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Expression of the ghrelin receptor gene in neurons of the medulla oblongata of the rat

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ABSTRACT

There is ambiguity concerning the distribution of neurons that express the ghrelin receptor (GHSR) in the medulla oblongata. In the current study we have used a sensitive non-radioactive method to investigate GHSR mRNA distribution by in situ hybridization. Strong expression of the GHSR gene was confirmed in neurons of the facial nucleus (FacN, 7), the dorsal vagal complex (DVC) and the semi-compact (but not compact) nucleus ambiguus (AmbSC and AmbC). In addition, expression of GHSR was found in other regions, where it had not been described before. GHSR-positive neurons were observed in the gustatory rostral nucleus tractus solitarius and in areas involved in vestibulo-ocular processing (such as the medial vestibular nucleus and the nucleus abducens). GHSR expression was also noted in ventral areas associated with cardio-respiratory control, including the gigantocellular reticular nucleus, the lateral paragigantocellular nucleus, the rostral and caudal ventrolateral medulla, the (pre)-Bötzinger complex and the rostral and caudal ventrolateral respiratory group. However, GHSR-positive neurons in ventrolateral areas did not express markers for cardiovascular presympathetic vasomotor neurons, respiratory propriobulbar rhythmogenic neurons or sensory interneurons. GHSR-positive cells were intermingled with catecholamine neurons in the dorsal vagal complex but these populations did not overlap.

Thus, the ghrelin receptor occurs in the medulla oblongata in i) second order sensory neurons processing gustatory, vestibulo-ocular and visceral sensation; ii) cholinergic somatomotor neurons of the FacN and autonomic preganglionic neurons of the DMNX and AmbSC; iii) cardiovascular neurons in the DVC, Gi and LPGi; iv) neurons of as yet unknown function in the ventrolateral medulla.

INTRODUCTION

Physiological studies indicate that ghrelin, or synthetic compounds that are agonists at ghrelin receptors, influence a range of physiological functions by their actions in the central nervous system. Effects in the hypothalamus, especially in relation to ingestive behavior, have been investigated in detail (Chaptini and Peikin, 2008; Chen et al., 2009; Higgins et al., 2007; Kojima and Kangawa, 2005). Consistent with this focus, several studies have investigated the distribution of the ghrelin receptor (growth hormone secretagogue receptor, GHSR) in the hypothalamus and rostral brain regions (Guan et al., 1997; Mitchell et al., 2001; Zigman et al., 2006). The description of receptor distribution has been extended into the medulla oblongata and cervical cord by Zigman et al. (2006), and receptor expression has been described in a sub-set of autonomic preganglionic neurons in the spinal cord (Ferens et al., 2010a; Ferens et al., 2010b). In both the medulla oblongata and the spinal cord, investigations using ghrelin receptor agonists have revealed physiological effects, notably on autonomic functions.

Several discrepancies occur in the published observations of ghrelin receptors in the medulla. Direct injection of ghrelin into the nucleus of the solitary tract (NTS) decreased blood pressure and heart rate, and reduced renal sympathetic nerve discharge (Lin et al., 2004). Consistent with this, expression of the ghrelin receptor gene has been demonstrated in NTS neurons (Zigman et al., 2006). However, it has been reported that the hypotensive effect of ghrelin in the NTS is mimicked by des-acyl ghrelin (Tsubota et al., 2005), although conventional ghrelin receptors are not efficiently stimulated by des-acyl ghrelin (Bednarek et al., 2000; Kojima et al., 1999). Moreover, ghrelin receptor immunoreactivity has been reported in the cardiovascular regulating centers in the rostral and caudal ventrolateral medulla, (RVLM and CVLM, respectively (Lin et al., 2004)), despite the observation made by these authors that micro-injection of ghrelin into these regions did not affect blood pressure or heart rate. In contrast, Zigman et al. (2006) did not report receptor expressing neurons in either RVLM or CVLM.

Receptor expressing neurons have been described in the nucleus ambiguus (Zigman et al., 2006), and could have a role in regulation of heart rate and the esophagus. There is also functional evidence of effects on the digestive system. Injection of ghrelin into the 4th ventricle or into the caudal region of the dorsal vagal complex relaxed the stomach, which suggests that receptors might be present on vagal preganglionic neurons (Kobashi et al., 2009). Consistent with this hypothesis, the effects of injection into the 4th ventricle were prevented by vagotomy.

The present work aimed to determine the distribution of ghrelin receptor expressing neurons in the medulla oblongata of the rat and relate these to physiological studies of the sites of ghrelin

actions. We sought to resolve the apparent discrepancy concerning receptor expression in the RVLM and CVLM by combining *in situ* hybridization with localization of tyrosine hydroxylase (TH), a marker of the catecholaminergic neurons of the C1 group of the RVLM and the A1 group of the CVLM.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (300-350 g) were maintained on a 12:12 hour light-dark cycle and were provided with food and water ad libitum. Rats were deeply anesthetised and subsequently transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PFA/PBS), pH7.4. Brains were removed and post-fixed overnight in 4% PFA/PBS at 4^{o}C. A total of 21 rats was used for the experiments described in this paper. The procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Combined in situ hybridization (ISH) and immunohistochemistry (IHC)

A plasmid to prepare antisense cRNA complimentary to the rat ghrelin receptor mRNA was constructed using a partial rat ghrelin receptor cDNA of 913 bp that was amplified by PCR using forward primer TGTGGTGGTGTTTGCTTTCATCC and reverse primer GGACCTACTTTTCCATGCTCAAATT. The amplified product spans the predicted exon 2 and 352 bp of the 3´ untranslated region of the rat ghrelin receptor gene. The PCR product was cloned into the pCRII-TOPO vector (Invitrogen, Melbourne, Australia). The sequence of the insert was confirmed and its orientation was established using M13 forward and reverse primers. Linear templates were prepared by PCR using M13 forward and reverse primers. Antisense and control sense digoxigenin (DIG)-labeled cRNA were prepared by *in vitro* transcription with T7 and SP6 RNA polymerases (Roche Products, Dee Why, NSW, Australia), respectively.

The fixed brain tissue was washed twice in ice-cold PBS, pretreated with diethylpyrocarbonate (DEPC-PBS) and the medulla was divided into rostral and caudal segments, which were then equilibrated overnight at 4 °C in 30% (w/v) sucrose (molecular grade; Sigma) in DEPC-PBS before being embedded in optimal cutting temperature compound (OCT; Tissue Tek, Elkhart, IN, USA) and snap-frozen in isopentane cooled by liquid nitrogen. Transverse cryostat sections (30 μm) were cut and floated in individual wells of an unused, sterile 24 well tray (Costar, Corning Inc, Corning, NY), containing ice-cold DEPC-PBS. Sections were subsequently transferred to a fresh 24 well tray, containing DEPC-PBS. Care was taken to minimise carry-over of OCT, as residual OCT may react with the alkaline phosphatase substrate used to detect the mRNA probe (see below). Sections were then transferred to unused, sterile 48 well trays (Costar) containing 150 μL hybridization buffer per well, consisting of 50% formamide, 4x SSC (standard salt concentration), pH 7.5, 10% dextran sulphate, 1x Denhardt's solution (Sigma-Aldrich, Sydney, Australia) and 0.1 mg mL^{-1} yeast RNA. Sections were prehybridized at 60 °C for 1 h, before hybridization with the GHSR antisense or control sense cRNA in hybridization buffer, overnight at 60 °C. Following hybridization,

sections were transferred to 24 well trays and washed for 2 times 30 min at 60 °C in 1x SSC, 50% formamide, 0.1% (v/v) Tween-20, followed by 5 washes (10 min) in MABT (150 mmol.L^{-1} NaCl containing 100 mmol.L^{-1} maleic acid and 0.1% Tween20, pH 7.5) at room temperature. Washes were performed while using a dissection microscope to locate the sections in the wells and carefully aspirating the buffers around them with a 21G needle attached to a 5 ml syringe.

Sections were incubated for 2 h at room temperature in blocking solution, consisting of 20% sheep serum plus 10% Roche Blocking Reagent (Roche Diagnostics) in MABT and then incubated overnight at 4 °C in a combination of alkaline phosphatase (AP)-conjugated sheep-anti-digoxigenin (DIG) Fab' (Roche Diagnostics) plus primary antibodies at the dilutions indicated in Table I. Two different mixtures were used: 1) Sheep α DIG plus Mouse α CART and Rabbit α TH (Figures 5 and 7) or, 2) Sheep α DIG plus Mouse α TH and Rabbit α NK1R (Figures 6 and 7). After primary antibody incubation, sections were washed 3 times 10 min in MABT followed by incubation in Donkey α Mouse Alexa 594 and Donkey α Rabbit Alexa488. Subsequently, sections were washed 5 times 10 min with MABT, 2 times 10 min with detection buffer (100 mM NaCl, 50 mM MgCl2, 100 mM Tris, pH9.5 and 0.1% Tween20) and incubated with NBT/BCIP (nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate; Roche #11681451001) substrate in detection buffer (20 µL per mL) for detection of AP activity.

For ChAT labelling combined with *in situ* hybridization (Figures 4 and 6) a modified protocol was used: Following the post-hybridization washes, sections were incubated for 2 h at room temperature in 10% horse serum in MABT and then incubated overnight at 4 °C in Goat α ChAT and rabbit α TH, followed by 3x10 min washes with MABT and a 2 h incubation with Donkey α Sheep Alexa594 and Donkey α Rabbit Alexa488 at room temperature. After 3x10 min washes with MABT, sections were incubated for 2h in 20% sheep serum plus 10% Roche Blocking Reagent in MABT, followed by an overnight incubation with Sheep α DIG-AP and AP detection, as described above. In control experiments, involving sense GHSR cRNA probes, AP activity was not detected. Sections were transferred to Superfrost Plus slides (Menzel GmbH, Braunschweig, Germany) and coverslipped using a mixture of Mowiol (polyvinylalcohol 44-88, Sigma-Aldrich, St Louis, MO) in 24 (v/v)%glycerol in 100 mM Tris/HCl, pH 8.5(Heimer and Taylor, 1974).

At the time of writing, except for the mouse α CART antibody, details for all antibodies used in this study can be found in the JCN antibody database, justifying the use of these reagents for the purposes described here. The manufacturer (R&D Systems) reports that the mouse monoclonal anti-CART antibody detects recombinant human CART (both the full length mature 88 AA peptide and a truncated 48 AA C-terminal peptide, found predominantly in the hypothalamus) in ELISA assay and Western blot. The CART staining patterns we describe in the rat medulla are

identical to those described before (Burman et al., 2004); (Abraham et al., 2009) with a rabbit α CART antibody manufactured by Phoenix Pharmaceuticals, details of which can be found in the JCN database (Kirouac et al., 2006). Furthermore, in a direct comparison, Gonsalvez et al., (2010) reported that in their hands the staining obtained with the R&D Mouse α CART antibody was indistinguishable from the Phoenix Pharmaceuticals Rabbit α CART antibody.

Nissl staining

Thirty µm cryostat sections were collected in PBS, mounted on Superfrost Plus slides and airdried. Slides were placed in 10% neutral buffered formalin for 5 min, and then incubated in a 0.025% cresyl violet acetate solution (pH3.8) at 37°C for 10 min, and rinsed in distilled water. Slides were differentiated in freshly made 95% ethanol for 1~2min, then dehydrated in 100% ethanol (3x 0.5min), cleared in Histolene (3 x 0.5 min) and mounted in DPX mounting medium.

Microscopy and image collection

Slides processed for *in situ* hybridization and Nissl staining were first scanned using a Mirax automated histological slide scanner (Zeiss, Sydney, Australia) and, after assessment of the staining, low power images were captured from the Mirax scan files using the Zeiss Mirax Viewer software. Images were subsequently processed with Adobe Photoshop CS4, using rotation and crop tools.

High resolution images were collected with a Zeiss AxioImager M2 microscope equipped with a motorised Z-stage and an Apotome module. Z-stacks were collected with either an HrC AxioCam (for color brightfield images) or an MRm AxioCam digital camera (for grayscale brightfield and fluorescence Apotome images). Fluorescence images of immunolabeled cells in triple-labeled sections were collected with the Apotome module, using the optical sectioning mode, in parallel with grayscale brightfield images of the *in situ* hybridization staining in the same fields. Z-stacks were collapsed using the extended focus function in the Zeiss Axiovision software and exported in TIFF format. To visualise the relationships between neighbouring cell groups, high resolution panoramas were created by stitching together images obtained from adjacent fields in Adobe Photoshop CS4, using the auto-align and auto-blend functions. To facilitate comparison of brightfield ISH staining with fluorescent IHC staining in the same field, brightfield images were inverted, using Adobe Photoshop CS4, so that the stained cells appear bright in a dark background. These images where then layered upon the fluorescence images and blended using the "exclusion" style.

Micrographs were adjusted for brightness and contrast and combined in montages in Adobe Photoshop CS4. Plates were annotated using the Corel Draw X3 graphics suite (Corel Corporation, Dublin, Ireland).

RESULTS

A non-radioactive *in situ* hybridization protocol identifies novel sites of GHSR mRNA expression

Expression of the gene for the ghrelin receptor, GHSR, was analysed in rat brain by non-radioactive *in situ* hybridization. The cRNA probe that was used was 913 bp long and comprises all of the second exon and 352 bp of the 3' UTR of the rat GHSR gene (Ferens et al., 2010a). It thus specifically recognizes the functional GHSR1a splice variant and gives no information regarding expression of the non-functional truncated GHSR1b variant. Following the non-radioactive protocol, the staining patterns that were observed with this probe in the rat hypothalamus were identical to those reported earlier with radioactive methods (Bennett et al., 1997; Zigman et al., 2006); results not shown). When GHSR expression was examined in the medulla oblongata the most conspicuous labeling was found in various aspects of the dorsal vagal complex (Fig. 1A-F), i.e. the dorsal motor nucleus of the vagus (DMNX, 1C and D), the nucleus tractus solitarius (NTS, 1A and C) and the area postrema (AP, 1A, C and F), the nucleus ambiguus (Fig. 1A and B) and the facial nucleus (Fig.1H and I), as also previously reported (Zigman et al., 2006). The background staining was very low and specific GHSR mRNA staining could be localized with high resolution to intracellular perinuclear structures (Fig.1E and G). The low background of our method, combined with its high resolution (i.e. the limited restriction of the staining to specific intracellular structures), allowed us to confidently identify less intensely stained cells as being positive for expression of the GHSR gene. For example, medial to the facial nucleus we noted expression in gigantocellular nucleus (Gi) and lateral paragigantocellular nucleus (LPGi; Fig.1I and J). The previously unreported GHSR mRNA labeling of structures that is described in this paper was not detected in control experiments using sense GHSR cRNA probes.

Novel sites of GHSR mRNA expression in the medulla oblongata

Prompted by the observation of GHRS mRNA expression at a site where it had not been described before we surveyed expression of this gene from the border between the pons and the rostral medulla (~ Bregma -10.30 mm) to just caudal of the obex (~Bregma-14.60 mm) using coronal sections of 30 µm thickness. This survey indicated a mixture of known and novel sites of GHSR mRNA expression and the data are summarised in maps at the end of this paper (Figure 7). Thus, again in agreement with (Zigman et al., 2006), we find GHSR expression in the superior salivatory nucleus (SuS;) and the raphe magnus at the most rostral medulla. On the other hand,

novel, distinct GHSR mRNA expression was found, for instance in the medial vestibular nucleus (MVe; Fig. 2A, C), the nucleus abducens (6) and the rostral nucleus tractus solitarius (rNTS).

Within and around ventral areas involved in cardiovascular control, such as the RVLM and CVLM, we noted the occurrence of small neurons expressing low-to-moderate levels of GHSR mRNA. These cells were found just ventral and medial to the compact formation of the nucleus ambiguus (AmbC; Fig 2E-L). The AmbC itself, which in brightfield appears as a tight cluster of neurons that are more translucent than their surroundings, does not contain significant levels of GHSR mRNA expression. Clusters of small GHSR mRNA expressing cells overlapped not only with areas containing the RVLM (Fig. 2E, G and H) and CVLM (Fig 2I-L), but also with areas containing neurons belonging to respiratory centers such as the Bötzinger (Bötz; Fig 2E) and pre Bötzinger (prB; Fig 2G) complexes, the rostral ventral respiratory group (RVRG; Fig 2H) and the caudal ventral respiratory group (CVRG; Fig. 2L).

Expression of GHSR mRNA in cholinergic motor neurons in the medulla.

Next, we set out to characterize the GHSR-expressing neurons identified in our survey in more detail, using antibodies detecting various functional neuronal markers and landmarks in the medulla. We performed combined *in situ* hybridization(ISH)/immunohistochemistry (IHC) for GHSR and choline acetyltransferase (ChAT) to investigate expression of the ghrelin receptor in cholinergic (pre)motor neurons (Figure 3). To facilitate comparison between the ISH signal and the ChAT IHC signal, the brightfield images of the GHSR ISH staining were inverted and the resulting images blended with the ChAT immunofluorescence images. The results shown in Fig 3A-F indicate that virtually all GHSR expressing neurons in the facial nucleus express ChAT.

As mentioned earlier, the AmbC, which appears as a tight cluster of round, ChAT-positive neurons, does not express appreciable amounts of GHSR mRNA (Fig G-I). However, in the same field two large GHSR expressing neurons and a small one are located ventro-laterally of the AmbC. Only one of these neurons expresses ChAT and presumably belongs to the AmbSC, whereas the ChAT-negative neurons may be part of either the AmbL or AmbE.

Likewise, GHSR-expressing neurons in the DMNX (Fig 3J-L) are either ChAT-positive or negative. Large, round-shaped, strongly GHSR-expressing neurons in the DMNX were invariably ChAT positive, whereas smaller, weaker GHSR-expressing neurons could be either ChAT-positive or -negative.

Expression of GHSR mRNA in relation to cardiovascular control centers in the ventral medulla

Although our survey did not find strong levels of expression of the ghrelin receptor in areas occupied by cardiovascular control centers in the ventrolateral medulla, such as the caudal A5 area, the RVLM and the CVLM, clusters of neurons expressing low-to-moderate levels of GHSR mRNA were occasionally detected near or within these areas. To investigate expression of the ghrelin receptor in specific ventrolateral neurons involved in cardiovascular regulation, we combined IHC for tyrosine hydroxylase (TH, the rate-limiting enzyme in catecholamine biosynthesis) and cocaine and amphetamine related transcript (CART) peptide with GHSR ISH. Together, CART and TH are expressed by a large proportion of neurons in these centers (Burman et al., 2004), and for the RVLM in particular it has been demonstrated that all presympathetic vasomotor neurons (both catecholaminergic C1 neurons and non-C1 neurons) express CART (Burman et al., 2004).

Figure 4A-C shows ventral expression of GHSR mRNA at the boundary with the pons (~Bregma-10.40 mm). The A5 nucleus is clearly identified by TH and CART-expressing neurons. The TH and /or CART positive neurons in the A5 area do not express GHSR mRNA, nor do any of their neighboring cells. The nearest GHSR mRNA expression is found medially to the A5 nucleus, in the SuS (Fig 4B). More medially, GHSR mRNA is expressed in the Gi and LPGi, which contain neurons that have been implicated in the regulation of circulation (Babic and Ciriello, 2004; Dergacheva et al., 2010; Stornetta et al., 2004), but are not catecholaminergic.

Figure 4D-G represents images taken from a section at ~Bregma -11.80, caudal to the facial nucleus. Also here, there is no expression of GHSR mRNA in the TH or CART expressing neurons of the RVLM. However, ventral to the AmbC, there are scattered neurons expressing low-to-moderate levels of GHSR mRNA (Fig 4E, F; yellow arrows). Some of these neurons intermingle with the TH/CART expressing neurons of the RVLM (Fig 4F). GHSR mRNA continues to be expressed in the Gi and LPGi (Fig 4D; yellow arrows), but is not expressed by a number of CART-positive neurons (red arrows) found in these areas. The AmbC, which contains CART-positive neurons and is heavily innervated by CART-expressing fibers (Fig 4D, G), again does not appear to express significant amounts of GHSR mRNA, but a few GHSR-positive neurons are found at or near the periphery of this nucleus (Fig 4G; yellow arrows).

This pattern continues until the caudal level of the RVLM, as illustrated in the images of Figure 4G and H, taken from a section at ~12.50 mm caudal from Bregma. The number of GHSR-expressing neurons directly intermingling with the TH/CART neurons of the RVLM varies strongly along the rostro-caudal axis, and at some levels they are not observed at all, suggesting that they appear in clusters.

At caudal levels of the medulla (Fig 4J-M; ~Bregma -13.30 to -14.80 mm) strong GHSR mRNA expression is found associated with the nucleus ambiguus. At these levels there appears to

be a continuum of neurons that moderately-to-strongly express GHSR, extending ventrally from the Amb or nucleus retroambiguus (RAmb) into the CVLM, but not as ventral as the lateral reticular nucleus (LRt; Fig 4J-L). In the CVLM/A1 area TH and CART are not co-expressed to the same extent as in the RVLM/C1 area (Burman et al., 2004) and TH and/or CART expressing neurons in the CVLM area generally do not express GHSR mRNA. However, a rare example of a neuron expressing GHSR mRNA and TH and CART is shown in Figure 4L-P.

Expression of GHSR mRNA in relation to NK1R expressing respiratory control centers and cholinergic sensory interneurons in the ventral medulla

To address the possibility that small GHSR mRNA expressing neurons in areas ventral to the AmbC could be involved in respiratory control we combined GHSR ISH with IHC for the neurokinin 1 receptor (NK1R) on sections taken from Bregma -11.80 mm (just caudal to the facial nucleus) to Bregma -14.30 mm (the level of the obex). NK1R marks important subsets of respiratory neurons in the medulla, specifically propiobulbar neurons in the preBötzinger complex (prB), associated with the generation of respiratory rhythms (Gray et al., 1999; Guyenet and Wang, 2001), and excitatory inspiration augmenting (I-Aug) bulbospinal neurons in the rostral ventral respiratory group (RVRG) (Guyenet et al., 2002). To facilitate localization of the RVLM and CVLM in these sections we also included anti-TH immunohistochemistry.

In these sections (shown in Figure 5), NK1R is distinctly expressed in the AmbC, the RVLM and the CVLM, whereas along the rostro-caudal axis NK1R expression between the AmbC and RVLM/CVLM varies (see also Alheid and McCrimmon (2008)). It is particularly strong at ~ Bregma -12.30 mm (Fig 6C, H), where the prB is found and at ~Bregma -13.00 (Fig 6E), in the RVRG. However, no expression of GHSR mRNA in any kind of NK1R expressing neuron was detected in the ventral medulla, although GHSR mRNA expressing neurons were found intermingled with NK1R expressing neurons in the prB (Fig 5C, H), the RVLM (Fig 5G, I), the CVLM (Fig 5F, J), the RVRG (Fig 5E) and the CVRG (Fig 5F, J).

Another candidate for the small GHSR expressing neurons in the ventral medulla could be a set of cholinergic neurons that are localized in the medial portion of the RVLM (Stornetta et al., 2012). Recently, these neurons were surprisingly identified as interneurons regulating somatosensory and auditory stimuli, rather than circulatory or respiratory functions (Stornetta et al., 2012). To investigate this option we combined GHSR ISH with ChAT and TH IHC. The results (Fig 5G, K) indicate that GHSR expressing neurons in the RVLM area are distinct from both cholinergic sensory interneurons and C1 presympathetic vasomotor neurons.

Expression of GHSR mRNA in the dorsal medulla in relation to CART-innervated structures, and TH and NK1R expressing neurons

Next, we investigated the identity of GHSR expressing neurons in the dorsal medulla, using TH and CART IHC in combination with GHSR ISH. First, we addressed the precise location of GHSR mRNA expressing neurons we assigned to the rostral NTS (see Fig. 2A and B) in relation to the solitary tract (tractus solitarius; TS). The TS is revealed by CART-positive axon bundles, running in rostro-caudal direction (Fig 6A). The GHSR mRNA expressing neurons are found in an area that is lightly innervated by branching CART fibers, lateral to the TS, which defines the location of these cells as the rostrolateral NTS, which has been implicated in the processing of tactile information arising from the oral cavity and the tongue (see Corson et al. (2012) and references therein).

CART IHC proved very informative for the identification of the dorso-vagal complex (DVC) and its boundaries, as all aspects of the DVC (NTS, DMNX, AP) are innervated by CART-expressing fibres (Fig 6B-D; see also Abraham et al. (2009)). In the more rostral portion of the DVC, IHC for TH identifies the C2 adrenergic neurons (Fig 6B, F), whereas more caudally the A2 noradrenergic neurons are marked by TH (Fig 6C-E, G-I). In addition, TH is strongly expressed by a population of neurons in the AP (6J-L; see e.g. Faulconbridge et al. (2008)).

Combined GHSR ISH and TH/CART IHC showed that neither C2 adrenergic neurons, nor the more caudal A2 noradrenergic neurons express GHSR mRNA (Fig 6A-I). GHSR positive neurons in the cNTS are either found in clusters away from C2/A2 neurons or intermingling with these neurons (Fig 6F-I).

Likewise, in the AP clusters of GHSR mRNA expressing neurons and TH expressing neurons are predominantly segregated at the more rostral levels, where GHSR mRNA is expressed in a collar/V-shaped fashion and most TH-neurons are found dorsally to the GHSR neurons, with a few TH neurons intermingling with the GHSR collar (Fig 6J). More caudally, there is a higher degree of intermingling of GHSR mRNA and TH expressing neurons (Fig 6K). Importantly, also in the AP, neurons expressing both GHSR mRNA and TH were not found. In addition, we investigated the expression of GHSR mRNA in the AP in relation to NK1R and TH (Fig 6L-O). The results show there is no overlap between GHSR mRNA and NK1R expression in the AP, as these receptors are expressed by distinct populations of neurons. There is, however, a considerable overlap between NK1R and TH expression, with >80% of NK1R expressing neurons also expressing TH.

Together, our data show that GHSR mRNA expressing neurons in the DVC are not catecholaminergic.

Maps of GHSR mRNA expression in the medulla, in relation to TH expression

Our observations on GHSR mRNA expression in the rat medulla are summarized in the maps of Fig. 7A-L that were generated from representative transverse cryosections along the rostro-caudal axis of the medulla. The immunohistochemistry for TH that was performed together with GHSR *in situ* hybridization on these sections identified catecholamine synthesizing neurons that are also represented in these maps.

The maps indicate expression of the ghrelin receptor in two main categories: i) sensory neurons (mostly second order) and ii) motor efferents. Falling within the first category, would be dorsal neurons found in the rostral (gustatory) and caudal (visceral) NTS, the area postrema (AP), the medial vestibular nucleus (MVe) and the epifascicular nucleus (EF). Included in the latter category would be neurons of the facial nucleus, the nucleus abducens (6), the DMNX, the semicompact and loose formations of the nucleus ambiguus (AmbSC and AmbL) and the nucleus retroambiguus (RAmb).

Furthermore, clusters of ventro-lateral neurons of as yet unknown identity are found, expressing low-to-moderate levels of GHSR mRNA, in the vicinity of the RVLM, CVLM, Bötzinger compex and preBötzinger complex. GHSR mRNA is also expressed at moderate levels in ventro-medial areas, such as the Gi, LPGi and low levels in the ventral medullary reticular nucleus (MdV) and in medial areas like the intermediate reticular nucleus (IRt) and the paramedian nucleus (PMn)

In addition, the maps show distinct areas where the ghrelin receptor is notably absent. These include the hypoglossal (HGN, 12), the gracile (Gr), the cuneate (Cu), external cuneate (ECu) and the spinal trigeminal (Sp5) nuclei. Furthermore, there is no significant expression of the ghrelin receptor in the compact formation of the nucleus ambiguus (AmbC).

Clusters of ghrelin receptor expression are found in the vicinity of catecholamine producing centers within the medulla and in some areas they overlap with these centers, such as in the NTS, the AP and the RVLM and CVLM. However, co-expression of GHSR and TH was almost never observed, indicating that GHSR expressing neurons in these areas are distinct from catecholamine producing neurons.

DISCUSSION

In the medulla oblongata, the distribution of strongly stained neurons that expressed ghrelin receptor mRNA was quite similar to that described by Zigman et al. (2006). The concordance applies to the major nuclei, SuS, FacN/7, cNTS, DMNX/10, AP, and Amb. However, a more detailed rostro-caudal survey of the GHSR mRNA expression associated with the nucleus ambiguus in our study revealed that most of the GHSR gene expression is found in neurons of the more caudal and ventral formations of the Amb, including the AmbSC, AmbL, AmbE and RAmb, with little or no expression in the AmbC. In addition, we found more faintly reactive neurons in the regions adjacent to and partially overlapping with the C1 and A1 catecholamine groups and in the LPGi, Gi, IRt, MdV, Abd/6, EF, MVe, PMn, and rNTS nuclei. The validity of the localization is supported by the lack of reaction using a sense probe, and the congruence of localization with the work of Zigman et al. (2006), who used shorter probes and a different detection system. The fact that we report more extensive expression of the ghrelin receptor in the medulla than previously described is due to two reasons: First, the non-radio-active detection system we employed has higher resolution, allowing localization of mRNA at sub-cellular level. This enables the confident identification of weaker and more sparsely labeled structures. Second, our study focuses on a small area of the rat brain, whereas the Zigman study presented surveys of whole brain in both rat and mouse. A full description of all weaker labeled structures would not have been within the scope of that study. In evidence, the GHSR mRNA expression that we describe in the Gi (e.g. Fig. 1I, J) is also apparent in the Zigman study (see Fig. 1R in Zigman et al. (2006)), but was not commented on.

As previously discussed, the distribution of cells expressing ghrelin receptor mRNA does not match the distribution revealed by receptor immunohistochemistry (Zigman et al., 2006). Ghrelin receptor immunoreactivity was reported to occur in the NTS and DMNX, but not in the AP in one study (Lin et al., 2004). In another study the receptor was localized to the DMNX, but the NTS and AP were not commented on (Zhang et al., 2004). As we discuss below, there is convincing functional evidence that the receptor protein is expressed by AP neurons, suggesting deficiencies in these immunohistochemical studies in their detection of ghrelin receptor expression. Findings in the ventro-lateral medulla also differ between in situ hybridization and immunohistochemistry (see below).

Ghrelin receptor expressing neurons in cardio-respiratory control centers

Ghrelin receptor immunoreactivity has been reported in the cardio-respiratory regulating centers of the RVLM and CVLM (Lin et al., 2004), although whether the reactivity was in catecholamine neurons was not investigated. In contrast, Zigman et al. (2006) did not report receptor expressing neurons in the regions of the RVLM or CVLM, although they did determine co-localization of GHSR and TH in the hypothalamus. We have found small weak-to-moderately intensely stained neurons throughout the RVLM and CVLM, and in the respiratory regions that lie dorsal to the RVLM (Kanjhan et al., 1995; Smith et al., 2009). The intensity of the staining and the frequency of occurrence of these small cells are such that they may have been ignored in a whole brain survey, as performed by Zigman et al. The pattern of GHSR mRNA expression that we determined in the RVLM and CVLM areas is quite dissimilar to the dense, strong labeling of large neurons described in the immunohistochemical analysis by Lin et al. (2004).

Co-staining with TH, CART, and NK1R antibodies failed to detect significant levels of GHSR mRNA expression in the important functional subclasses of neurons marked by expression of these proteins (Fig. 5 and 6). Thus, GHSR is absent from catecholamine neurons of cardiovascular control pathways; it is absent from the A5 cell group (Kanbar et al., 2011), the C1 presympathetic neurons of RVLM, and the A1 group in the CVLM, that have significant roles in cardiovascular control (Dampney, 2009). In addition, GHSR was absent from CART expressing neurons of the RVLM, which is a marker of all presympathetic neurons in this nucleus (Burman et al., 2004). Furthermore, GHSR mRNA did not colocalize with ChAT in small neurons in the medial RVLM (Fig. 6G, K) that have recently been implicated in the modulation of sensory functions (Stornetta et al., 2012). GHSR expression was also absent from NK1R expressing neuron of the preBötzinger complex (propriobulbar rhythmogenic neurons; (Alheid and McCrimmon, 2008; Gray et al., 1999) and the RVRG (excitatory inspiration augmenting (I-Aug) glutamatergic bulbospinal neurons (Guyenet et al., 2002).

Other candidates for GHSR expressing neurons in the ventrolateral medulla are various subclasses of ventral respiratory neurons that are NK1R-negative (see Alheid and McCrimmon (2008) for a review), and non-cholinergic cardiac vagal preganglionic neurons that have been identified as part of the AmbE (Takanaga et al., 2003). Unequivocal identification of these neurons will require further histochemical, functional and retrograde tracing studies.

We consistently found clusters of GHSR mRNA expressing neurons in the ventromedial medulla, i.e. the region of the RMg, LPGi, Gi and GiV nuclei, dorsal and medial to the RVLM neurons (Fig.1F, Suppl. Fig 2E, K, Fig. 4A, C, D, H). This area includes the rostral ventro-medial medulla or RVMM (Varner et al., 1994). It is noteworthy that the pattern of GHSR mRNA expression in the ventromedial medulla that we determined resembles the retrograde tracing results

of Stornetta et al. (2004), who found labeling in the RVLM, RPa, Gi, and GiV after injection of the trans-synaptic tracer pseudo-rabies virus (PRV) into the adrenal gland. The back-labeled neurons in the ventromedial areas were identified as GABAergic and glycinergic neurons that provide inhibitory inputs to sympathetic preganglionic neurons (Stornetta et al., 2004). The possibility that these inhibitory inputs to presympathetic neurons express the ghrelin receptor and may be modulated by ghrelin is intriguing and warrants future investigation. At this time there are no published data on the effects of ghrelin on LPGi, Gi or GiV neurons.

Finally, another cardiovascular control region that contains ghrelin receptor expressing neurons is the cNTS; injection of ghrelin into this nucleus lowers blood pressure and decreases heart rate (Lin et al., 2004; Tsubota et al., 2005). We confirmed expression of GHSR mRNA in this nucleus and furthermore established that GHSR expressing neurons are distinct from the C2 and A2 catecholaminergic neurons in the cNTS. This result was anticipated by a recent study by Cui et al. (2011) that suggested that modulation of electrophysiological properties of TH-expressing neurons in the cNTS by ghrelin involved a presynaptic mechanism, with GHSR-expressing vagal afferent neurons synapsing on TH-expressing neurons in the visceral NTS. Whether the effects of ghrelin injection into the visceral NTS on blood pressure involve a pre-synaptic or a post-synaptic mechanism is unknown at the present time.

GHSR expression in the area postrema

The area postrema (AP) is a circumventricular organ (CVO), lacking a complete blood-brain-barrier (BBB). Neurons in the AP are accessible to circulating ghrelin and have been implicated in the ghrelin-mediated feeding response (see Fry and Ferguson (2010) for a review) and modulation of gastrointestinal motility (Kobashi et al., 2009, Fujino et al., 2003). As it detects toxins, circulating in the blood or cerebro-spinal fluid (CSF), the AP is considered a chemoreceptor trigger zone (CTZ) for the emetic (vomiting) reflex (Sanger and Andrews, 2006). In suitable animal models, ghrelin has been implicated in the moderation of emesis, which may in part occur through central GHSR (Rudd et al., 2006; Sanger and Andrews, 2006). NK1R antagonists have been successfully introduced into the clinic as broad spectrum anti-emetics, which is likely due to their actions on NK1R expressed in brainstem nuclei, such as the AP (see Sanger and Andrews (2006) for a review). Our findings (shown in Fig. 6L) indicate that GHSR neurons in the AP are distinct from NK1R expressing neurons in that area. They further show that NK1R is expressed by catecholaminergic neurons in the AP and that GHSR expressing neurons are not catecholaminergic (Fig 6J-L). The distribution of the TH/NK1R double-positive cells in the AP resembles that of the noradrenergic neurons that have been implicated in amylin-mediated satiety (Potes et al., 2010).

Although further analysis on the chemical coding and functions of AP neurons is needed to confirm this, it appears that the AP may contain two separate, closely apposed populations of neurons, involved in the control of appetite and emesis: One ghrelin-responsive, and the other noradrenergic and amylin-responsive, expressing also NK1R.

A direct action of ghrelin on AP neurons has been confirmed by electrophysiological recording (Fry and Ferguson, 2009). In that study, 19% of AP neurons were excited and 19% were inhibited by ghrelin, with the remaining neurons being unresponsive (Fry and Ferguson, 2009). Which of these neurons are involved in the different effects mediated via the AP needs to be determined. We did not detect any conspicuous morphological distinctions that would characterize sub-groups of ghrelin receptor expressing neurons in the AP.

Modulation of foraging and feeding behavior by GHSR and ghrelin through the medulla

We discovered expression of the ghrelin receptor in a number of nuclei in the dorsal medulla that are involved in sensory processing or innervation of organs of sensation, such as the rostrolateral NTS , the medial vestibular nucleus (MVe), and more weakly in the nucleus abducens (Abd/6) and the epifascicular nucleus (EF). The MVe, Abd/6, and EF (Baizer and Broussard, 2010; Pompeiano et al., 2002) are all involved in the vestibulo-ocular reflex (VOR; (Cullen, 2012)). Amongst its roles, the VOR is essential for foraging behaviour (Zheng et al., 2006) and ghrelin injection stimulates foraging behavior in rodents (Keen-Rhinehart and Bartness, 2007). It would be of interest to investigate how ghrelin affects navigational performance during foraging, which likely involves these dorsal medullary nuclei. In addition, these nuclei are linked to the hippocampus (Zheng et al., 2006), another site of GHSR expression (Zigman et al., 2006) where ghrelin has been shown to act to improve spatial learning (Diano et al., 2006). Modulation of the VOR by ghrelin would also be in line with the effects this hormone has on olfaction and exploratory sniffing (Tong et al., 2011). Furthermore, the expression of GHSR in those aspects of the facial nucleus that innervate eyelids and outer ears (Zigman et al. (2006); confirmed in this paper) may similarly be linked to modulation by ghrelin of navigational performance and exploratory behavior.

The rostral NTS, where we found expression of the ghrelin receptor, processes gustatory information. The ghrelin/GHSR system has been implicated in gustation before (Disse et al., 2010; Overduin et al., 2012; Shin et al., 2010). Both ghrelin and GHSR are expressed in tastebuds and GHSR null mice exhibited reduced responsiveness to sour and salty tastes (Shin et al., 2010). Although ghrelin increased the motivation of rats to eat, it did not alter the palatability of the food on offer (Overduin et al., 2012). The precise location of GHSR mRNA in the rostrolateral NTS suggests that these neurons are part of the circuitry that processes tactile information in the oral

cavity (Corson et al., 2012). Thus, GHSR signaling in the rNTS may "prime" the circuitry involved in detecting the arrival of food in the oral cavity. Furthermore, rostrolateral NTS neurons project to the external lateral parabrachial nucleus (LPBE) in the pons (Halsell and Travers, 1997), another site of GHSR expression in the brain (Zigman et al., 2006). The parabrachial nucleus has recently been identified as a hub that integrates input from several brain regions (AGRP neurons in the arcuate nucleus of the hypothalamus, the raphe magnus, and the rNTS) to modulate appetite (Wu et al., 2012). It is intriguing that the ghrelin receptor is expressed in all aspects of this circuit. Future studies determining the impacts of GHSR signaling on the output of this circuit are warranted and should aid the understanding of the generation of appetite and its control.

Expression of GHSR in the nucleus ambiguus and retroambiguus

We found strong GHRS mRNA expression in the caudal and ventral aspects of the Amb (AmbSC, AmbL, AmbE), including the RAmb, from Bregma -13.00 mm to -14.60 mm (the caudal-most site examined in this study). Rostral to that, GHSR-positive neurons were found more sporadically just caudal to the AmbC, presumably in the AmbSC and AmbL, and possibly in the AmbE (small neurons intermingling with the RVLM). This pattern of expression is consistent with the GHSR-positive neurons innervating pharyngeal muscles (from ~ Bregma -13.00 to -14.00 mm),, and possibly innervation of cardiac ganglia, but not with innervations of the esophagus (Bieger and Hopkins, 1987). Caudal to the obex (within the RAmb), GHSR mRNA expressing neurons are most likely interneurons that may innervate motor neurons that supply a variety of targets, such as cricothyroid muscle, diaphragm and abdominal organs, and may be involved in processes that include vocalisation, vomiting, coughing, sneezing and the ventilatory response to exercise (Subramanian and Holstege, 2009). Retrograde tracing combined with GHSR ISH will be required to determine the connections of GHSR expressing neurons in the Amb and RAmb.

GHSR signaling in the brain in the absence of ghrelin

The GHSR gene is widely expressed in the brain (Bennett et al., 1997; Guan et al., 1997; Zigman et al., 2006), yet its only known natural ligand, acylated ghrelin (Kojima et al., 1999), is not found there (Furness et al., 2011; Sakata et al., 2009; Wortley et al., 2004; Grouselle et al., 2008). Thus, it has been a conundrum how the activity of the receptor is modulated in the brain, apart from at a few selected sites near circumventricular organs (CVO), like the area postrema that are readily accessed by circulating ghrelin.

For neurons whose terminals are outside the CNS, for example FacN motor neurons and DMNX preganglionic neurons, the site of ghrelin activity may be at receptors that are expressed in

the axon terminals that are accessible to circulating ghrelin, for example through a presynaptic inhibition (Cui et al., 2011). Alternatively, there may be an as yet undiscovered endogenous ligand, acting as a receptor agonist, or as an inverse agonist (Zigman et al., 2006) that modulates the constitutive activity of GHSR (Holst et al., 2003). A role for constitutive activity is suggested by the finding that the basal activity of GHSR enhances the signaling efficiency of the MC3R, which is co-expressed with GHSR in neurons in the arcuate nucleus and that unmodified substance P can act as an inverse agonist of GHSR (Rediger et al., 2011). Furthermore, the constitutive GHSR signal may be regulated by alteration of the expression levels of the receptor. For example, GHSR expression in nodose ganglia cycles following a circadian rhythm (Sato et al., 2007) and is affected by feeding status in nodose (Sato et al., 2007), hypothalamus and pituitary (Abizaid et al., 2008).

Finally, the recent discovery that GHSR1a forms functional and physiologically relevant heteromers with dopamine DRD2 receptors (Kern et al., 2012) provides another mechanism through which a physiological role of GHSR may be exerted. However, the GHSR expression pattern in the medulla is in many areas quite dissimilar to DRD2 expression (as determined from the Allen Brain Atlas (Henry and Hohmann, 2012)). The findings of Kern et al. (2012) suggest that further investigation of partner receptors and matching ligands for the GHSR needs to be undertaken.

Conclusions

We have confirmed earlier observations of the distribution of neurons with strong GHSR mRNA signals in specific cell groups of the medulla oblongata. In addition, we have discovered neurons with lower levels of receptor expression in several regions, either involved in sensory processing, such as the MVe, and the nucleus abducens and the rNTS, or in cardiovascular control, such as the Gi, GiV and LPGi. GHSR is not significantly expressed in some important cardiovascular neuronal subpopulations, such as the presympathetic vasomotor neurons (C1 and non-C1) of the RVLM or the A1 noradrenergic neurons of the CVLM. We found that GHSR expressing neurons in the dorsal vagal complex are distinct from the various catecholaminergic neurons found in this area. A more detailed analysis of GHSR expression in the nucleus ambiguus, suggests an involvement of this receptor in the modulation of the activity of the pharyngeal musculature, but rules out effects of GHSR signaling on the esophagus.

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Conflict of Interest

The authors declare no conflicts of interest

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: RB and JBF. Acquisition of data: RB and LY. Analysis and interpretation of data: RB, LY, DR and JBF. Drafting of the manuscript: RB, LY and JBF. Critical revision of the manuscript for important intellectual content: RB, DR and JBF. Obtained funding: JBF. Study supervision: RB and JBF.

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Figure Legends

Fig. 1. Non-radioactive *in situ* hybridization reveals novel sites of GHSR mRNA expression in the medulla, alongside known GHSR-positive structures. Low power images reveal GHSR expression in components of the dorsal vagal complex (DVC; 1A, C), i.e. the dorsal motor nucleus of the vagus (DMNX; 1A-D), the nucleus tractus solitarius (NTS; 1A, C), and the area postrema (AP; 1D, F, I), the ambiguus nucleus (Amb; 1A, B) and the facial nucleus (1H, I). Less intensely stained and more sparsely distributed cells are also readily observed in the gigantocellular nucleus (Gi, H) and the lateral paragigantocellular nucleus (LPGi; H), particularly at higher power (I, J). High power insets (E, G) reveal the subcellular distribution of the GHSR mRNA in perinuclear structures, that can be observed with the non-radioactive method. Sections taken at Bregma -13.60 mm (1A-F) and Bregma -11.45 mm (1H, I). Scale bars, 1 mm in 1A and H; 200 μm in 1C, I and B; 100 μm in 1D and J ; 20 μm in 1E and G.

Fig. 2. Novel sites of GHSR mRNA expression in the medulla. Following non-radioactive *in situ* hybridization, GHSR mRNA is detected in dorsal areas of medulla in the medial vestibular nucleus (MVe; 2 A, C), the gustatory rostral nucleus tractus solitarius (rNTS; 2A, B) and the nucleus abducens (6; 2C, D). In the ventrolateral medulla, small neurons expressing low-to-intermediate levels of GHSR mRNA, are found ventral to the nucleus ambiguus through the Bötzinger complex (Bötz; 2E), preBötzinger complex (prB, 2G, the rostral ventrolateral medulla (RVLM; 2E, G, H), the rostral ventral respiratory group (RVRG; 2H), the caudal ventral respiratory group (CVRG, 2L), and the caudal ventrolateral medulla (CVLM; 2J, L). Sections were taken at Bregma -10.90 mm (2A), -11.20 mm (2C), -11.80 mm (2E), -12.30 mm (2G), -12. 70 mm (2H), -12.80 mm (2I), -13.30 mm (2I, J) -13.80 mm (2K, L). Scale bars, 500 μm in 2I and K; 200 μm in 2A, C, E, G, H and J; 100 μm in 2L; 50 μm in 2B and D; 20 μm in 2F.

Fig. 3. Expression of GHSR mRNA in cholinergic neurons in the medulla. Combined immunohistochemistry for choline acetyl transferase (ChAT) and *in situ* hybridization (ISH) for GHSR on coronal sections of rat medulla. To allow direct comparison of the brightfield ISH signal (3A) with the ChAT immunofluorescence signal (3B) the brightfield image is inverted (3C). 3A-C , Expression of GHSR in ChAT expressing neurons in the facial nucleus. Virtually all GHSR mRNA expressing neurons in the facial nucleus express ChAT. 3G-I, Expression of GHSR and ChAT in the nucleus ambiguus (Amb). The AmbC appears as a tight cluster of ChAT expressing neurons

which do not express appreciable amounts of GHSR (3I). 3 neurons appear just adjacent to the AmbC, presumably belonging to the AmbSC or AmbL, with one expressing ChAT only, one GHSR mRNA only (yellow arrowhead), and one expressing both GHSR and ChAT (white arrowhead). 3J-L, Expression of GHSR mRNA and ChAT in the DMNX. GHSR mRNA expressing neurons in the DMNX are either ChAT-positive (white arrowheads) or ChAT-negative (yellow arrowhead). Scale bars: 50 μm (J-L), 100 μm (D-I), 500 μm (A-C)

Fig. 4. GHSR mRNA localization in the ventral medulla in relation to cardiovascular control centers, marked by TH and CART expression. Combined immunohistochemistry for Tyrosine Hydroxylase (TH) and Cocaine and Amphetamine Regulated Transcript (CART) and *in situ* hybridization (ISH) for GHSR on coronal sections of rat medulla. Sections at Bregma -10.80 mm (5A-C), -12.50 mm (5D-G), -12.80 mm (5H, I), -13.35 mm (J), -13.80 mm (K), -14.10 mm (L).

To allow direct comparison of the brightfield ISH signal with the immunofluorescence signals the brightfield image is inverted. GHSR mRNA is not expressed in the A5 area, which is identified by neurons that strongly express TH and CART (4A, B). The nearest GHSR mRNA expression is in the Superior Salivary nucleus (SuS, 5A, B). More caudally, GHSR mRNA expressing cells (yellow arrowheads in 4E, F, H and I), can occasionally be found near or within the area occupied by the rostroventrolateral medulla (RVLM), which is apparent by TH and CART expressing neurons (4D, E, F, H, I). However, GHSR mRNA is never co-localised with TH or CART in the RVLM. GHSR-positive neurons within the RVLM appear to be part of clusters of small cells that express to weak-to-moderate levels of GHSR mRNA and are frequently observed ventral to the AmbC (apparent by strong CART labelling; 4D, E, G, H). More caudally, the AmbC is no longer present, but similar clusters of cells are found dorsally from and intermingling with TH and CART expressing neurons in the caudoventrolateral medulla (CVLM; 4J, K, L, M). The majority of these cells presumably belong to the semi-compact (AmbC), loose (AmbL) and external (AmbE) formations of the nucleus ambiguus or the nucleus retroambiguus (RAmb). They generally do not express TH or CART (yellow arrowheads), with an exception (white arrowhead) shown in Fig 4L, M-O. Scale bars: 50 μm (M), 100 μm (J, K, L), 200 μm (B, C, E, F, G, I), 500 μm (A, D, H)

Fig. 5. GHSR mRNA localization in the ventral medulla in relation to NK1R-expressing respiratory nuclei and cholinergic sensory interneurons. To allow direct comparison of the brightfield ISH signal with the immunofluorescence signals the brightfield image is inverted. Combined *in situ* hybridization (ISH) for GHSR and immunohistochemistry for neurokinin-1

receptor (NK1R; 5A-F, H, I, J) or acetyl choline transferase (ChAT; 5G, K) on coronal sections of rat medulla. Sections at Bregma -11.80 mm (A), -12.10 mm (B), -12.30 mm (C), -12.70 mm (D), -13.60 mm (E), -14.10 mm (F), -12.70 mm (G). Clusters of cells expressing weak-to-moderate levels of GHSR mRNA are found ventral to the AmbC and throughout areas associated with respiratory control, such as the Bötzinger complex (Bötz; 5A, B) , the preBötzinger complex (prB; 5C, H), the rostral and caudal ventrolateral respiratory group (RVRG; 5D, E) and (CVRG; 5F, J). NK1R expression varies along the rostral-caudal axis, with low levels in the rostral Bötz and RVRG, increasing levels toward the caudal Bötz (5A, B), and very high levels in (propriobulbar) neurons in the prB (5C, H) and in (bulbospinal) neurons in the RVRG (5E). NK1R-expressing neurons are often intermingled with TH-expressing neurons of the RVLM and CVLM (5B-F, I, J). No examples of co-expression of GHSR mRNA in NK1R expressing neurons were identified in the ventrolateral medulla. ChAT-staining marks small sensory interneurons in the medial RVLM (5G, K), in addition to large motor neurons in the AmbC (5G). GHSR mRNA expressing small neurons (yellow arrows) are intermingling with ChAT (red arrows) and TH expressing neurons in the RVLM, but GHSR mRNA is not co-expressed with either of these markers (5K). Scale bars: 50 μm (H, I, J), 100 μm (B, D, F, K), 200 μm (A, C, E, G)

Fig. 6. GHSR mRNA localization in the rostral NTS (rNTS) and the dorsal vagal complex (DVC) in relation to TH, CART and NK1R expression. Sections at Bregma -11.50 mm (6A), -13.20 mm (6B, F), -13.60 mm (6C, G, J), -13.85 mm (6D, H, K, L), -14.20 mm (6E, H). To allow direct comparison of the brightfield ISH signal with the immunofluorescence signals the brightfield image is inverted. NB: The tissues surrounding the DVC are more opaque (see Fig. 3), resulting in a higher relative background in the inverted image in these areas (e.g. Fig. 6C). CART immunoreactivity is very prominent in the solitary tract (TS; 6A) and in fibre projections throughout the DVC, clearly defining the boundaries of the NTS and DMNX (10). TH marks C2 adrenergic neurons (6B, F), A2 noradrenergic neurons (6C, D, E, G, H, I) in the cNTS and noradrenergic neurons in the area postrema (AP; C, D, J, K-O). In the rNTS, GHSR mRNA expressing neurons are found lateral to the TS, in the rostrolateral NTS (6A). In the DVC, neurons expressing GHSR mRNA are found in the DMNX (10; 6B, C, D, G, H), cNTS (6B-I) and the AP (6C, D, J, K, L). GHSR mRNA is not found in TH expressing neurons throughout the DVC, or in NK1R-expressing neurons in the AP (6L). A very large proportion of NK1R-positive neurons in the AP also express TH (6L-O). Scale bars: 50 μm (A, F, L-O'), 100 μm (B, G, H, I, J, K), 200 μm (C, D, E)

Fig. 7. Maps documenting GHSR mRNA expression in the medulla oblongata, in relation to tyrosine hydroxylase (TH) expression. Circles depict GHSR mRNA expressing cells and x depicts TH expressing cells. The diameter and the darkness of the circles reflect the sizes and the intensities of staining of the GHSR-positive cells, respectively. Maps were constructed from images of triple-labeled transverse cryosections, cut along the rostro-caudal axis, processed for GHSR *in situ* hybridization and TH and CART immunohistochemistry and adjacent sections processed for Nissl staining. In some occasions, ChAT and NK1R immunostaining were used to precisely define the location of structures such as the AmbC. Landmarks were drawn in using maps from the Rat Brain Atlas by Paxinos and Watson (Paxinos G, Watson C. 1998. The rat brain in stereotaxic coordinates, 4th ed. San Diego: Academic Press).

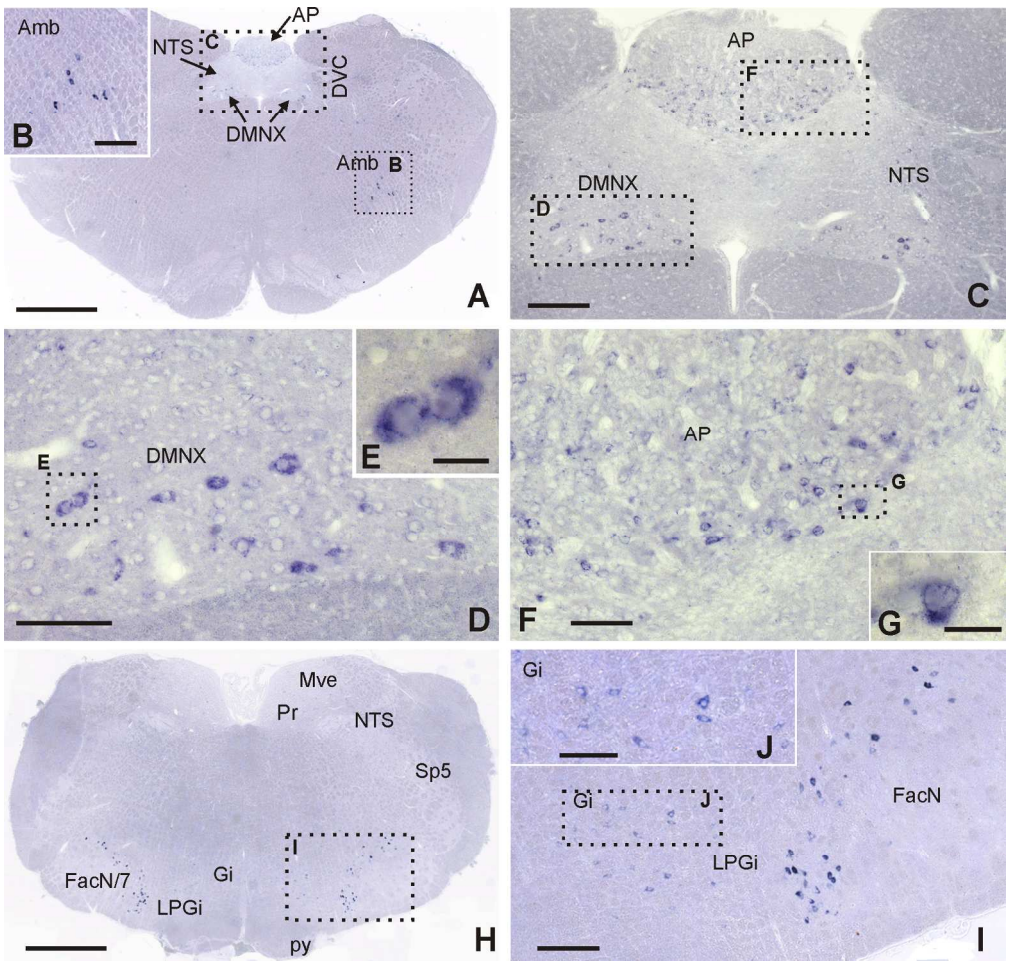
Abbreviations for anatomical structures used in this paper

6, nucleus abducens; 7, facial nucleus; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; 4V, 4th ventricle; A1, A1 noradrenaline cells; A2, A2 noradrenaline cells; Amb, Ambiguus nucleus; AmbC, Ambiguus nucleus , compact formation; AmbSC, Ambiguus nucleus , semi-compact formation; AmbL, Ambiguus nucleus, loose formation, AmbE, Ambiguus nucleus, external formation; AP, area postrema; Bo, Bötzinger complex; C1, C1 adrenaline cells; C2, C2 adrenaline cells; C3, C3 adrenaline cells; CC, central canal; CN, cochlear nucleus; Cu, cuneate nucleus; CVLM; caudal ventrolateral medulla; CVRG, rostral ventral respiratory group; DPGi, dorsal paragigantocellular nucleus; DMNX, dorsal motor nucleus of the vagus; DMSp5, dorsomedial spinal trigeminal nucleus; DVC, dorsal vagal complex; ECu, external cuneate nucleus; EF, epifascicular nucleus, FacN, facial nucleus; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus, alpha part; GiV, gigantocellular reticular nucleus, ventral part; HGN, hypoglossal nucleus; icp, inferior cerebellar peduncle; IO, inferior olive; IRt intermediate reticular nucleus; LPGi, lateral paragigantocellular nucleus; MdD, medullary reticular nucleus, dorsal part; MdV, medullary reticular nucleus, ventral part; ml, medial lemniscus; mlf, medial longitudinal fasciculus; MnA, median accessory nucleus of the medulla; MVe, medial vestibular nucleus; MVeMC, medial vestibular nucleus, magnocellular part; MVePC, medial vestibular nucleus, parvicellular part; NTS, nucleus tractus solitarius; PCRt, parvicellular reticular nucleus; PMn, paramedian reticular nucleus; PPy parapyramidal nucleus; Pr, prepositus nucleus; py, pyramidal tract; pyx, pyramidal decussation; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVLM, rostral ventrolateral medulla; RVMM, rostral ventromedial medulla; RVRG, rostral ventral respiratory group; sp5, spinal trigeminal tract; Sp5I, spinal trigeminal nucleus, interpolar part; Sp5C, spinal trigeminal nucleus, caudal part; TS, solitary tract.

TABLE 1

Source and concentrations of the primary and secondary antisera, used in this study

Primary Antibody Antigen	**Immunogen**	**Manufacturer**	**Dilution used**
Tyrosine Hydroxylase (TH)	TH purified from rat pheochromocytoma	Institut de Biotechnologies Jacques Boy (Reims, France); Rabbit polyclonal; #A52-512	1:800
Tyrosine Hydroxylase (TH)	TH purified from rat PC12 cells	Immunostar; Mouse Monoclonal; # 22941	1:1,000
Cocaine And Amphetamine Regulated Transcript (CART) Peptide	Synthetic peptide corresponding to human CART 28-116	R&D Systems; Mouse Monoclonal; #MAB163	1:2,000
Neurokinin 1 Receptor (NK1R)	Synthetic peptide corresponding to a 23 amino acid sequence (385-407) of the COOH terminus of the rat Substance P Receptor (NK-1).	Millipore; Rabbit Polyclonal; #AB5060	1:1,000
Choline Acetyltransferase (ChAT)	Human Placental Enzyme	Millipore; Goat Polyclonal; #AB144P	1:50
cRNA detection			
Sheep α digoxigenin (DIG) Fab' fragments, alkaline phosphatase (AP) conjugated		Roche; #11093274910	1:2,000
Secondary antibody		**Manufacturer**	**Dilution used**
Donkey α Rabbit Alexa 488		Molecular probes, Invitrogen; #A21206	1:1,000
Donkey α Sheep Alexa 488		Molecular probes, Invitrogen; #A11015	1:500
Donkey α Sheep Alexa 594		Molecular probes, Invitrogen; #A11016	1:500
Donkey α Mouse Alexa 594		Molecular probes, Invitrogen; #A21203	1:500



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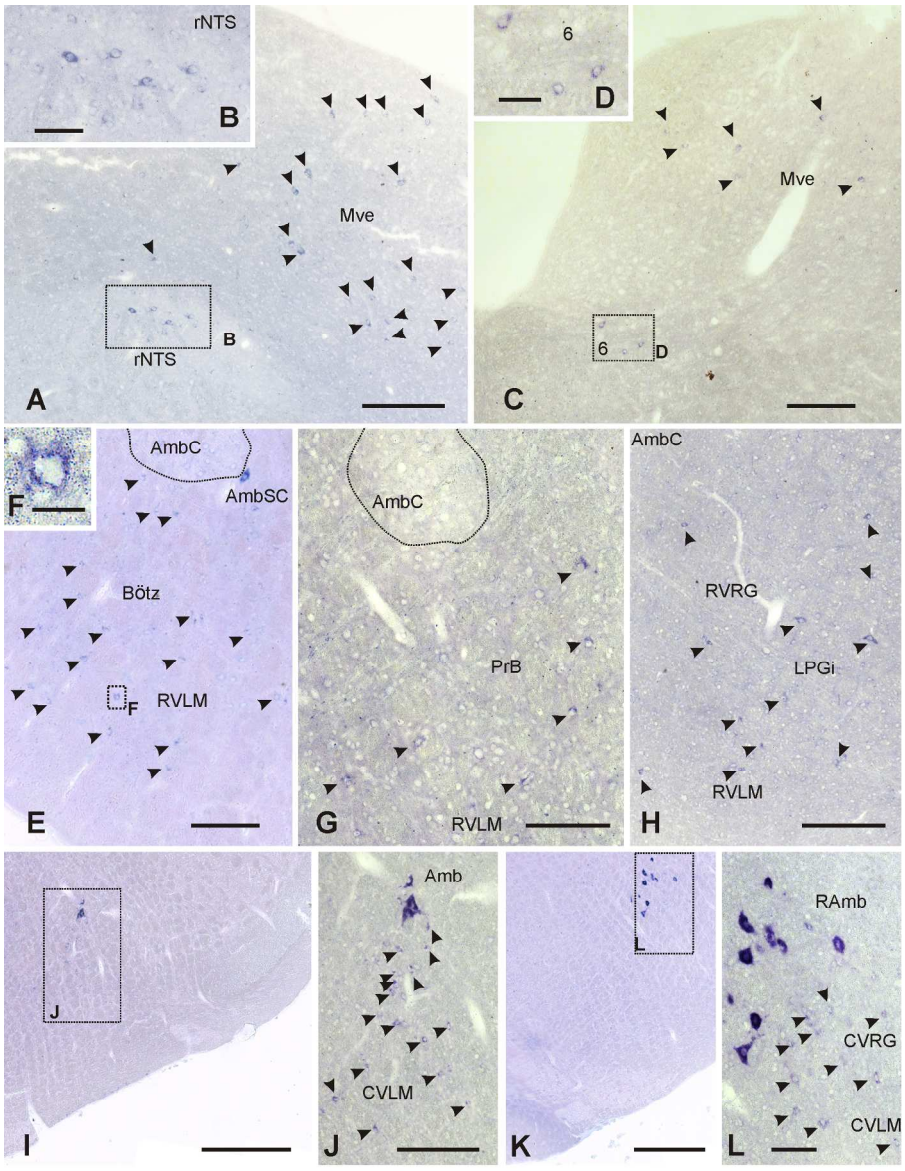

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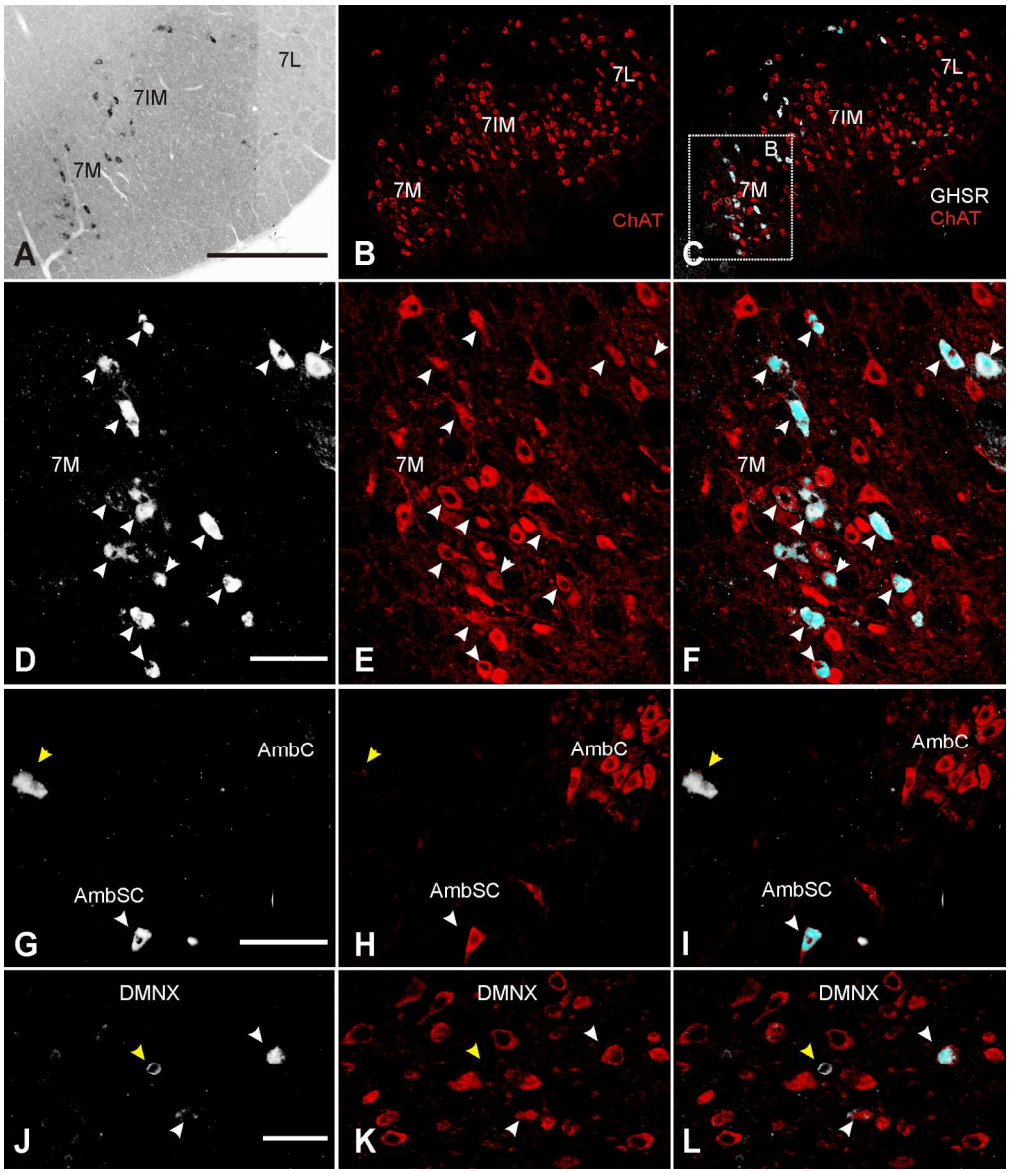

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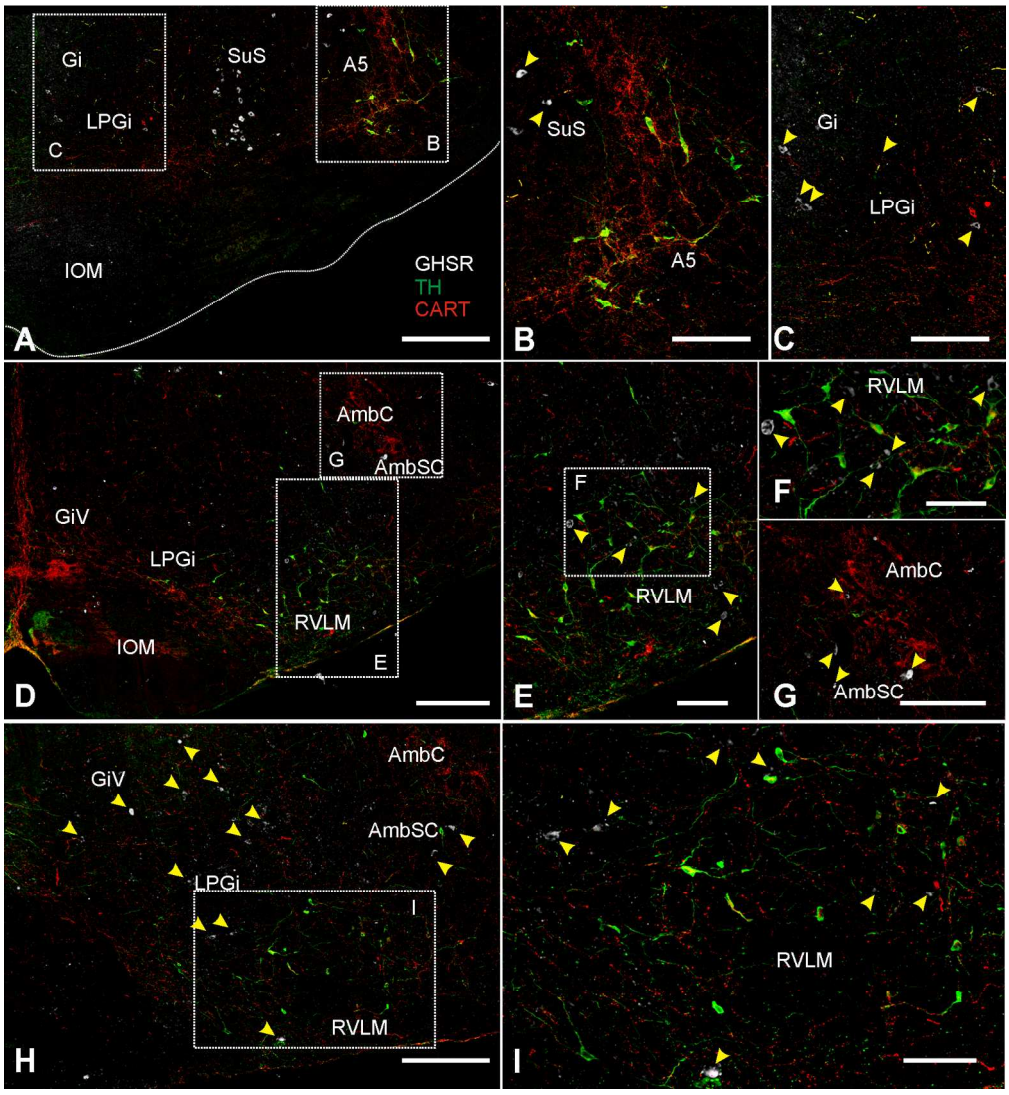

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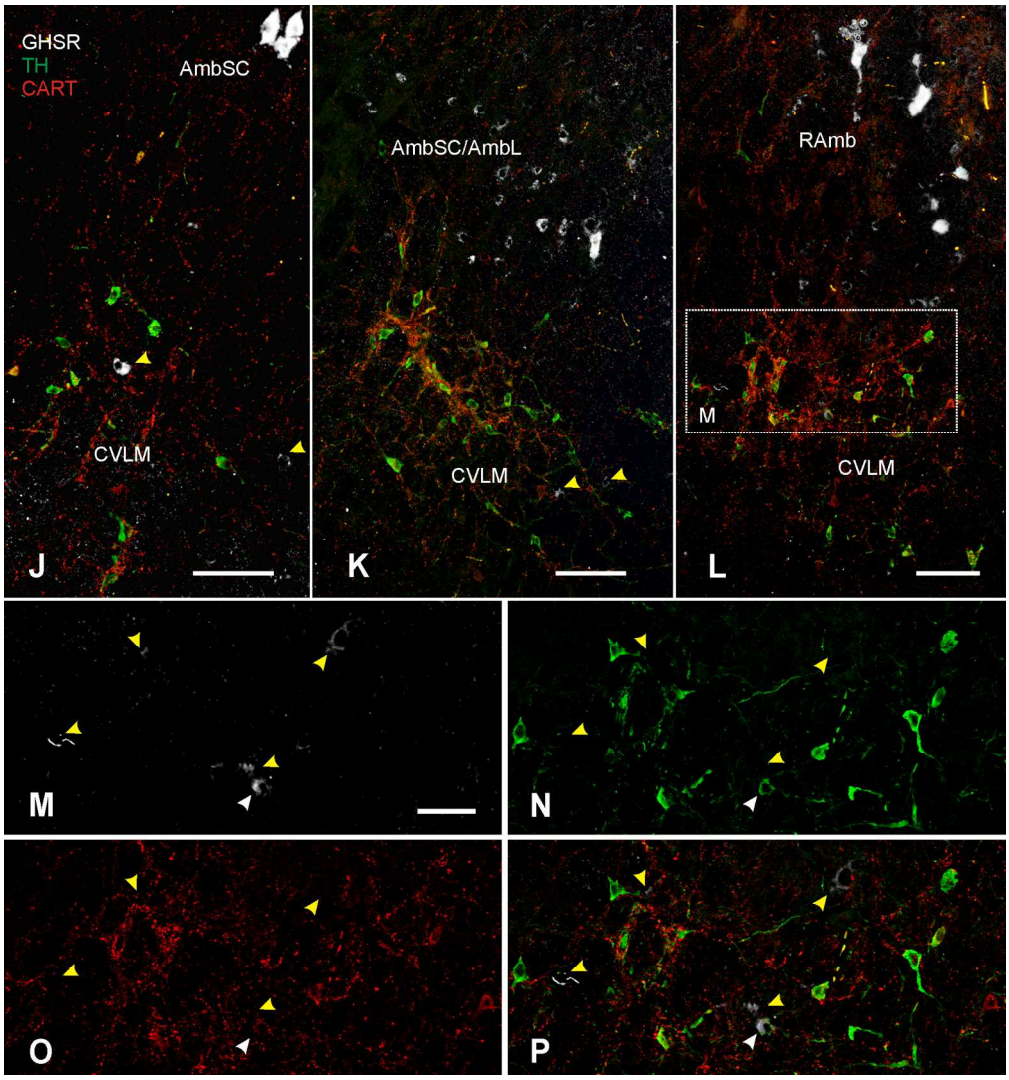

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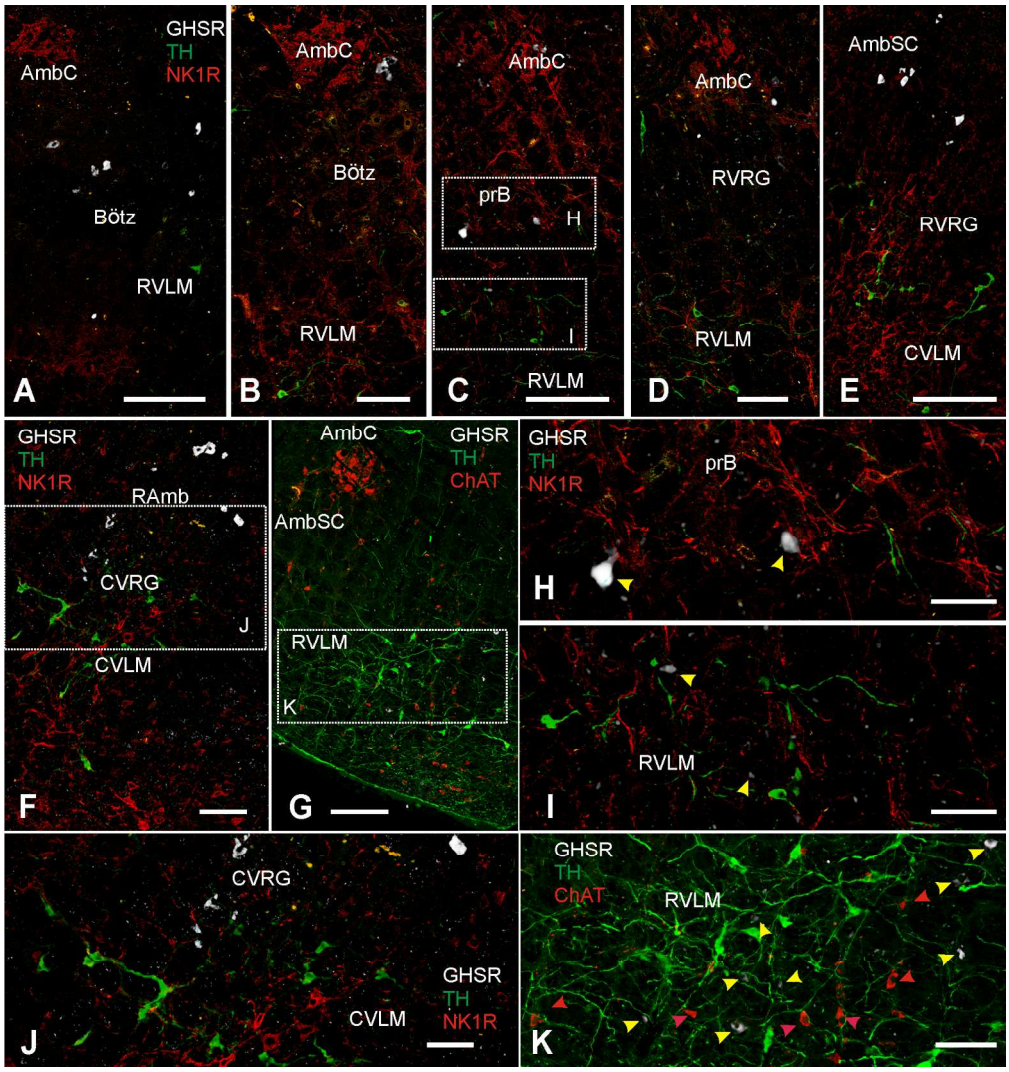

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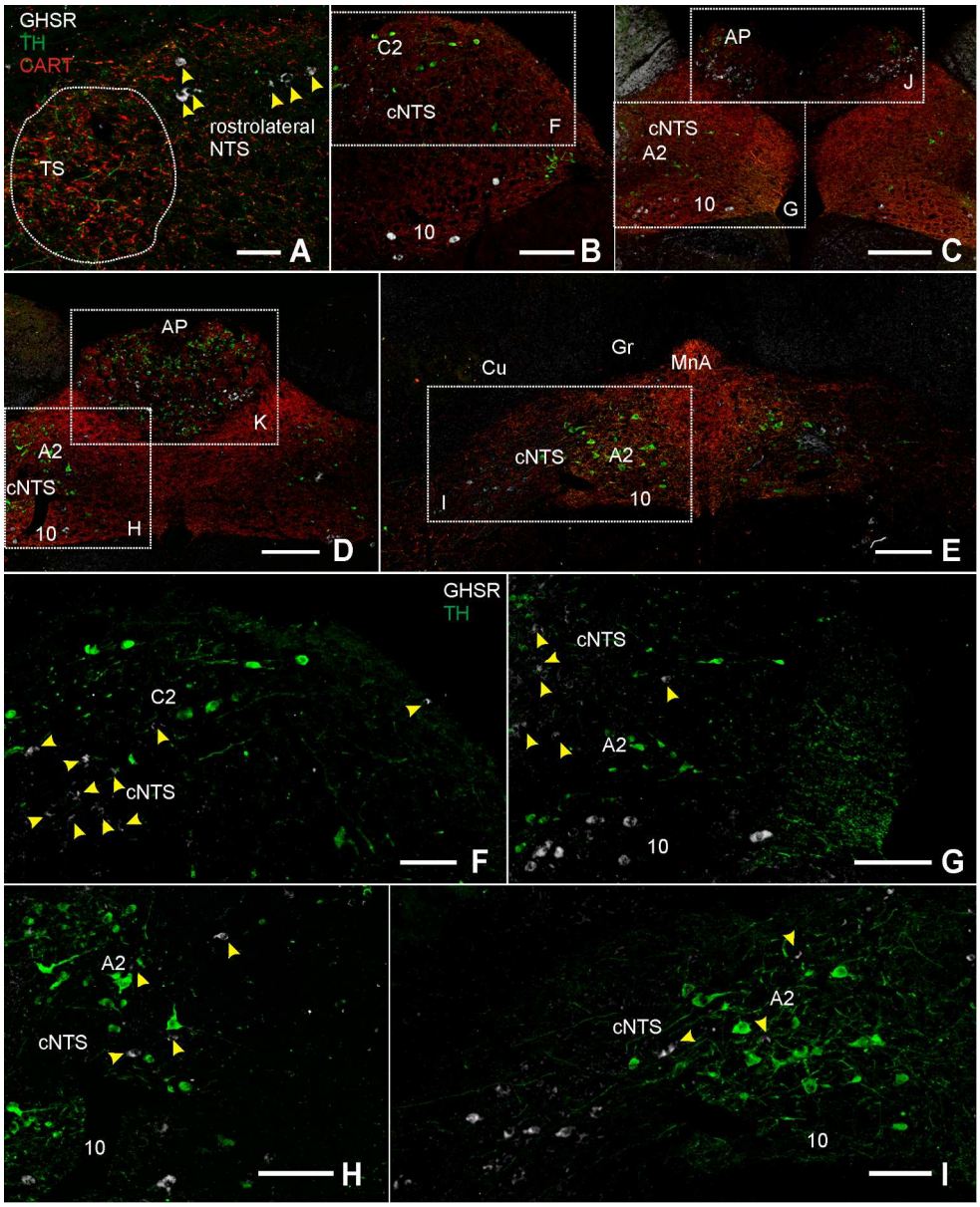

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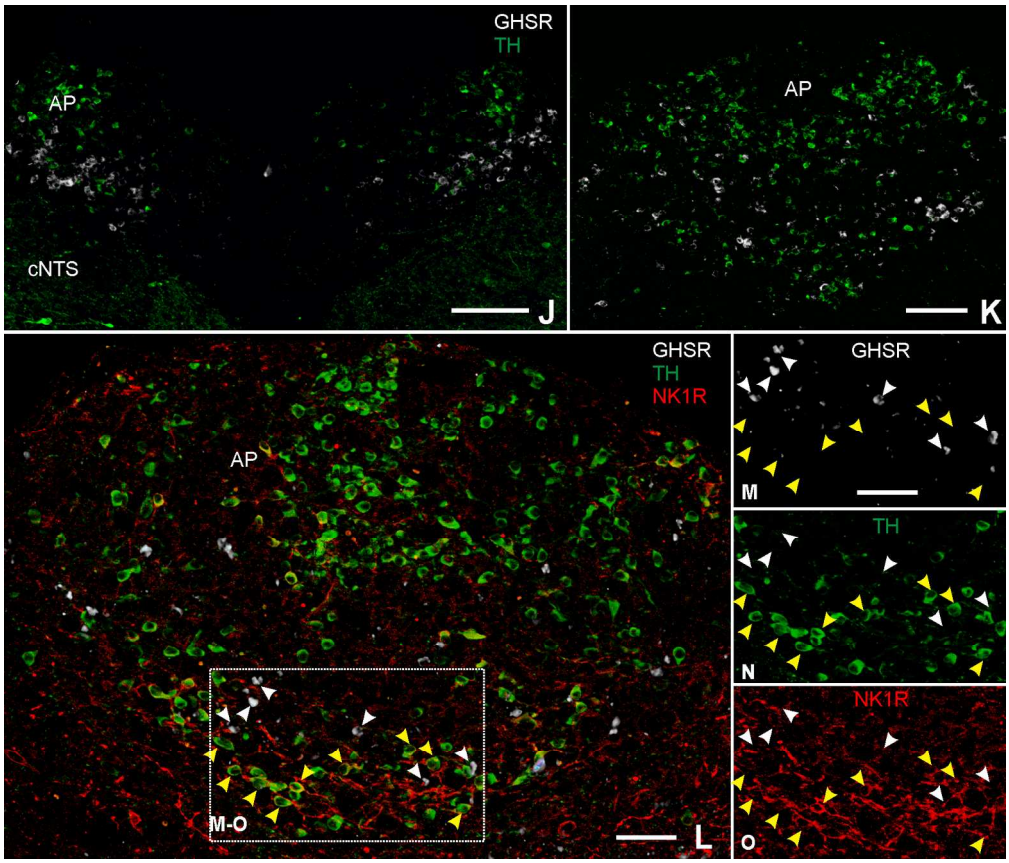

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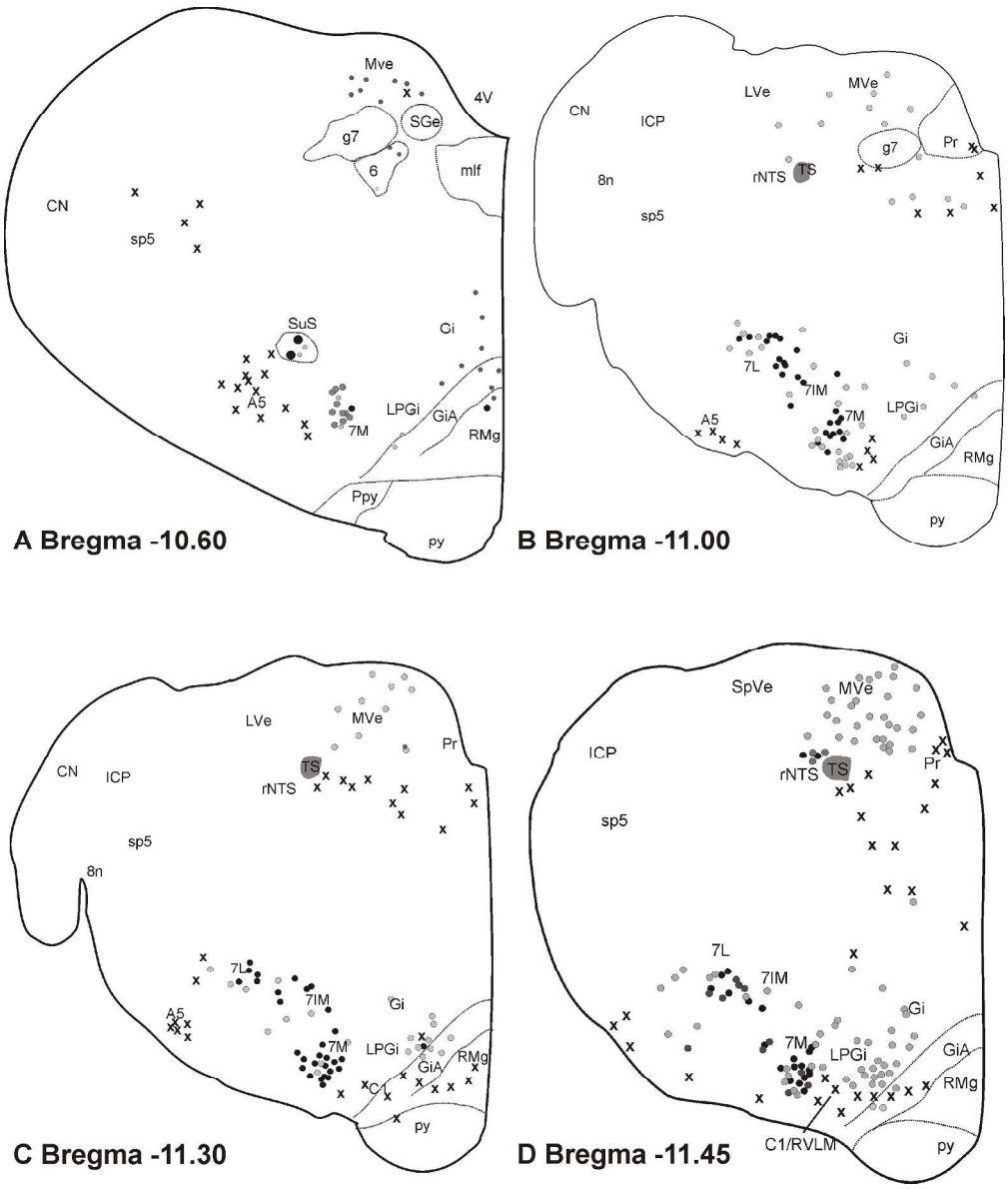

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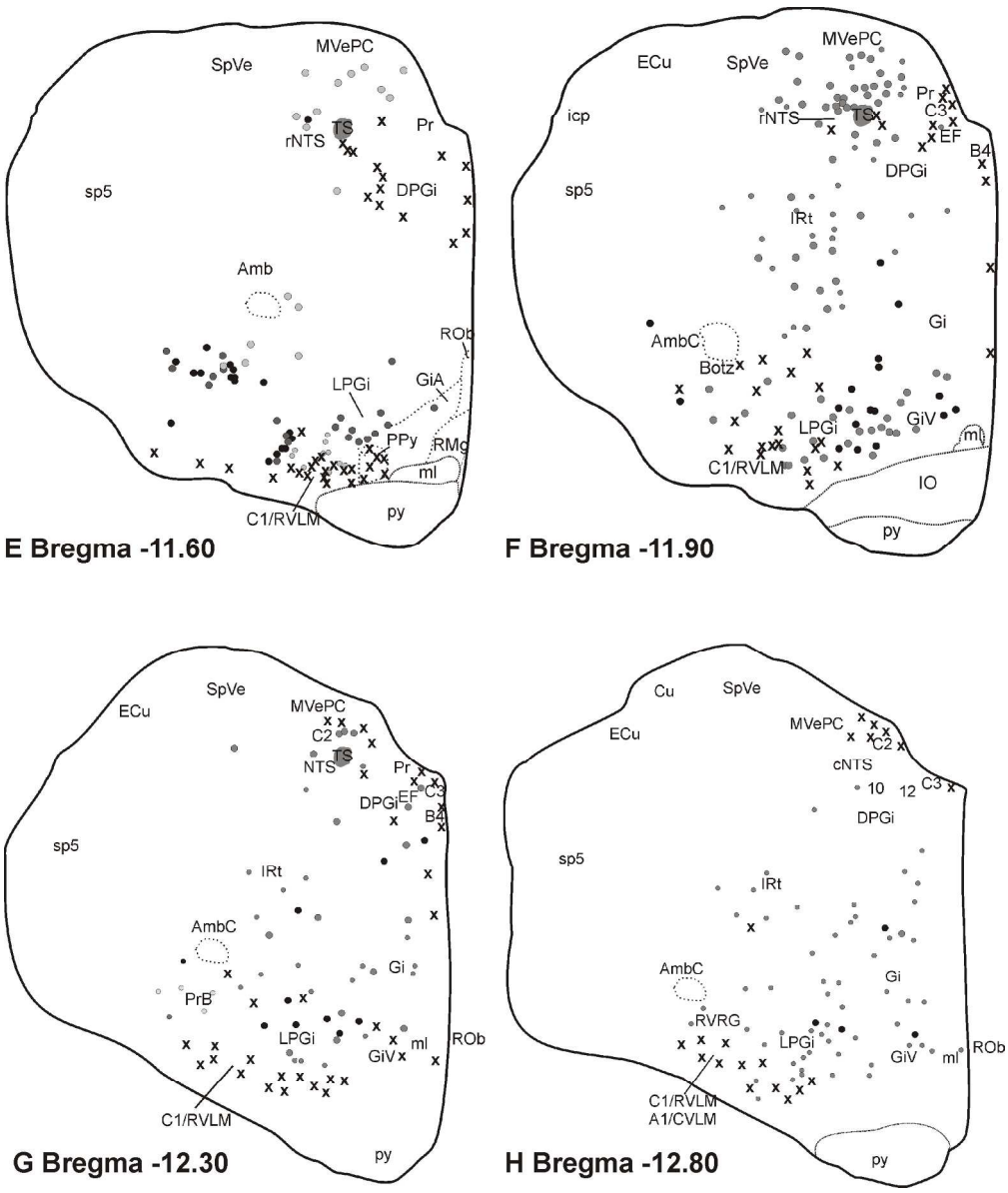

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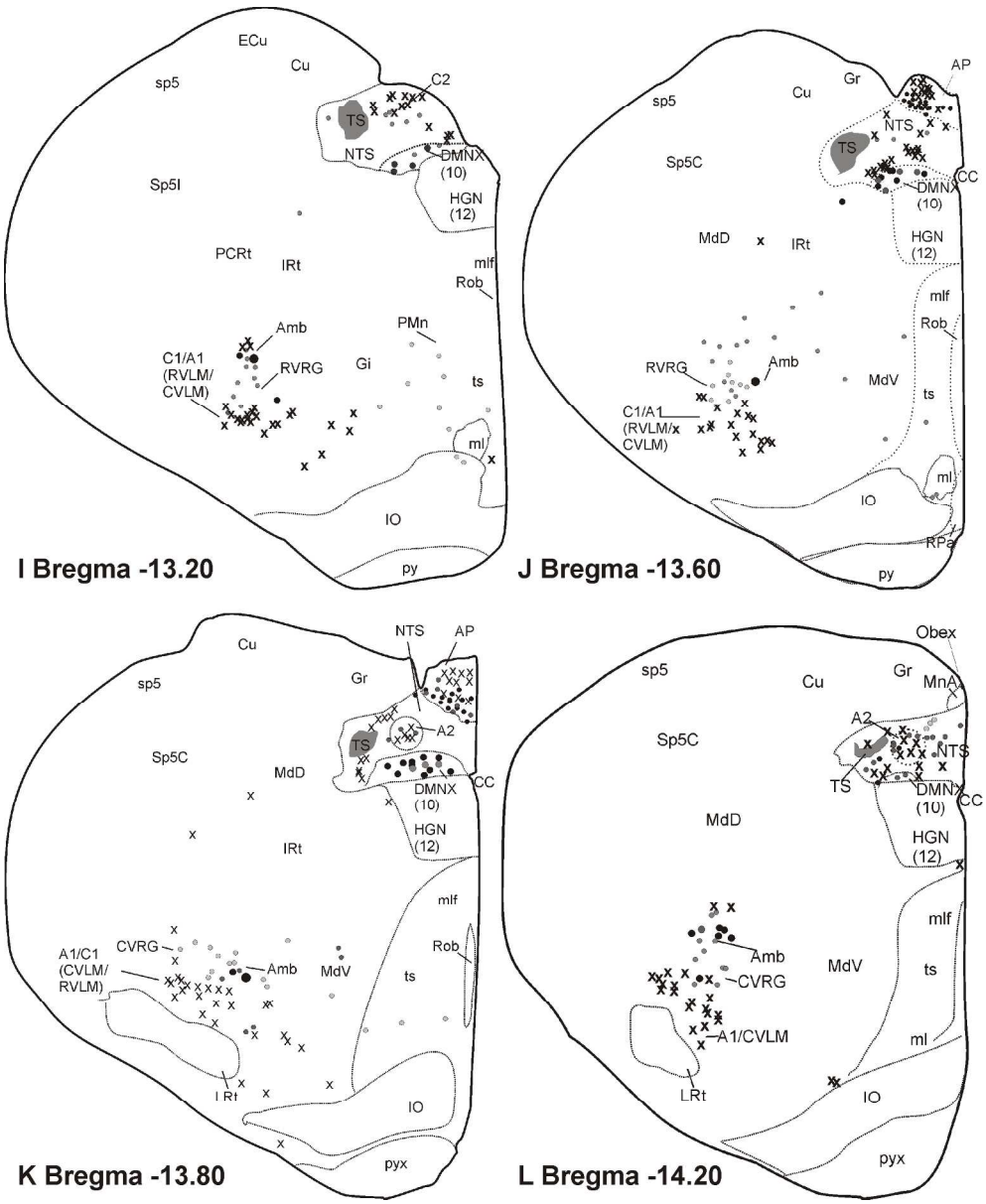

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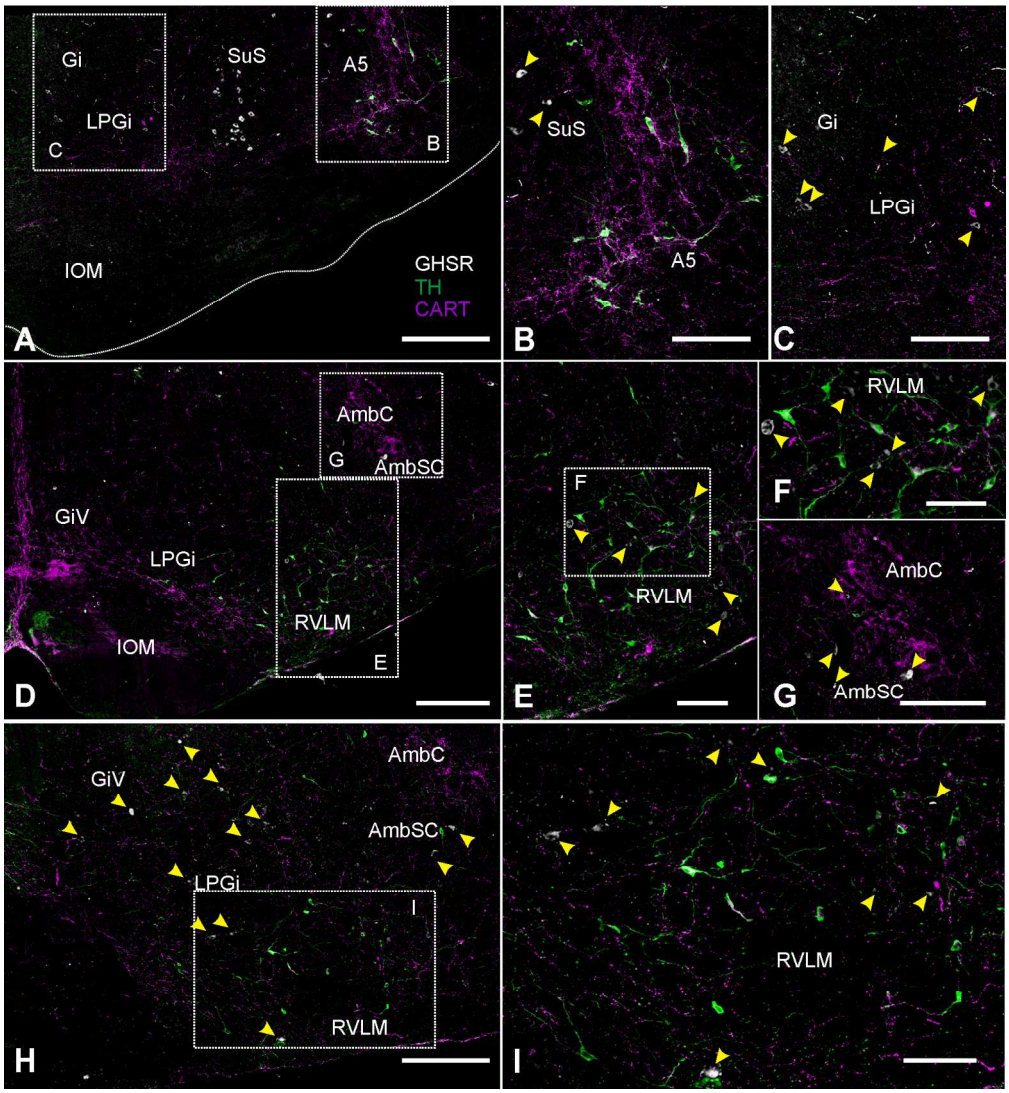

Suppl. Fig. 1 (magenta version of original Fig. 4). GHSR mRNA localization in the ventral medulla in relation to cardiovascular control centers, marked by TH and CART expression. Combined immunohistochemistry for Tyrosine Hydroxylase (TH) and Cocaine and Amphetamine Regulated Transcript (CART) and in situ hybridization (ISH) for GHSR on coronal sections of rat medulla. Sections at Bregma -10.80 mm (5A-C), -12.50 mm (5D-G), -12.80 mm (5H, I), -13.35 mm (J), -13.80 mm (K), -14.10 mm (L).
To allow direct comparison of the brightfield ISH signal with the immunofluorescence signals the brightfield image is inverted. GHSR mRNA is not expressed in the A5 area, which is identified by neurons that strongly express TH and CART (4A, B). The nearest GHSR mRNA expression is in the Superior Salivary nucleus (SuS, 5A, B). More caudally, GHSR mRNA expressing cells (yellow arrowheads in 4E, F, H and I), can occasionally be found near or within the area occupied by the rostroventrolateral medulla (RVLM), which is apparent by TH and CART expressing neurons (4D, E, F, H, I). However, GHSR mRNA is never co-localised with TH or CART in the RVLM. GHSR-positive neurons within the RVLM appear to be part of clusters of small cells that express to weak-to-moderate levels of GHSR mRNA and are frequently observed ventral to the AmbC (apparent by strong CART labelling; 4D, E, G, H). More caudally, the AmbC is no longer present, but similar clusters of cells are found dorsally from and intermingling with TH and CART expressing neurons in the caudoventrolateral medulla (CVLM; 4J, K, L, M). The majority of these cells presumably belong to the semi-compact (AmbC), loose (AmbL) and external (AmbE) formations of the nucleus ambiguus or the nucleus

retroambiguus (RAmb). They generally do not express TH or CART (yellow arrowheads), with an exception (white arrowhead) shown in Fig 4L, M-O. Scale bars: 50 μm (M), 100 μm (J, K, L), 200 μm (B, C, E, F, G, I), 500 μm (A, D, H).

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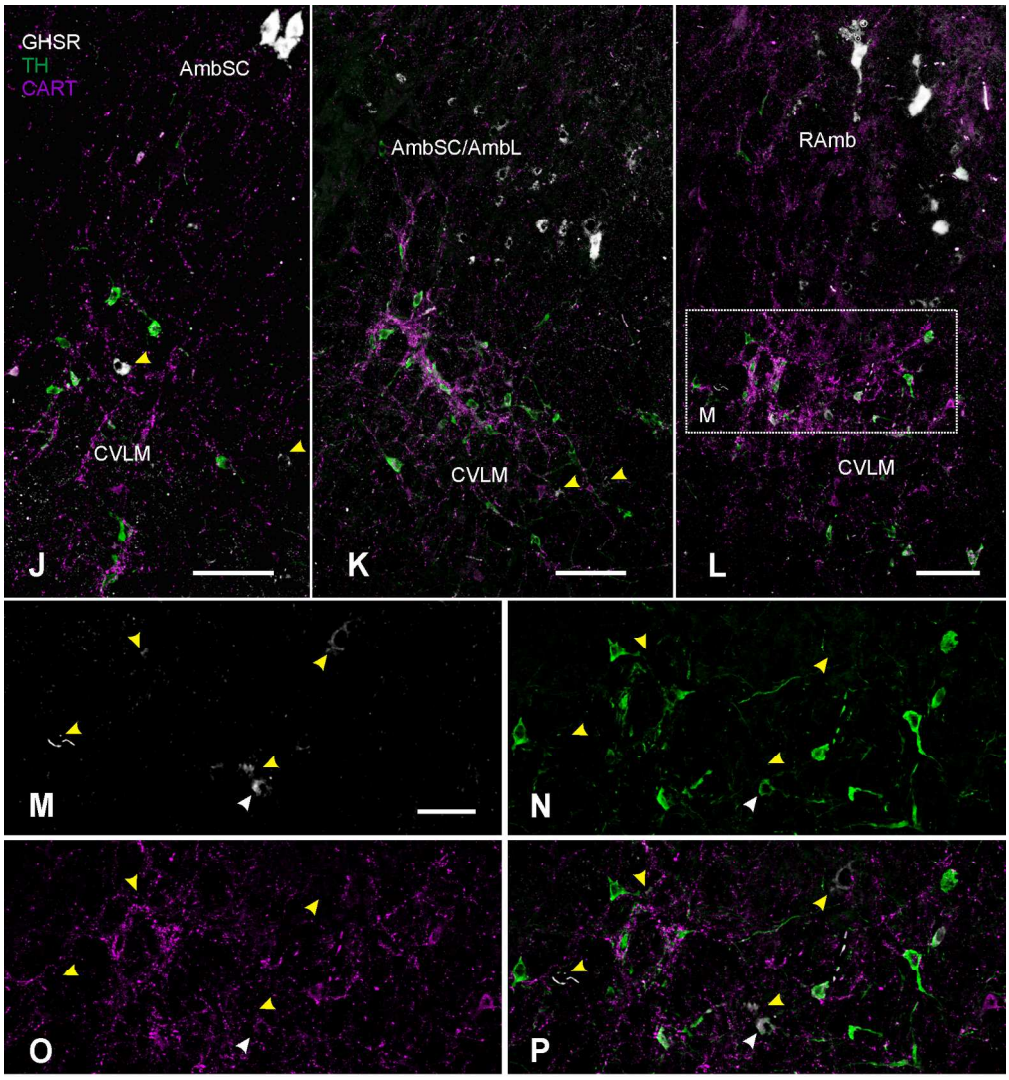

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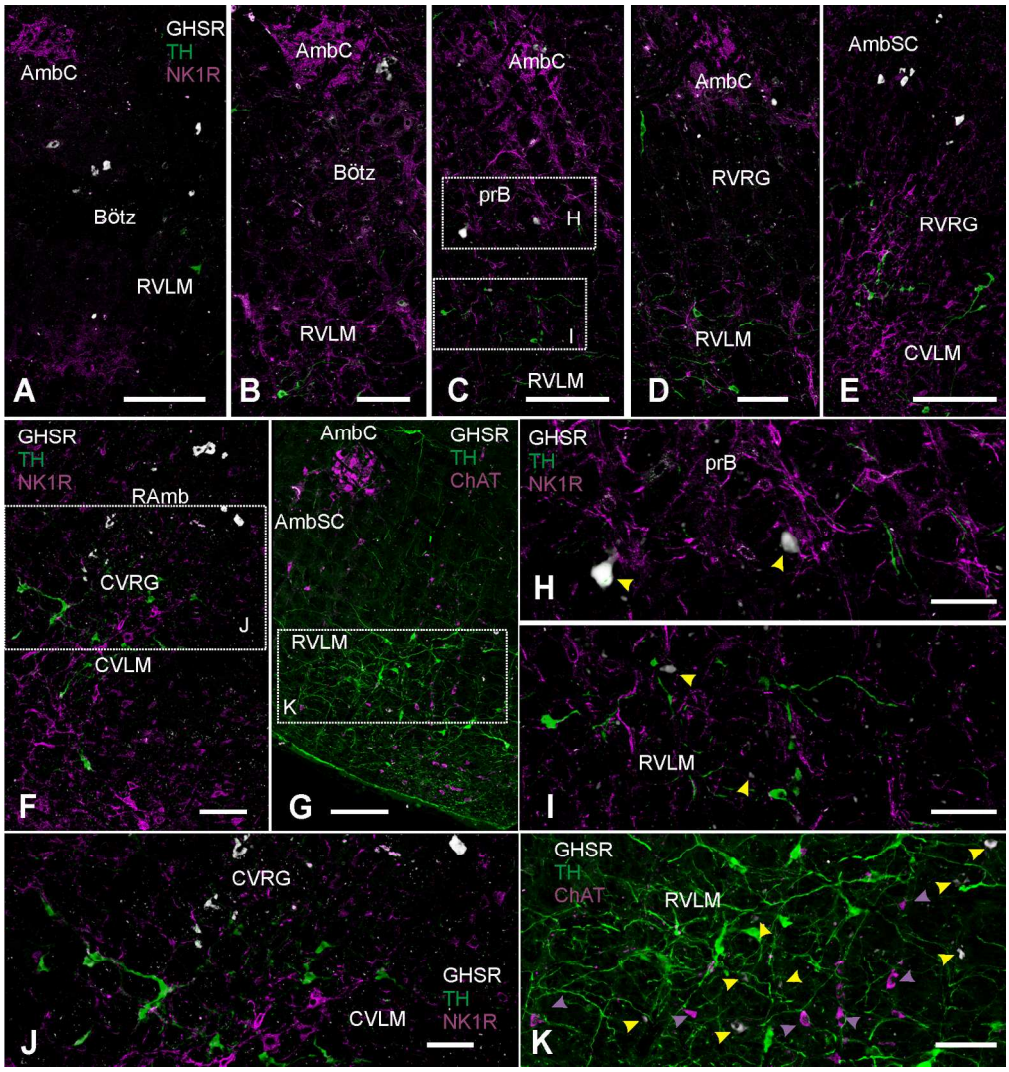

Suppl. Fig 2 (magenta version of original Fig. 5). GHSR mRNA localization in the ventral medulla in relation to NK1R-expressing respiratory nuclei and cholinergic sensory interneurons. To allow direct comparison of the brightfield ISH signal with the immunofluorescence signals the brightfield image is inverted. Combined in situ hybridization (ISH) for GHSR and immunohistochemistry for neurokinin-1 receptor (NK1R; 5A-F, H, I, J) or acetyl choline transferase (ChAT; 5G, K) on coronal sections of rat medulla. Sections at Bregma -11.80 mm (A), -12.10 mm (B), -12.30 mm (C), -12.70 mm (D), -13.60 mm (E), -14.10 mm (F), -12.70 mm (G). Clusters of cells expressing weak-to-moderate levels of GHSR mRNA are found ventral to the AmbC and throughout areas associated with respiratory control, such as the Bötzinger complex (Bötz; 6A, B) , the preBötzinger complex (prB; 5C, H), the rostral and caudal ventrolateral respiratory group (RVRG; 5D, E) and (CVRG; 5F, J). NK1R expression varies along the rostral-caudal axis, with low levels in the rostral Bötz and RVRG, increasing levels toward the caudal Bötz (5A, B), and very high levels in (propriobulbar) neurons in the prB (5C, H) and in (bulbospinal) neurons in the RVRG (5E). NK1R-expressing neurons are often intermingled with TH-expressing neurons of the RVLM and CVLM (5B-F, I, J). No examples of co-expression of GHSR mRNA in NK1R expressing neurons were identified in the ventrolateral medulla. ChAT-staining marks small sensory interneurons in the medial RVLM (5G, K), in addition to large motor neurons in the AmbC (5G). GHSR mRNA expressing small neurons (yellow arrows) are intermingling with ChAT (magenta arrows) and TH expressing neurons in the RVLM, but GHSR mRNA is not co-expressed with either of these markers (5K). Scale bars: 50 µm (H, I, J), 100 µm (B, D, F, K), 200 µm (A, C, E, G) .

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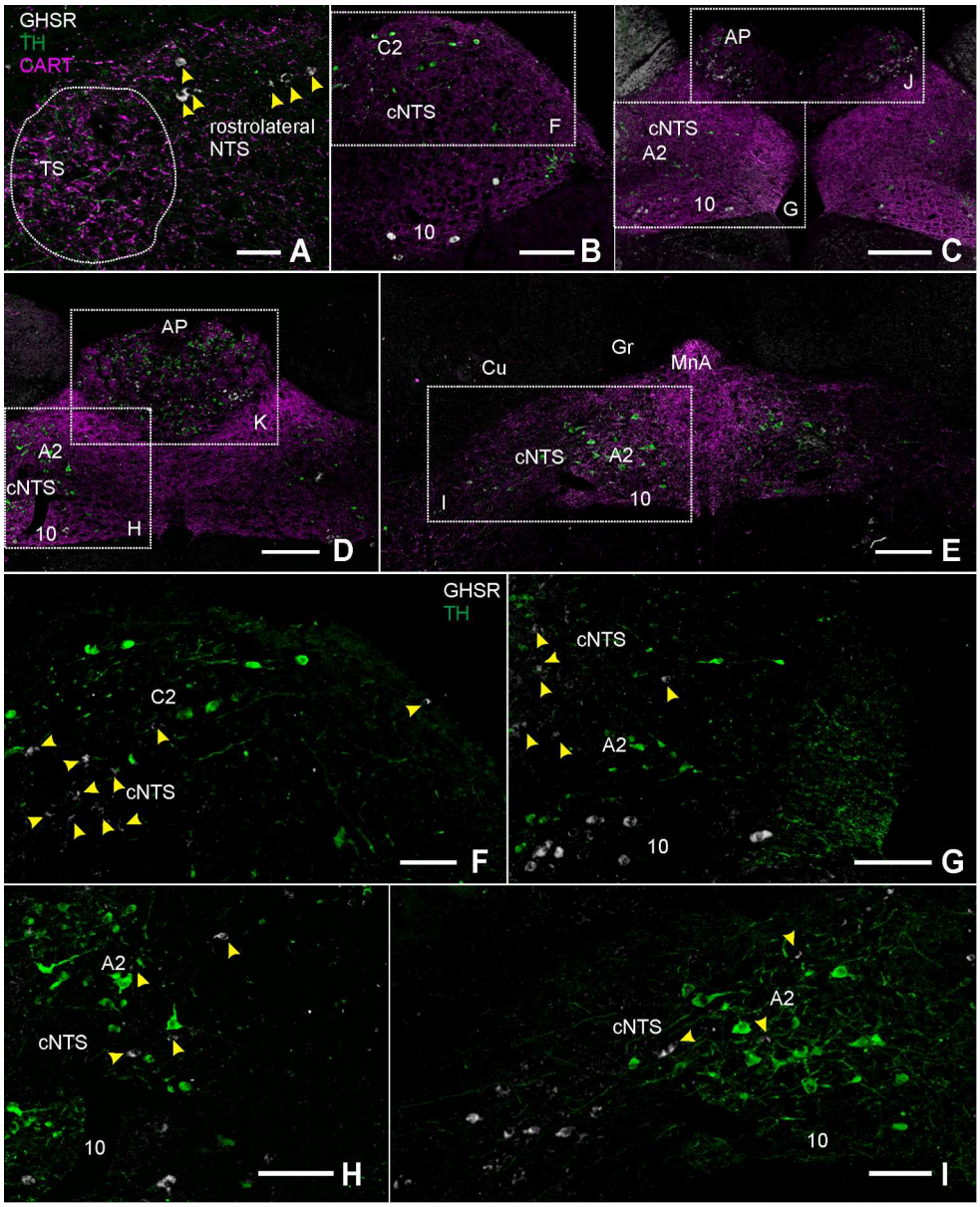

Suppl. Fig 3 (magenta version of original Fig 6). GHSR mRNA localization in the rostral NTS (rNTS) and the dorsal vagal complex (DVC) in relation to TH, CART and NK1R expression. Sections at Bregma -11.50 mm (6A), -13.20 mm (6B, F), -13.60 mm (6C, G, J), -13.85 mm (6D, H, K, L), -14.20 mm (6E, H). To allow direct comparison of the brightfield ISH signal with the immunofluorescence signals the brightfield image is inverted. NB: The tissues surrounding the DVC are more opaque (see Fig. 3), resulting in a higher relative background in the inverted image in these areas (e.g. Fig. 6C). CART immunoreactivity is very prominent in the solitary tract (TS; 6A) and in fibre projections throughout the DVC, clearly defining the boundaries of the NTS and DMNX (10). TH marks C2 adrenergic neurons (6B, F), A2 noradrenergic neurons (6C, D, E, G, H, I) in the cNTS and noradrenergic neurons in the area postrema (AP; C, D, J, K-O). In the rNTS, GHSR mRNA expressing neurons are found lateral to the TS, in the rostrolateral NTS (6A). In the DVC, neurons expressing GHSR mRNA are found in the DMNX (10; 6B, C, D, G, H), cNTS (6B-I) and the AP (6C, D, J, K, L). GHSR mRNA is not found in TH expressing neurons throughout the DVC, or in NK1R-expressing neurons in the AP (6L). A very large proportion of NK1R-positive neurons in the AP also express TH (6L-O). Scale

bars: 50 μm (A, F, L-O'), 100 μm (B, G, H, I, J, K), 200 μm (C, D, E)
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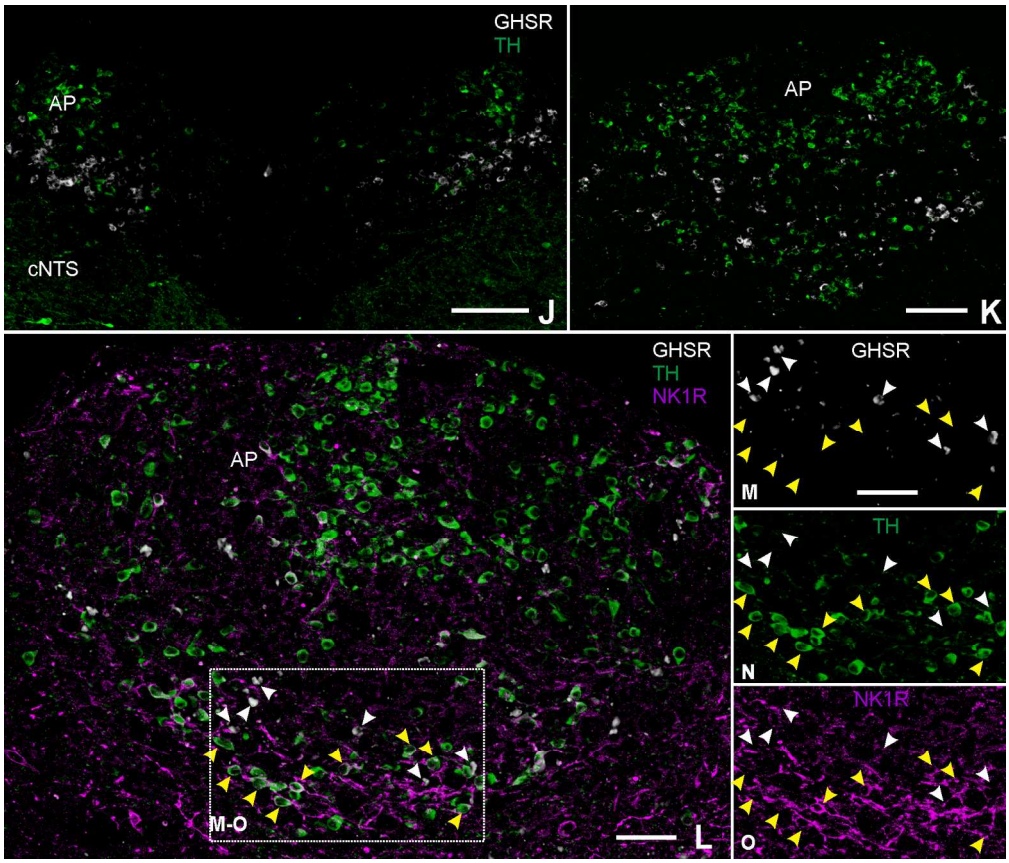

171x146mm (300 x 300 DPI)

Legends to Supplementary Figures

Suppl. Fig. 1 (magenta version of original Fig. 4). GHSR mRNA localization in the ventral medulla in relation to cardiovascular control centers, marked by TH and CART expression. Combined immunohistochemistry for Tyrosine Hydroxylase (TH) and Cocaine and Amphetamine Regulated Transcript (CART) and *in situ* hybridization (ISH) for GHSR on coronal sections of rat medulla. Sections at Bregma -10.80 mm (5A-C), -12.50 mm (5D-G), -12.80 mm (5H, I), -13.35 mm (J), -13.80 mm (K), -14.10 mm (L).

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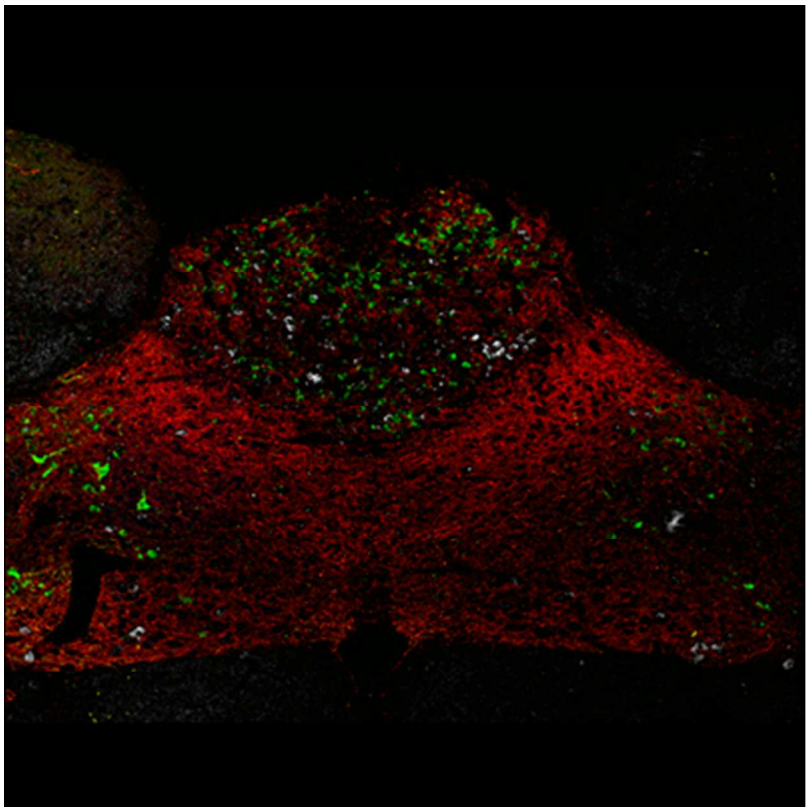

A detailed survey of ghrelin receptor (GHSR) mRNA expression in the rat medulla reveals expression in areas involved in gustatory, vestibulo-ocular, visceral sensory processing, and cardio-respiratory control. GHSR mRNA is found in the caudal and ventral aspects of the nucleus ambiguus and retroambiguus, but not in the rostral compact formation, ruling out control of esophagal musculature by ghrelin. GHSR mRNA expressing neurons (white) are distinct from catecholaminergic TH-expressing neurons (green) in the dorsal vagal complex (pictured) and the RVLM.
141x141mm (72 x 72 DPI)

A detailed survey of ghrelin receptor (GHSR) mRNA expression in the rat medulla reveals expression in areas involved in gustatory, vestibulo-ocular, visceral sensory processing, and cardio-respiratory control. GHSR mRNA is found in the caudal and ventral aspects of the nucleus ambiguus and retroambiguus, but not in the rostral compact formation, ruling out control of esophagal musculature by ghrelin. GHSR mRNA expressing neurons (white) are distinct from catecholaminergic TH-expressing neurons (green) in the dorsal vagal complex (pictured) and the RVLM.