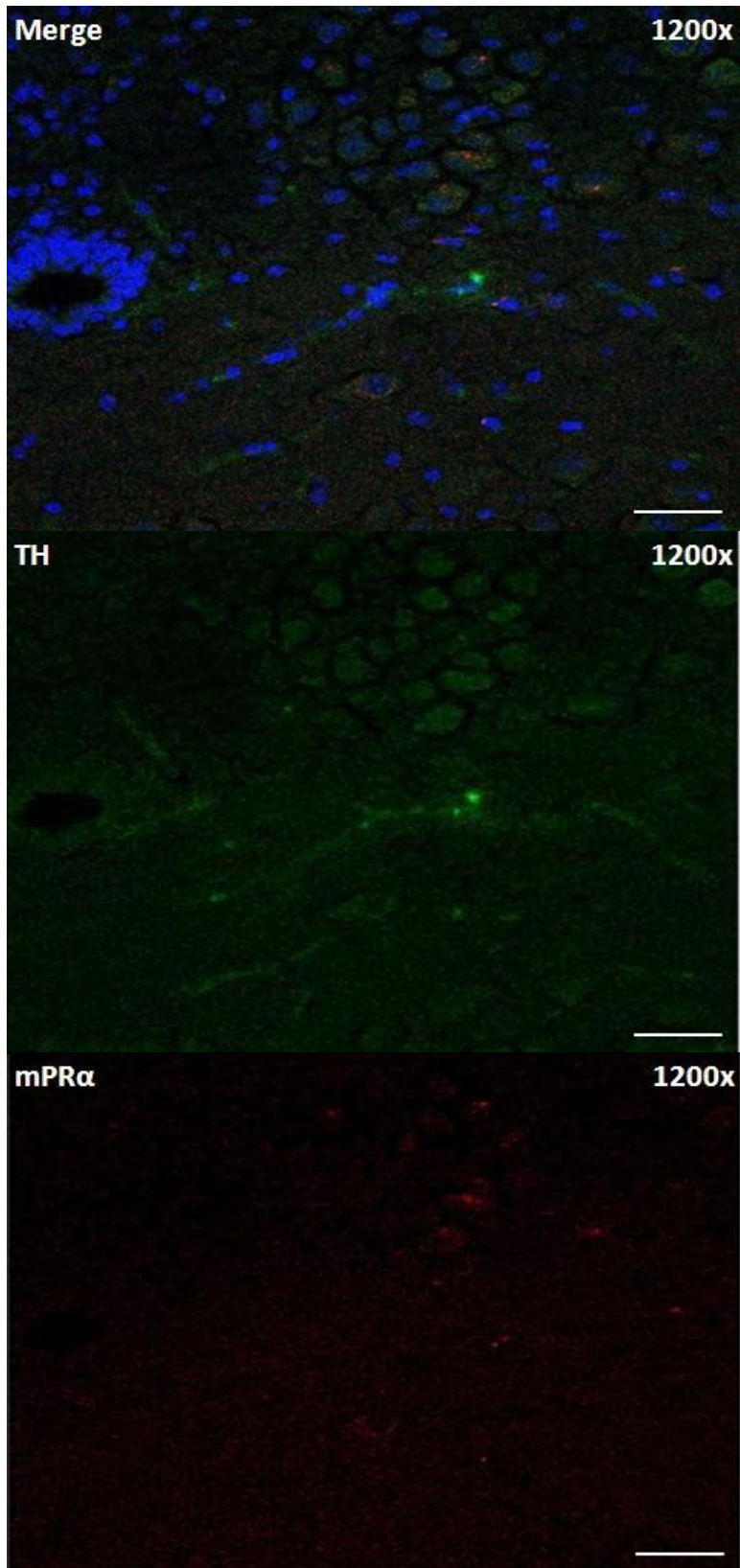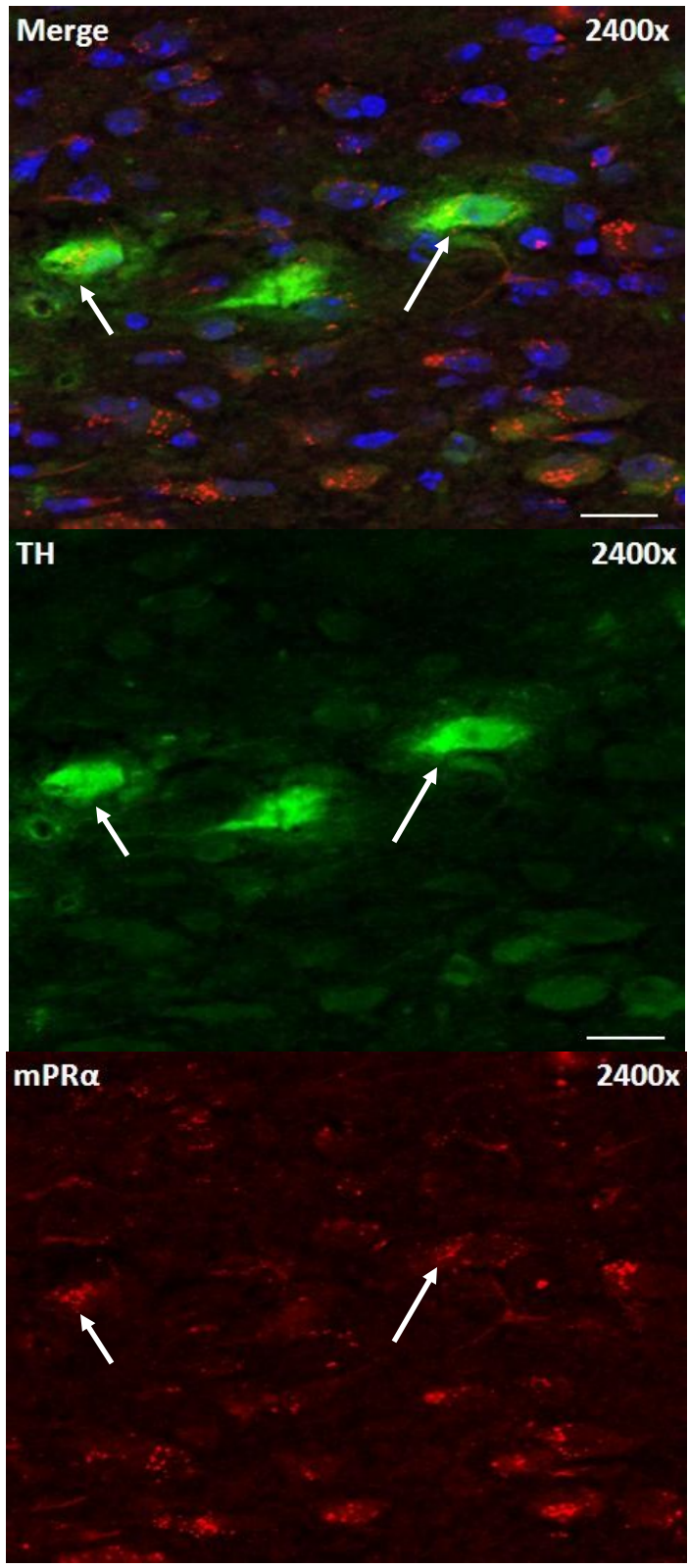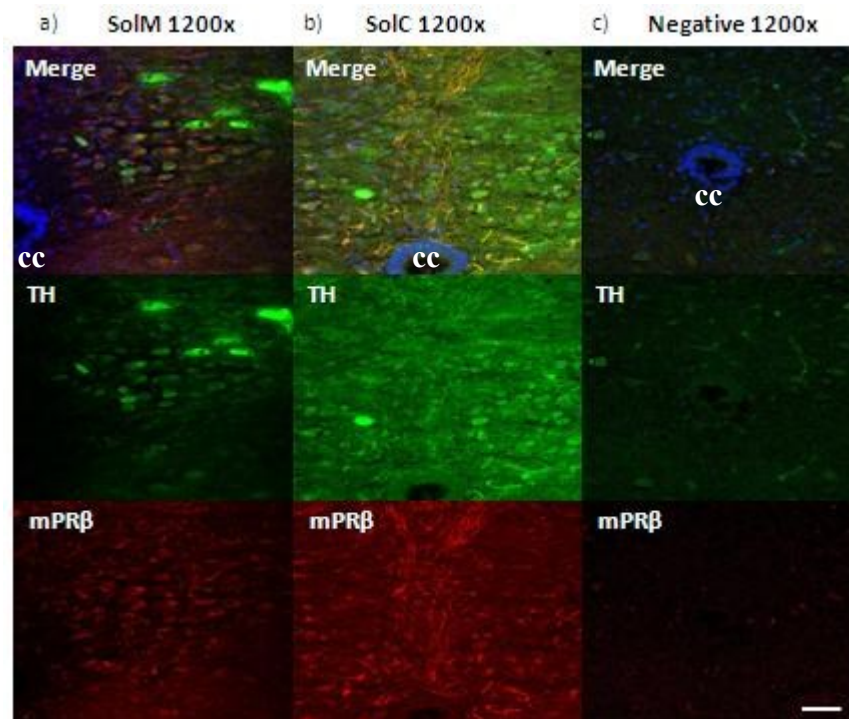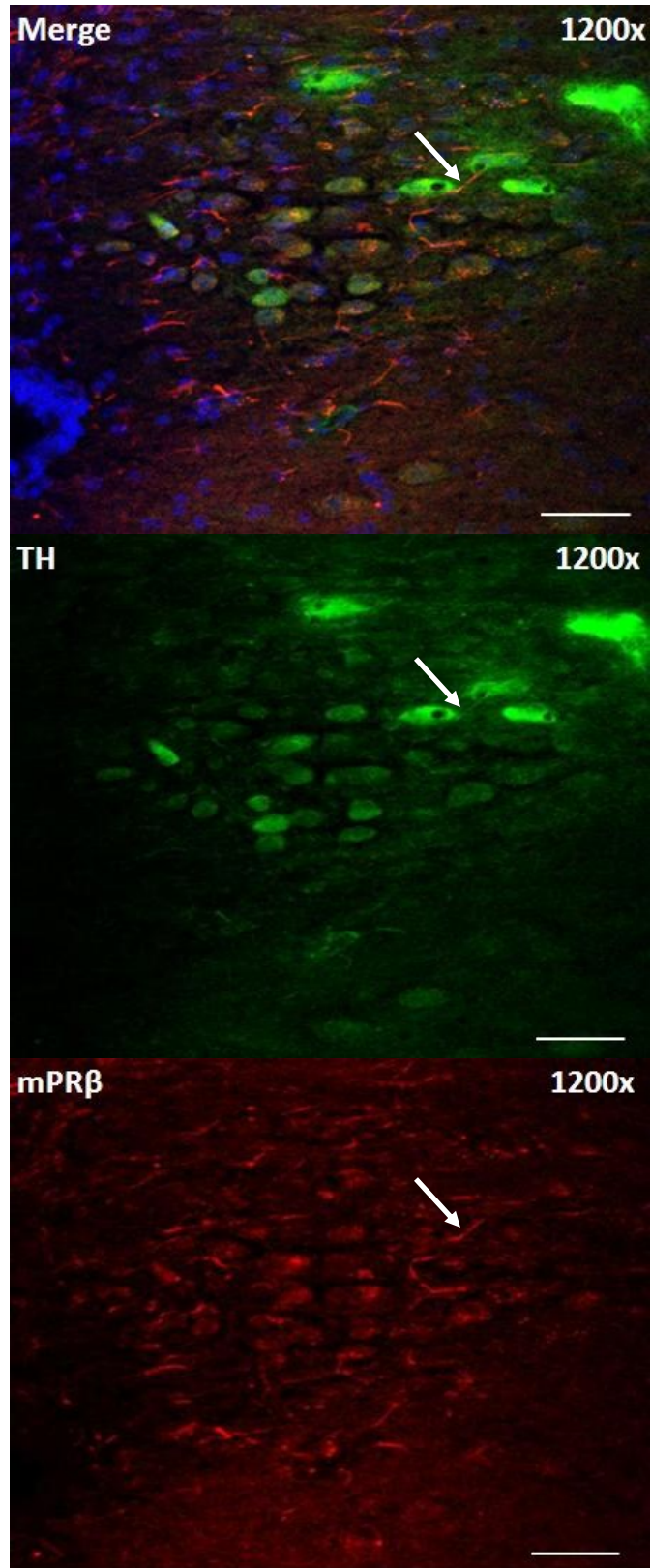

Figure 8 − Double Immunofluorescence for mPRβ and TH in the caudal region of the adult mouse brainstem. It is estimated that this region is between bregma − 7.56 mm and − 7.76 mm. a) mPRβ, TH and DAPI are merged at 1200x in a region corresponding to SolM based on anatomical atlas seen in Fig. 3b. b) mPRβ, TH and DAPI are merged at 1200x in a region corresponding to SolC based on anatomical schematic seen in Fig. 1b. c) Negative for mPRβ, TH an merge at 1200x. Scale bar at 1200x = 50 μm. Below is a magnified view of individual images. cc-central canal.
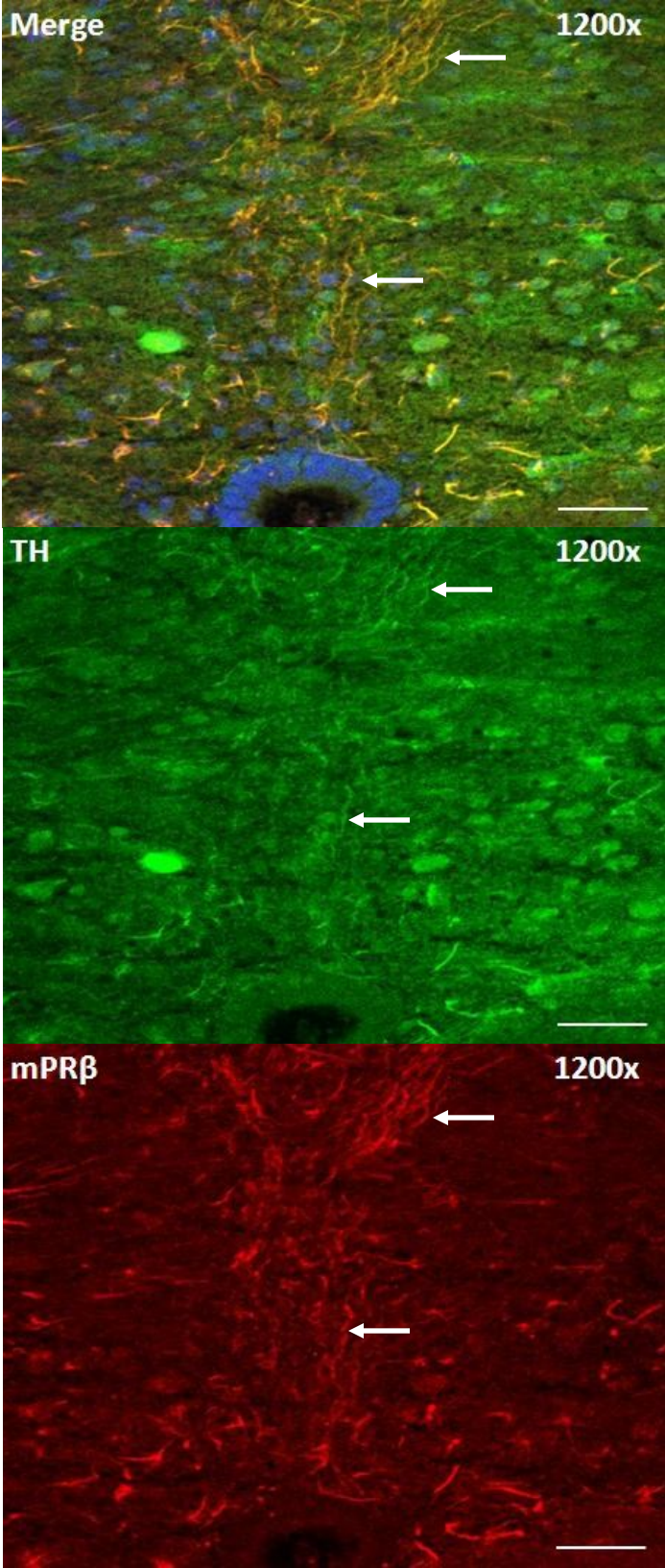
8a

8b
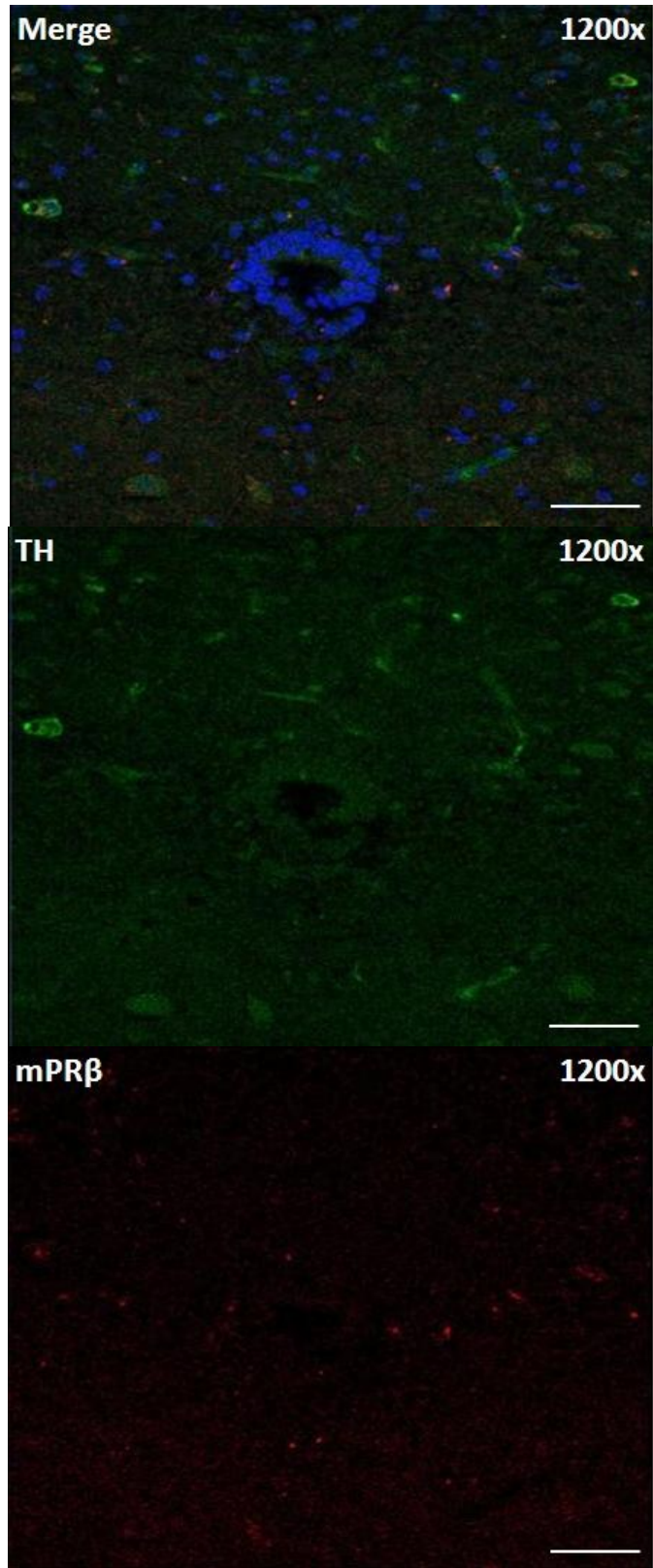
8c
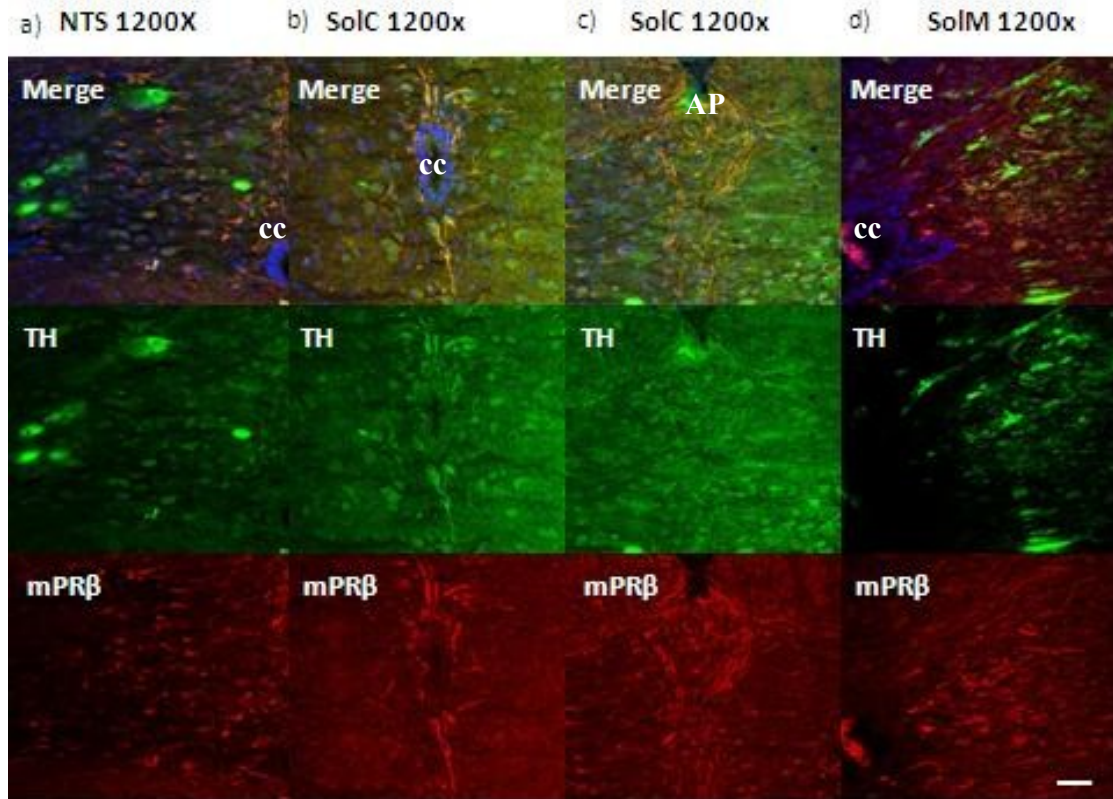
Figure 9 – **Double Immunofluorescence for mPRβ and TH in the caudal region of the adult mouse brainstem between bregma – 7.56 mm and – 7.76 mm.** a) mPRβ, TH and merge at 1200x in the NTS. b) mPRβ, TH and DAPI are merged at 1200x in a region corresponding to SolC. c) mPRβ, TH and merge at 1200x in a region corresponding to SolC and the AP. d) mPRβ, TH and merge at 1200x in a region corresponding to SolM. Structural identification was made with reference to the anatomical schematic seen in Fig. 3b and 1b. Scale bar at 1200x = 50 μm. Below is a magnified view of individual images. cc- central canal, AP- area postrema.
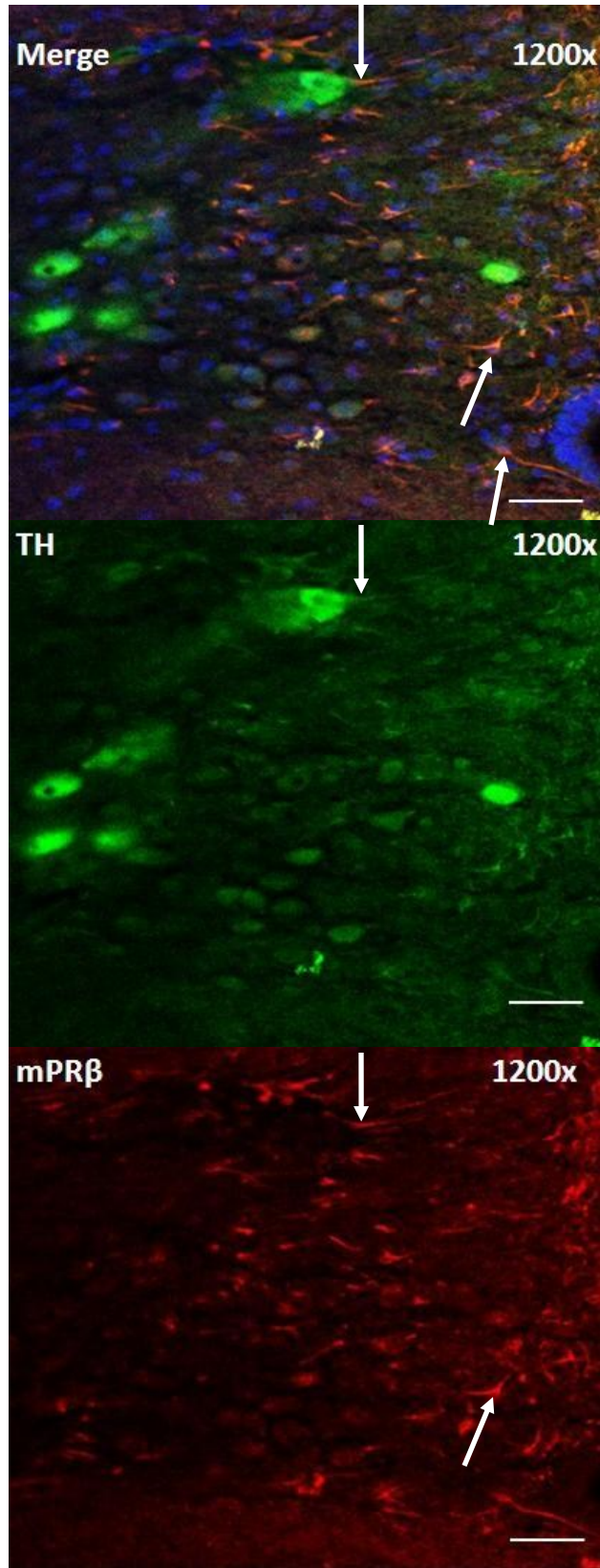
9a
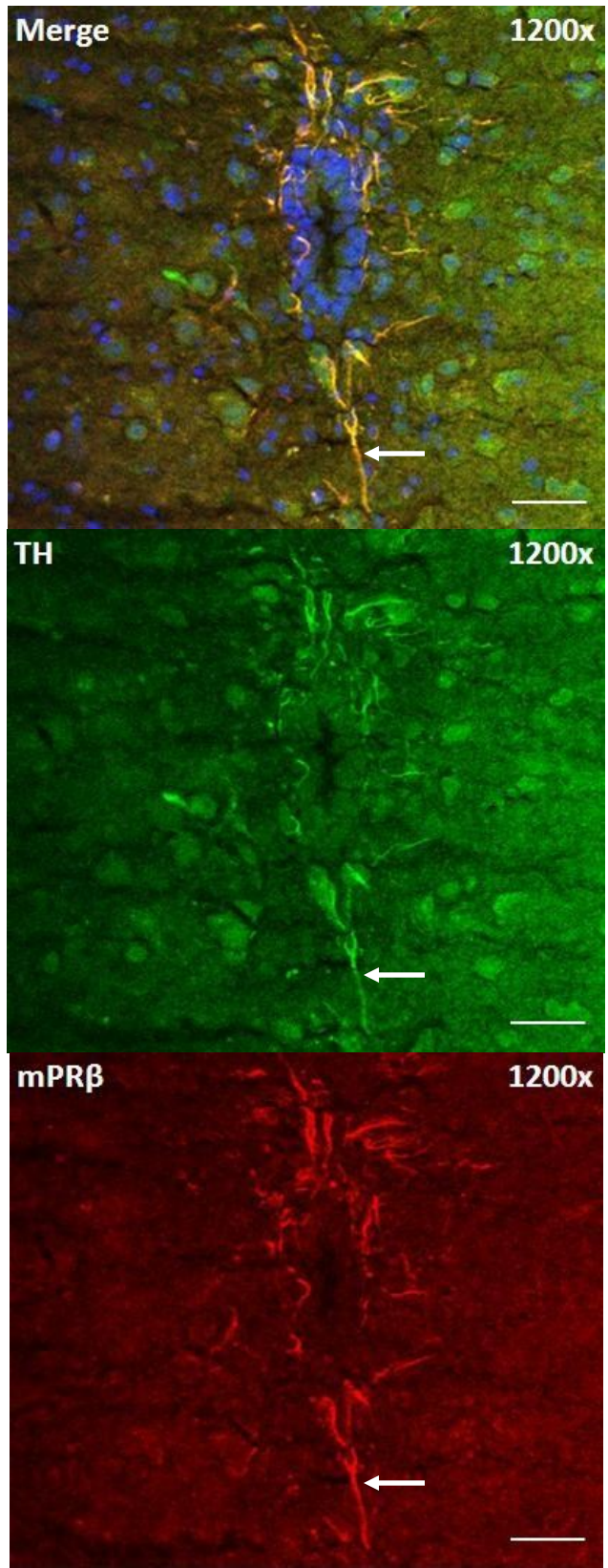
9b

9c

9d

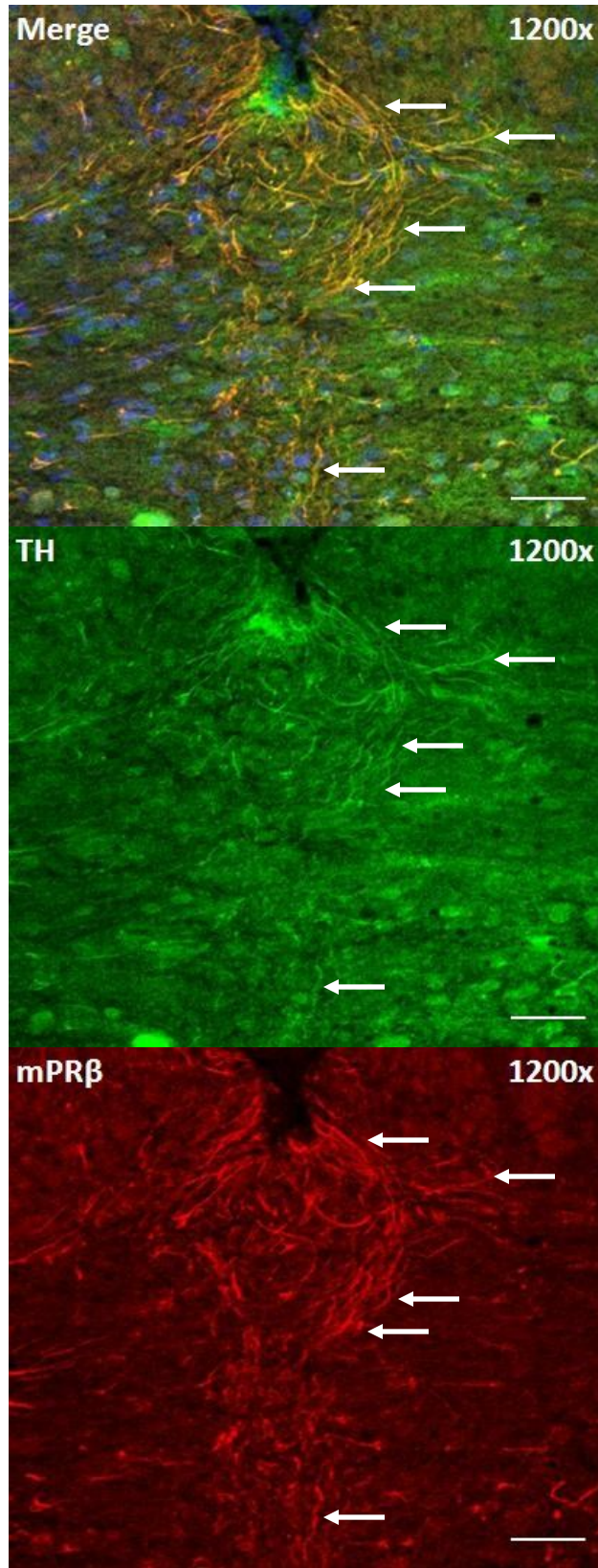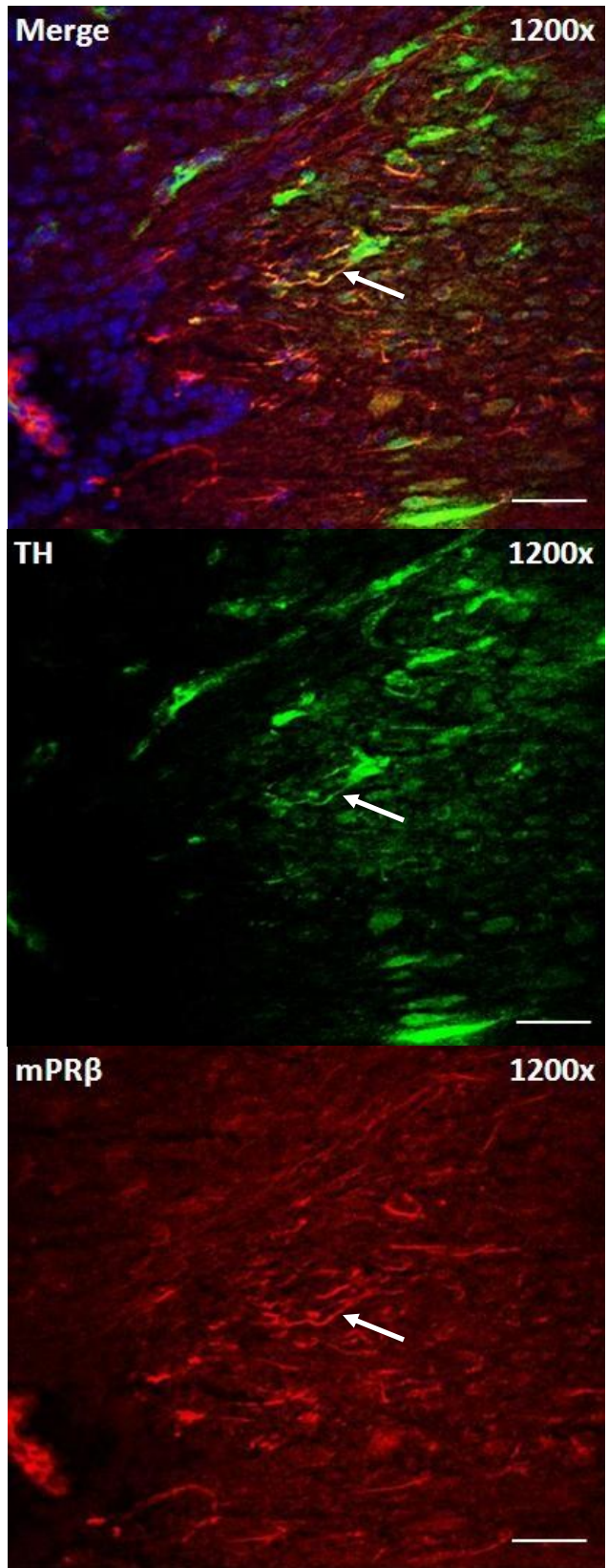| Brain Region	mPRα IR	mPRβ IR	3β-HSD IR	Neuron Type
AP	+++	++++	++	I
10N	+++	+++	+	II, III
12N	+++	-	+	II, III
Rob	+	-	+	n/a
SolC	+	++++	++++	I
SolM	+++	++	+++	I, II, III
SolIM	+++	-	-	II, III
SolV	++	++	+++	I, II, III
SolVL	+	+++	+++	II, III
SolL	+	-	-	I
SolI	++	++	++	I
SolDL	+++	++	+++	I, II, III
SubP	++	+++	++	I
sol	+++	+++	+++	I

Table 2 – Relative Immunoreactivity of mPRα, mPRβ and 3β-HSD in relevant respiratory structures of the caudal brainstem based on immunohistochemical analysis between bregma – 7.56 mm and – 7.76 mm. Staining strength is indicated as – for none, + for weak, ++ for medium, +++ for strong and ++++ for very strong staining. Type I neurons had small cell bodies and several axon collaterals surrounding them. Type II and III neurons had large cell bodies and a long projection axon. n/a – not available, IR – immunoreactivity, AP – area postrema, 10N – dorsal motor nucleus of vagus cranial nerve, 12N – nucleus of hypoglossal cranial nerve, Rob – raphe obscures, SolC – commissural solitary tract, SolM – medial solitary tract, SolIM – intramedial solitary tract, SolV – ventral solitary tract, SolVL – ventro-lateral solitary tract, SolL – lateral solitary tract, SolI intramedial solitary tract, SolDL - dorsolateral solitary tract, SubP - sub-Postrema, sol – solitary tract.

Brain Region	mPRα IR	mPRβ IR	TH IR	Neuron Type
AP	-	++++	++++	I
10N	++	++	++	II, III
SolC	-	++++	++++	I
SolM	+++	+++	+++	I, II, III

Table 3 – Relative Immunoreactivity of mPRα, mPRβ and TH in relevant respiratory structures of the caudal brainstem based on double immunofluorescence analysis between bregma – 7.56 mm and – 7.76 mm. Staining strength is indicated as – for none, + for weak, ++ for medium, +++ for strong and ++++ for very strong staining. Type I neurons had small cell bodies and several axon collaterals surrounding them. Type II and III neurons had large cell bodies and a long projection axon. IR – immunoreactivity, AP – area postrema, 10N – dorsal motor nucleus of vagus cranial nerve, SolC – commissural solitary tract, SolM – medial solitary tract.
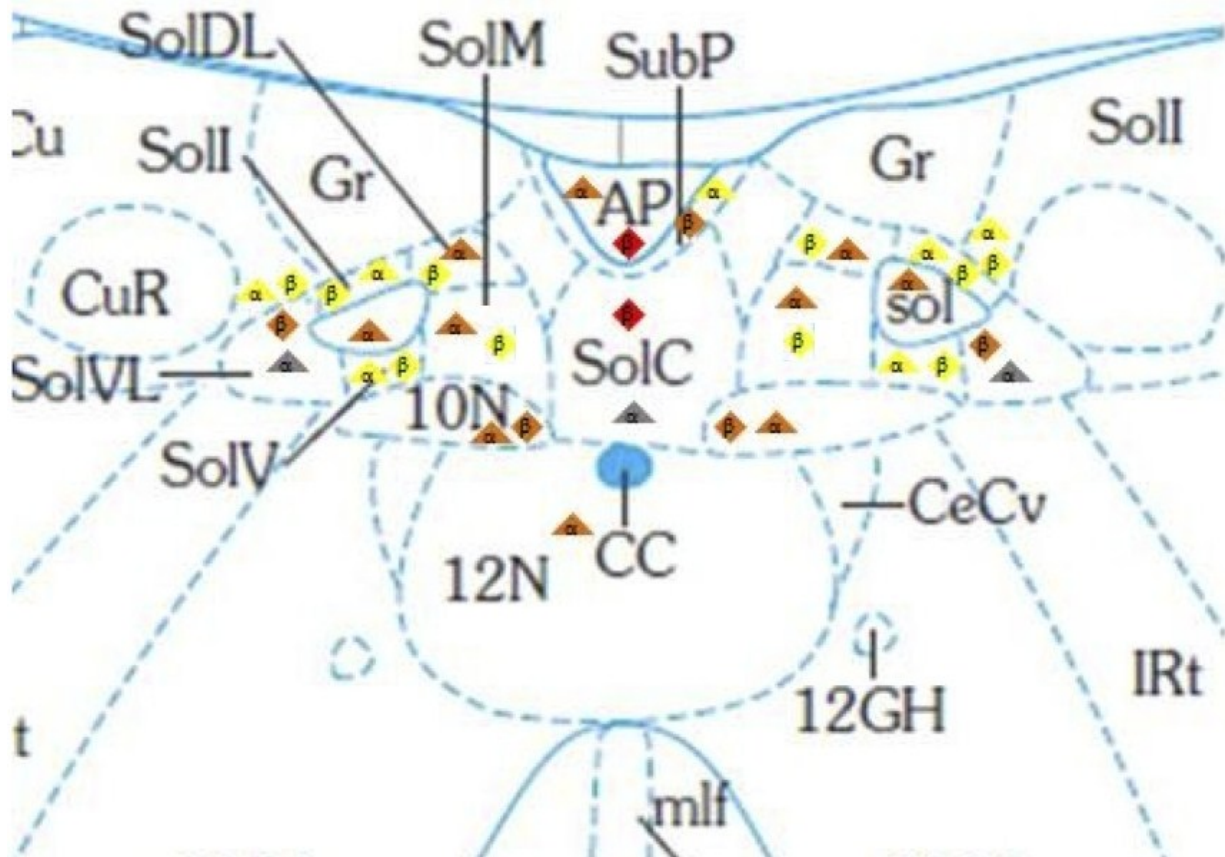
Figure 10 – Relative expression of mPRα and mPRβ in respiratory related structures of the caudal brainstem between bregma – 7.56 mm and 7.76 mm. Triangles are used to represent mPRα and diamonds are used for mPRβ. Triangles and diamonds are placed where expression was observed. For simplification purposes, the anatomical schematic at bregma – 7.76 mm was used. The intensity of mPR expression ranges from strongest to weakest. Red - very strong, orange – strong, yellow – medium and grey – weak. AP – area postrema, CC – central canal, 10N – dorsal motor nucleus of vagus cranial nerve, 12N – nucleus of hypoglossal cranial nerve, Rob – raphe obscures, SolC – commissural solitary tract, SolM – medial solitary tract, SolIM – intramedial solitary tract, SolV – ventral solitary tract, SolVL – ventro-lateral solitary tract, SolL – lateral solitary tract, SolI intramedial solitary tract, SolDL - dorsolateral solitary tract, SubP - sub-Postrema, sol – solitary tract, mlf – medial longitudinal fascicle, Gr – gracile nucleus, IRt – internal reticular nucleus, CeCv – central cervical nucleus, CuR – cuneate nucleus, rotundus part .

Figure 11 – Relative degree of colocalization between TH and mPRα or mPRβ in respiratory related structures of the caudal brainstem between bregma – 7.56 mm and 7.76 mm. Green squares are used to represent the location of TH and intentionally overlap triangles and diamonds which represent mPRα and mPRβ, respectively, to demonstrate colocalization. Squares, triangles and diamonds are placed where expression was observed. For simplification purposes, the anatomical schematic at bregma – 7.76 mm was used. The intensity of mPR expression ranges from strongest to weakest. Red - very strong, orange – strong, yellow – medium and grey – weak. AP – area postrema, CC – central canal, 10N – dorsal motor nucleus of vagus cranial nerve, 12N – nucleus of hypoglossal cranial nerve, Rob – raphe obscures, SolC – commissural solitary tract, SolM – medial solitary tract, SolIM – intramedial solitary tract, SolV – ventral solitary tract, SolVL – ventro-lateral solitary tract, SolL – lateral solitary tract, SolI intramedial solitary tract, SolDL - dorsolateral solitary tract, SubP - sub-Postrema, sol – solitary tract, mlf – medial longitudinal fascicle, Gr – gracile nucleus, IRt – internal reticular nucleus, CeCv – central cervical nucleus, CuR – cuneate nucleus, rotundus part .

Figure 12 – Expression of mPRα in immunohistochemical and immunofluorescence analysis. The top right panel demonstrates expression using immnohistochemistry (scale bar at 200x = 60 μm. The top left panel is a merge of TH and mPRα expression seen in the bottom two panels (scale bar at 1200x = 40 μm).
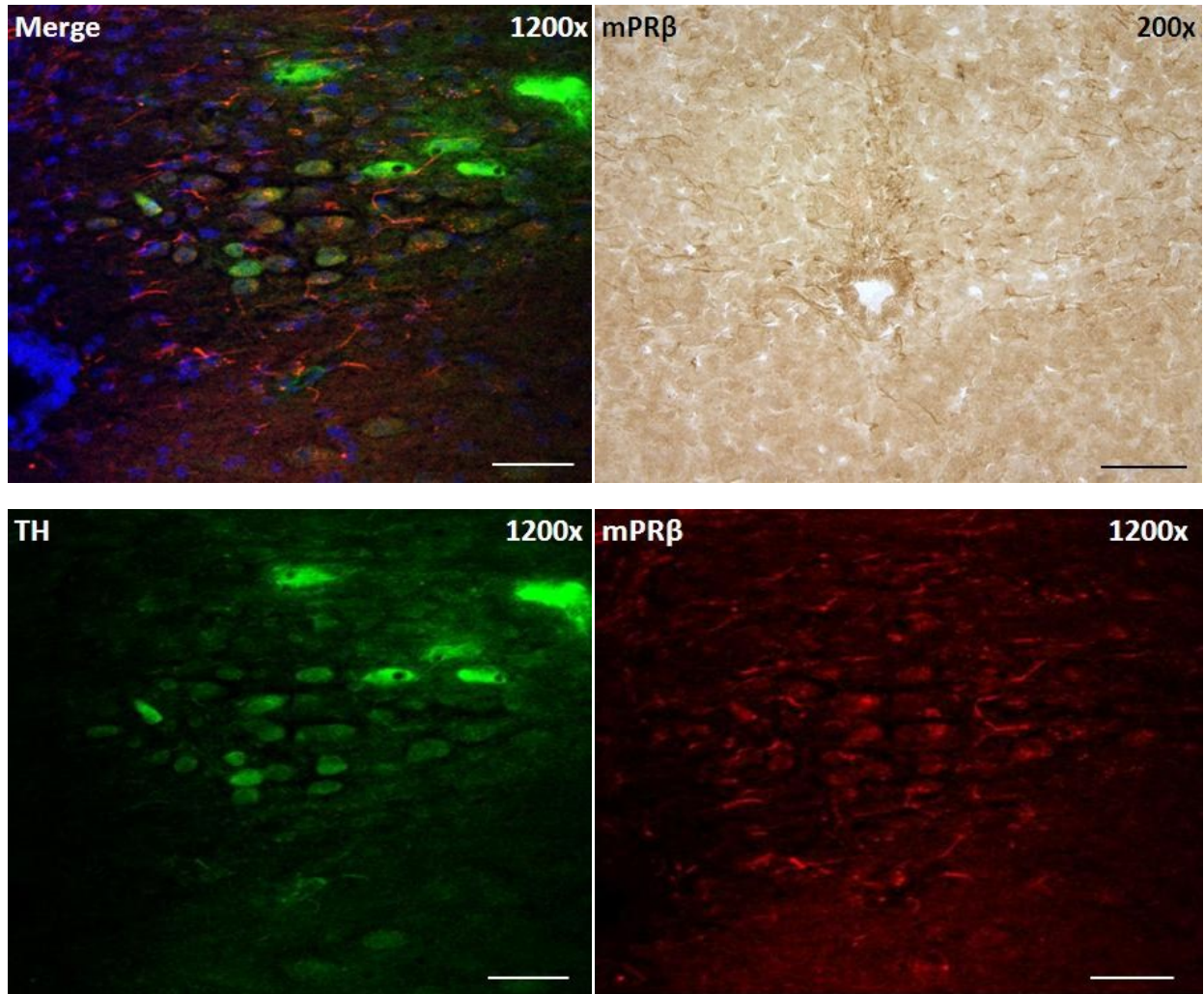
Figure 13 - **Expression of mPRβ in immunohistochemical and immunofluorescence analysis.** The top right panel demonstrates expression using immnohistochemistry (scale bar at 200x = 60 μm. The top left panel is a merge of TH and mPRβ expression seen in the bottom two panels (scale bar at 1200x = 40 μm).

4 Discussion

4.1 mPRα and mPRβ in the Brainstem

The present study demonstrates evidence for the presence of mPRα and mPRβ in the caudal and rostral brainstem. Immunoreactivity for the two receptors was greater in the caudal portion of the NTS, which is known to house structures involved in respiratory control. Receptor distribution was found to be centered and restricted to the NTS (Table 2 and 3) as well as key structures proximal to the NTS (10N, 12N and AP). Membrane receptors were observed at various locations throughout the neuron. mPRα primarily coated the cell body (Fig. 1, 2, 6 and 7) whereas mPRβ staining was most intense in the axon and cell body (Fig. 3, 4, 8 and 9). mPRβ had the most extensive distribution (Fig. 10), indicative of a larger role played by this receptor subtype in NTS respiratory control.

Also of interest with regards to structural receptor localization, is that mPR staining intensity is magnified by the presence of Estrogen. Thus the reproducibility of this study depends on the Estrogen content of the animals used. To confirm the validity of these findings, however, follow up studies reporting mPR localization in similar structures will be necessary. Never the less, because Estrogen content is relatively consistent in male adult mice, it is believed that mPR expression should vary minimally (Zuluogo D. et al., 2012).

The enzyme, 3β-HSD, which synthesizes the conversion of pregnenolone into progesterone was found to be distributed in structures expressing the mPR, consistent with previous studies stating local biosynthesis and expression of progesterone in the neuron (Micevych P. et al., 2008; Micevych P. and Sinchak K. 2008).

TH was observed using the double immunofluorescence technique in the 10N, SolC, SolM and AP. This technique allowed for simultaneous detection of TH and mPRα or mPRβ. TH was colocalized with both receptors in the 10N, SolC, SolM and AP (Fig. 11). Therefore, mPR's are localized alongside TH in: 10N, a structure which innervates various structures of the respiratory system, SolC, a structure known for passing chemoafferent nerve fibers, SolM, the largest subnuclei in the NTS and the AP, a region of great anatomical relevance due to central position and connectivity with surrounding subnucleis of the NTS (Kalia M. et al., 1984). The

significance of finding colocalization, means mPRα and mPRβ are found in catecholaminergic neurons, which play a large neuromodulatory role in respiratory control (Li, 2008). Figure 6 also indicates that in the following subnulei of the NTS, SolC and SolM, TH was also colocalized with mPR which suggests that catecholaminergic fibers project through these structures (Kalia M. et al., 1984; Kawai Y. and Senba E. 1999).

Colocalization between mPR and TH indicate that this might be a mechanism through which progesterone regulates TH expression and thus TH's neuromodulatory effects on respiration (Li, 2008). Progesterone may be regulating neurotransmitter synthesis as well as neurotransmission in order to exert its effects on respiratory control. It is also possible that progesterone may have paracrine signaling effects from neighboring catecholaminergic neurons as seen with the blue arrows in the enlarged panels of Fig. 6a.

It has been suggested from past morphological, chemical and electrophysiological studies of neuronal characteristics that: NTS neurons could be classified into subgroups based on similar patterns of synaptic responses and physical characteristics such as axonal trajectory, soma size and chemical properties (Kawai Y. and Senba E. 1999). Thus, the system of neuronal categorization used in this study. Using this system, previous studies found that NTS neurons with small cell bodies, exhibit polysynaptic excitatory responses after solitary tract (ST) stimulation. These are considered type I neurons and they bear extensive axonal collaterals, making them local circuit neurons (Kawai Y. and Senba E. 1999). Neurons bearing a large cell body and a long projection axon (Fig. S4) have a monosynaptic excitatory response to ST stimulation (type II neurons), or an excitatory then inhibitory response (type III neurons). Type II and III neurons however, were not possible to distinguish solely based on morphological characteristics in the current study. Type II and III neurons had few axon collaterals but a large projection axon that extended outside of the NTS, making these projection neurons. It is possible that such projection cells with large cell bodies are capable, in response to carotid body stimulation, of producing signals that could be projected to other regions of the brain such as the RVLM, a region involved in O_2 sensing and respiratory rhythm generation (PBC) which receives dense input from the NTS (Kawai Y. and Senba E. 1999; Powell F. et al., 2009).

4.2 NTS

Carotid chemoreceptor afferents predominantly innervate the caudal region of the NTS (Kline D. et al., 2010; Koshiya N. and Guyenet P. 1996). Based on their projection type function, CB chemoreceptor afferents are believed to innervate NTS subnuclei SolM, SolIM, SolV, SolVL and SolDL (Table 2). Past studies have indicated that stimulating these afferents activates respiratory and sympathetic outflow (Koshiya N. and Guyenet P. 1996). Research has also indicated that NTS neurons project to the central respiratory oscillator in the RVLM. Hypoxic stimulation of carotid body chemoreceptors was found to excite neurons in the caudal region of the NTS. A population of neurons in the caudal portion of the NTS is believed to project through the RVLM, acting as the central relay between peripheral chemoreceptors and the central respiratory oscillator in the RVLM (Kline D. et al., 2010; Koshiya N. and Guyenet P. 1996).

4.3 Functional Role of NTS Subnuclei

From the location of mPRs, it is possible to infer their function based on the known activity of the structures in which they were found. SolC is a passage way for afferent fibers of the vagus nerve (Kalia M. et al., 1984; Kawai Y. and Senba E. 1999). Chemoreceptor afferents also project to SolC. Past immunoreactivity experiments have indicated that cell perikarya, traversing fibers and their local collateral arborizations can be found in SolC (Kalia M. et al., 1984; Kawai Y. and Senba E. 1999). Our results show that extensive and dense mPRβ staining of fibers appeared here, colocalized with TH (Fig. 3c). Since projection fibers from the peripheral chemoreceptors are TH positive, it is tempting to speculate that mPRβ might modulate the function of these fibers to alter respiratory control. SolM is the largest subnucleus of the NTS and extends throughout the entire NTS rostro-caudally. Its functional role is to modulate cardiovascular, gastrointestinal and baroreceptor afferent input activity. Most of the modulatory effects of SolM occur in the dendritic zones of anuclear regions surrounding SolM (Kawai Y. and Senba E. 1999). The largest cell bodies in this study were observed in SolM (Fig. 1b and 6a) with the large projection axon projecting from the cell body seen in Fig. 8a, 9a and 9d. Thus SolM neurons meet the profile of type II and III projection neurons with neuromodulatory capabilities highly suited for receiving chemoafferent input, integrating it, and relaying it off to structures such as the PBC or RVLM (Kawai Y. and Senba E. 1999). mPRα had strong staining in the SolM,

perhaps indicative of a modulatory effect of progesterone on projection neurons found in this area (Finley J. and Katz D. 1992; Kalia M. et al., 1984; Kawai Y. and Senba E. 1999).

SolI neurons were observed to bear small cell bodies (Fig. 1c) and are mainly known to contain a dendritic zone of perikarya containing nerve terminals belonging to pulmonary and cardiac afferents (Kalia M. et al., 1984). SolIn receives the majority of afferents from the larynx. It also receives afferent nerve fibers from the extrathoracic trachea, the main bronchi and the lungs. The physiology of SolI neurons renders neurons in this structure capable of monosynaptic transmission as well as great local signal transduction within its proximal network of neurons including neurons from structures neighbouring the NTS (Kalia M. et al., 1984; Kawai Y. and Senba E. 1999).

SolDL has displayed perikaryal immunoreactivity in past experiments in the form of fine dots scattered throughout the SolDL region. This could represent terminals or preterminal processes of neuronal perikarya located in adjacent subnuclei. SolDL mainly receives baroreceptor and chemoreceptor afferents from the carotid sinus/aortic nerve as well as lung stretch receptor afferent nerves and is primarily involved in chemoreceptor and baroreceptor reflex (Kawai Y. and Senba E. 1999). Immunohistochemistry indicated that SolDL housed small to large sized neurons with short circuit and projection axons (Kalia M. et al., 1984). mPRα and mPRβ were observed here, which further supports a functional implication for mPR's in the direct integration of chemoreflex afferent input from the carotid bodies (Fig. 10). SolV which had type I, II and III neurons and SolVL which had type II and III neurons, are associated with the termination of vagal afferents from pulmonary stretch receptors (Kawai Y. and Senba E. 1999).

Finally, the AP region also displayed staining for both mPRα and mPRβ. AP has dense projections to the SolDL and SolDM. Lacking a blood brain barrier, the AP is highly susceptible to influence via circulating peptides and hormones such as progesterone. The ideal anatomical position of the AP also allows for significant effects to NTS neuronal activity and by this standard, effects on central respiratory control (Hay M. and Bishop V. 1991).

4.4   Previous Localization of mPR

The cloning of mPR has provided researchers with an excellent method for understanding the actions of progesterone in the nervous system. Previous studies have found mPRα to be found in neurons, astrocytes, oligodendrocytes and NG^{2+} progenitor cells of the mouse spinal cord, which has hinted at progesterone's role in promyelation and neuronal protection. The study was conducted by Labombarda et al, who also sought to determine the expression patterns of mPRβ. They discovered that the distribution of mPRα was greater than that of mPRβ in the mouse spinal cord, counter to the current observations in the brainstem. The study also found that both receptors had stable expression patterns regardless of sex or the presence of the classical PR. However, mPRβ was not seen in glial cells, but instead in ventral horn motoneurons and neurites, which was indicative of a role in neuronal transmission and plasticity. Such studies are paving the way for future in vivo molecular and pharmacological strategies at uncovering the exact function of mPR's (Labombarda F. et al., 2010).

Using northern blot analysis, Zhu et al 2008 also found broad mPRβ expression but not mPRα or mPRγ in the nervous system. This expression was found in the spinal cord, cerebral cortex, cerebellum, thalamus, pituitary gland and caudate nucleus. Using a more sensitive assay, real-time polymerase chain reaction (RT-PCR), Tang et al 2005 found mRNA of all three mPR's in the human brain. mPRα and mPRβ expression fluctuated in the brain with hormonal changes. Highest expression occurred at proestrous followed by a decline during the luteal phase when progesterone levels were at their peak (Dressing G. et al., 2011). mPRα and mPRβ have been generally found to colocalize and have high expression in the brain. A bulk of evidence points to the involvement of mPR's in non-genomic progesterone action in target tissues (Zhu Y. et al., 2003, 2008).

4.5   Neural Expression of Classical Progesterone Receptor

Aside from membrane receptors, the "classical" and most widely known progesterone receptors are cytosolic receptors belonging to the super-family of steroid receptors, acting as transcription factors to regulate gene expression (Gonzales-Flores O. et al., 2011). PR containing neurons in the pre-optic area and hypothalamus contain enzymes that synthesize neurotransmitters. Studies have indicated that neurons of the NTS contain various neuropeptides, catecholamines as well as

excitatory and inhibitory amino acids (Kawai Y. and Senba E. 1999). A high percentage of the PR containing neurons express TH, Gamma amino-butyric acid (GABA), decarboxylase (GAD) and tryptophan hydroxylase (TPH - the key enzymes in the synthesis of 5-HT). Sex steroid hormones can modulate neurotransmission by regulating expression of neurotransmitter synthesizing enzymes/receptors in the brain. One study indicated that progesterone was involved in the regulation of these enzymes through interaction with PRA and PRB, and that PR isoforms can differentially regulate TPH, TH and GAD expression (Gonzales-Flores O. et al., 2011). A study conducted by Haywood et al in 1999 discovered that PR expression in the rat brainstem NE neurons fluctuated throughout the estrous cycle. Using the double labeling immunofluorescence technique, Haywood et al showed that there exist NE neurons in the NTS expressing the PR. Interestingly, the NTS is where TH colocalized with mPR in the current study (Fig. 11). Not only was PR expressed in these neurons, but it also exhibited a cyclical expression pattern driven by circulating estrogen levels. Such changes to progesterone receptor expression in NTS neurons may have represented the molecular events that underlie the ability of progesterone to alter NE transmission (Fig. S5; Haywood S. et al., 1999).

4.6   Crosstalk between Membrane and Nuclear Progesterone Receptors

A question recently asked by researchers is: does progesterone act in a 2-step manner combining genomic and non-genomic mechanisms of action to render its biological effects? Extrapolating knowledge from cell growth studies, it can be said that there lies a link in between non-genomic activation of signal transduction pathways and long-term effects on the regulation of cell growth (Kawata M. et al., 2008). The colocalization of mPR's and classic PR's raises the potential for crosstalk to occur (Cutini P. et al., 2009). A main outcome of such an interaction could be an alteration in receptor signalling. Stimulation by progesterone produces rapid non-genomic actions initiated by mPRs at the plasma membrane (Zhu Y. et al., 2003, 2008). Changes in these signalling factors can lead to long-term changes to cellular transcription and regulation of PR transcriptional activities (Kawata M. et al., 2008). Insight into crosstalk taking place between the mPR and PR provides researchers with a greater understanding of how progesterone functions in target tissues such as those involved in central respiratory control (Cutini P. et al., 2009). Figure S6 demonstrates a visual example of what crosstalk may resemble.

5 Conclusion

In conclusion, mPRα and mPRβ were found in various subnuclei of the caudal NTS implicated in respiratory control suggesting that these receptors too have a role to contribute in central respiratory control. mPRα and mPRβ were also found in catecholaminergic neurons in the caudal NTS expressing TH. Lastly, expression of 3β-HSD, an enzyme involved in progesterone synthesis, was reported in areas involved in respiratory control.

6 Appendix: Supplementary Figures
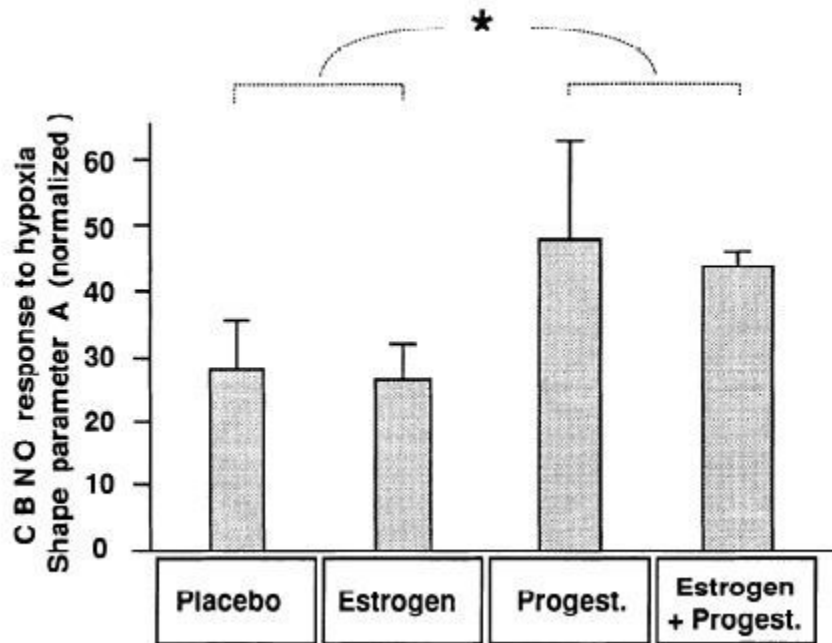
Figure S1 – The CBNO response to hypoxia in 2 groups of cats receiving progesterone treatment was stronger than response in 2 groups not receiving progesterone treatment. *P<0.05 (Adapted from Hannhart B. et al., 1990).
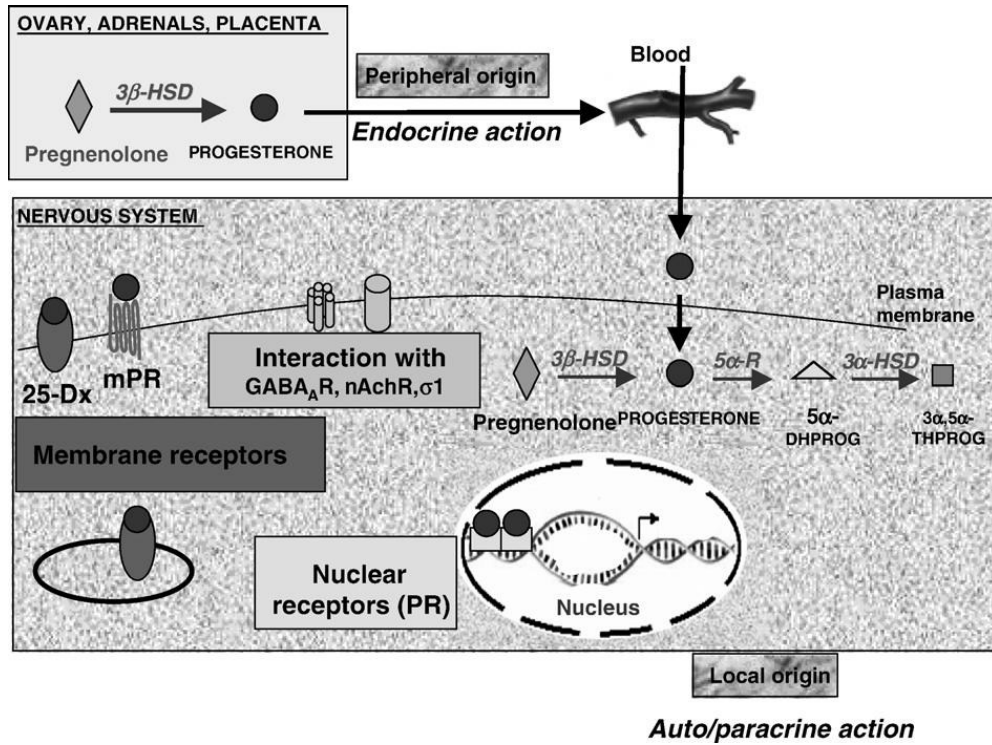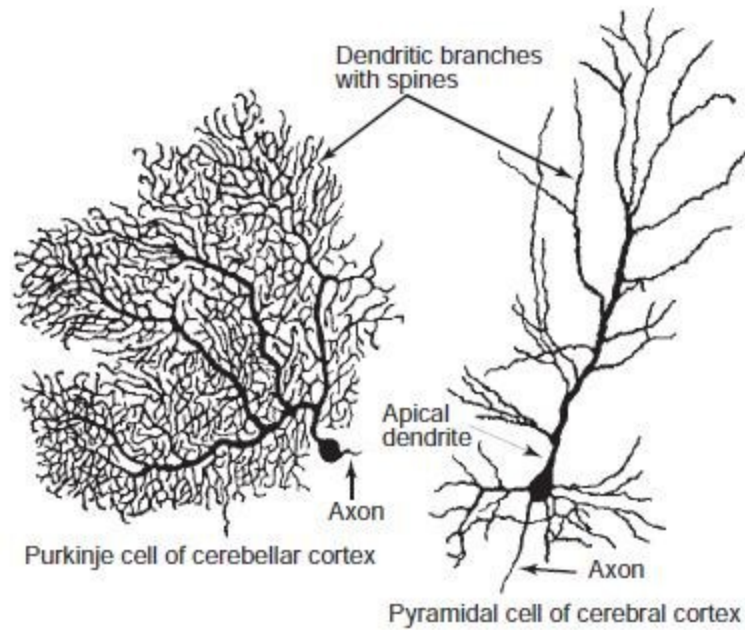
Figure S2 – **Progesterone in the nervous system: metabolism and different mechanisms of action.** (Adapted from Guennoun R. et al., 2008)
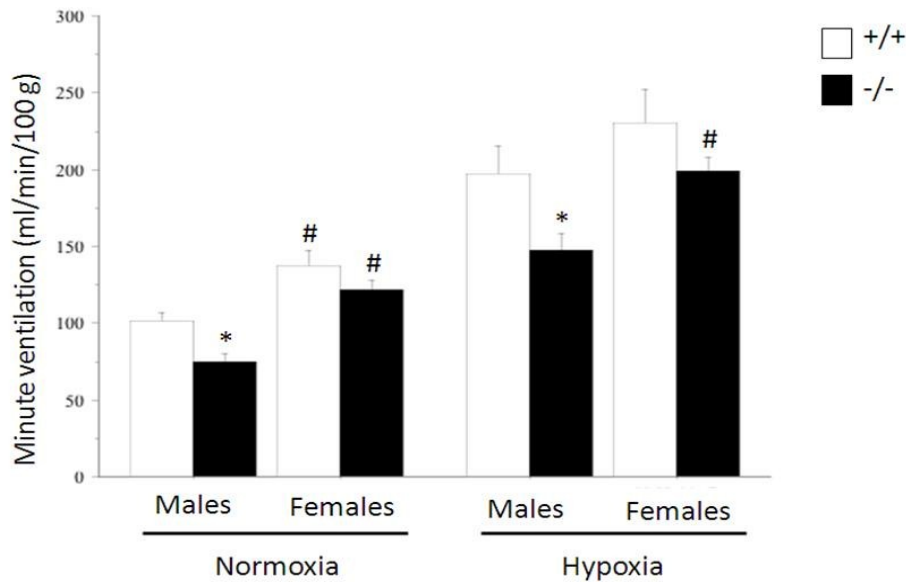
Figure S3 – **Minute ventilation during normoxia and hypoxia in wild type and PRKO (-/-) adult male and female mice. +/+ Homozygous for PR; -/- Homozygous PRKO. *: p<0.05 -/- vs. +/+, #: p<0.05 females vs. males**

Figure S4 – **Typical morphology of projection neurons.** (Left) A Purkinje cell of the cerebellar cortex and (right) a pyramidal neuron of the neocortex. These neurons are highly polarized. Each has an extensively branched, spiny apical dendrite, shorter basal dendrites, and a single axon emerging from the basal pole of the cell (Adapted from Fundamental Neuroscience 3rd edition).
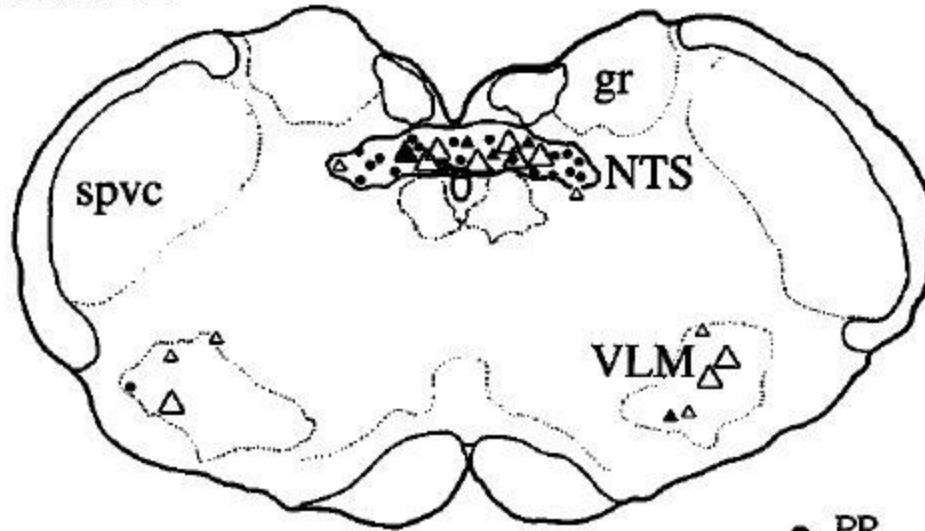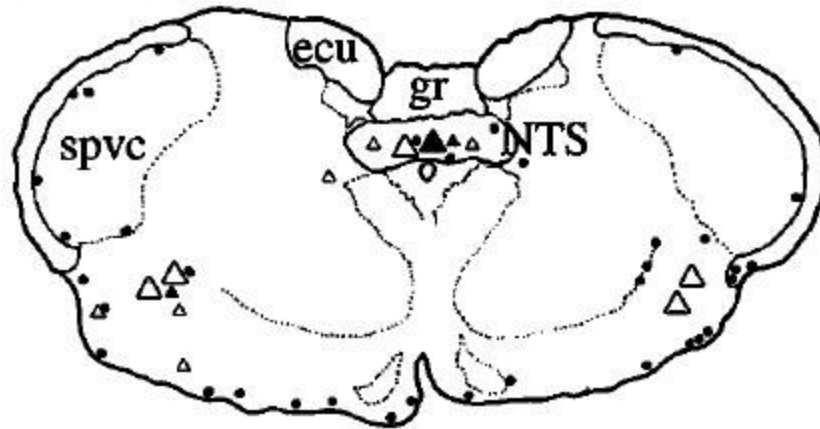
Figure S5 – Camera lucida diagrams of single and double labeled cells exhibiting TH and PR immunoreactivity at two AP levels of the caudal brainstem of the rat. Large triangles denote three to five TH-immunoreactive neurons, whereas small triangles represent one or two neurons. ap, Area postrema; ecu, cuneate nucleus; g, gracile nucleus; spvc, caudal part of the spinal nucleus of the trigeminal nerve; xii, hypoglossal nerve (Adapted from Haywood S. et al., 1999).
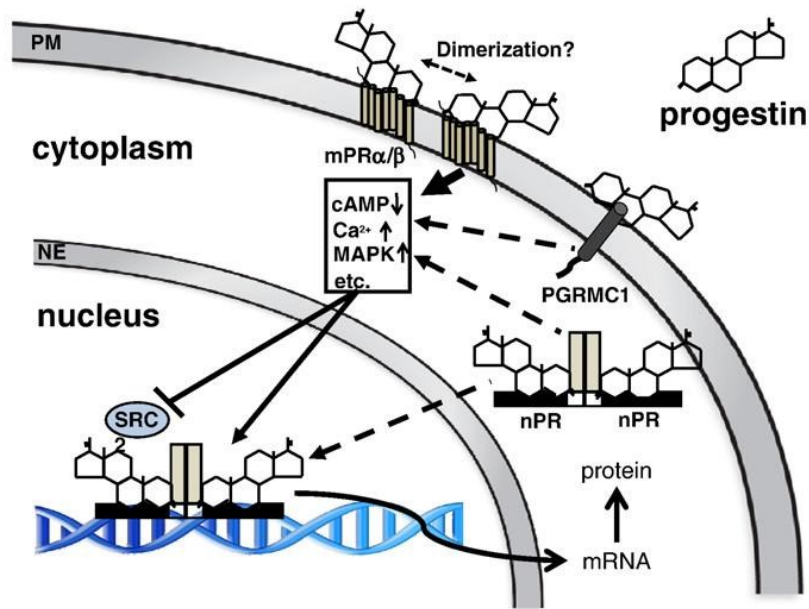
Figure S6 – **Potential nongenomic and genomic signaling pathways of progestins via membrane progestin receptors (mPRs), progestin receptor membrane components (PGRMC) and nuclear progestin receptors (nPR)**. NE=nuclear envelope, PM=plasma membrane and SRC2=steroid receptor coactivator 2 (Adapted from Zhu Y. et al., 2008).

7 Acknowledgements

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