Localization of serotonergic neurons in the Parapyramidal Region in the mouse activated during locomotion on a treadmill using c-fos as a neuronal activity marker

by

Erika Couto Roldan

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Department of Physiology and Pathophysiology
University of Manitoba
Winnipeg

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List of abbreviations

5-HT	5 hydroxytryptamine - Serotonine
CnF	Cuneiform nucleus
CPG	Central Pattern Generator
CTB	Cholera toxin subunit B
DAPI	4',6-diamidino-2-phenylindole
eYFP	Enhanced yellow fluorescent protein
FTG	Gigantocellular tegmental field
FTM	Magnocellular tegmental field
LPGi	Lateral paragigantocellular nucleus
MLR	Mesencephalic Locomotor Region
MRF	Medial reticular formation
mRT	Mesencephalic reticular formation
NMDA	N-methyl-D-aspartate
NRG	Nucleus reticularis gigantocellularis
PPR	Parapyramidal region
PrCnF	Precuneiform area
PTg	Pedunculotegmental nucleus
Rmg	Raphe magnus
Rob	Raphe obscurus
Rpa	Raphe pallidus
VLF	Ventrolateral funiculus

WGA-HRP	Wheat germ agglutinin-horseradish peroxidase

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Figure 12. Sampling scheme used in this study.

Abstract

The role of the serotonergic system in locomotion has been extensively studied. Most of the serotonin in the spinal cord is produced in cells located in the brainstem that belong to the reticulospinal system. It has been demonstrated that a specific group of serotonergic cells in the parapyramidal region (PPR) can produce locomotor-like activity in the neonatal rat. Here we studied the expression of c-fos in medullary serotonergic neurons after a locomotor task on a treadmill in adult mice. We used a transgenically modified line in which serotonergic cells expressed enhanced yellow fluorescent protein (eYFP). We counted and plotted cells using Micro Bright Field Co. software (Stereo Investigator and Neurolucida). We determined the location of the serotonergic cells in this mouse line in the adult. Serotonergic cell localization corresponds with previous findings pertaining to the mouse. We found an increase in the number of eYFP-positive cells expressing c-fos after a locomotor task in the raphe and PPR (LPGi, according to the Franklin and Paxinos mouse adult brain atlas) between bregma -6.8 and bregma -6.48 in the mouse (flanked rostrally by the seventh cranial nerve and caudally by the inferior olive). The percentage of eYFP-positive cells that expressed c-fos after the locomotor task in the raphe was 4%, whereas in the PPR the percentage was13.3%. Note that the total number of cells expressing c-fos in the raphe exceeded those located in the PPR. Methodological shortcomings prevented us from assessing our data with statistic tools. Such difficulties are discussed. Based on the increased c-fos labelling observed in serotonergic cells in the PPR, our results corroborate the observation that a specific group of serotonergic neurons located in that region are involved in locomotion.

Introduction

Locomotion is a rhythmic motor activity that is controlled by a network of spinal neurons referred to as the Central Pattern Generator (Wilson and Wyman, 1965; reviewed in Grillner et al., 1997; Rossignol and Dubuc, 1994). Locomotion can be initiated by several pathways that originate in the brain expressing different neurotransmitters including glutamate, noradrenaline, dopamine, and serotonin (reviewed in Grillner et al., 1997; Jordan, 1991, 1998; Rossignol, 1996). Several researchers have reported the existence of the so called Mesencephalic Locomotor Region (MLR) in many species, including cats and dogs (Shik and Orlovsky, 1976), rats (Skinner and Garcia-Rill, 1984), mice (Liang et al., 2011), birds (Steeves et al., 1987), stingray (Livingstone, 1981) and lamprey (McClellan and Grillner, 1984). When electrical or chemical stimulation is applied to this region, locomotor-like activity can be induced *in vivo* and *in vitro* (for review see Schmidt and Jordan, 2000; Jordan et al., 2008). One important f eature is that the speed of locomotion is correlated with the intensity of the stimulus applied to the MLR. Candidate nuclei included in the MLR are the pedunculotegmental nucleus (PTg) (Coles et al., 1989; Garcia-Rill and Skinner, 1987b; Jordan, 1998; Skinner and Garcia-Rill, 1984), the mesencephalic reticular formation (mRt) (Garcia-Rill et al., 1985), the precuneiform area (PrCnF) (Liang et al., 2011), the cuneiform nucleus (CnF) (Allen et al., 1996; Coles et al., 1989; Garcia Rill et al., 1983b; Jordan, 1998; Skinner and Garcia-Rill, 1984), the periaqueductal gray and the locus coeruleus (Jordan, 1998).

Evidence supporting the involvement of the Reticulospinal System in locomotion.

Anatomical

There is a considerable amount of evidence pointing to the involvement of the reticulospinal system as one "relay station" from superior centers, including the MLR, to the spinal cord through which locomotion is elicited. Studies in the cat (Armstrong, 1986; Garcia-Rill and Skinner, 1987b; Garcia-Rill et al., 1985; Orlovskii, 1969), rats and other mammals (Atsuta et al., 1988; Jordan, 1991, 1998; Mamiya et al., 2005), as well as lower vertebrates (Grillner et al., 1998; Le Ray et al., 2011) have consistently found this to be the case. Studies of spinal lesions in cats (Steeves and Jordan, 1980), as well as labelling studies showing projections from the brainstem to the lumbar intermediate gray matter (Reed et al., 2008), point to the fact that this pathway is localized within the spinal cord in the ventral and ventrolateral funiculus (Steeves and Jordan, 1980). Furthermore, lesion studies in primates (including humans) and non-primates support this idea (Eidelberg, 1981; Eidelberg et al., 1981). Using retrograde and anterograde labelling in the MLR (Garcia Rill et al., 1983b; Steeves and Jordan, 1984) as well as reversible cooling and irreversible lesions of the dorsal half of the brainstem and spinal cord (C2-C3) (Noga et al., 1991), early studies provided anatomical evidence to support the idea that the MLR does not project directly to the spinal cord; it does so indirectly by projecting to the brainstem reticulospinal areas such as the gigantocellular (FTG) and magnocellular (FTM) tegmental fields and the descending serotonergic nuclei. Similar observations have been made in other species, including the rat (Garcia-Rill et al., 1986) and stingray (Williams et al., 1984). It has also been shown that a retrograde tracer injected into the

medial reticular formation travelled to the MLR and hypothalamic locomotor region (Bayev et al., 1988). Jordan and colleagues (Steeves and Jordan, 1984) studied the projections from the MLR and found that "the majority of the descending projections travelled to the medial reticular nuclei, specifically the n. gigantocellularis, magnocellularis and raphe magnus, with the heaviest labeling on the ipsilateral side and much less contralateral labeling". Finally, when lesions in the FTG and FTM are performed, rats show substantial locomotor deficits (Zemlan et al., 1983).

Physiological

Jordan and coworkers, using reversible local cooling of several brainstem areas, demonstrated that the MLR projects to the medial reticular nucleus (Shefchyk et al., 1984). Since the late 1980s, it was known that reticulospinal cells located in the caudal raphe nuclei and the adjacent reticular formation project to all regions of the spinal cord gray matter and send direct projections to motoneurons in the spinal cord in the rat (Martin et al., 1985; Zemlan et al., 1984) as well as other species (Martin et al., 1979; Holstege and Kuypers, 1987b). Since the 1970s, it has been known that stimulation of the MLR induced activation of the medial reticular formation (MRF) bilaterally (Orlovsky, 1970a). They also demonstrated that such stimulation increased the activity of fibers within the ventrolateral funiculus (VLF) of the spinal cord (both, ipsi and contralaterally) (Orlovsky, 1969). Finally, Orlovsky and colleagues showed that when locomotion was evoked by stimulation in the MLR, reticulospinal cells became rhythmically active (Orlovsky, 1970b).

Later studies have expanded on this: locomotor movements can be evoked when these reticulospinal cells are stimulated (Mori et al., 1978). During spontaneous locomotion, MLR cells are rhythmically active (Garcia Rill et al., 1983a). The case is the same with reticulospinal cells assessed in cats under several experimental conditions: unrestrained cats walking on a treadmill (Drew et al., 1986), during fictive locomotion (Perreault et al., 1993), with cats after a precollicular-premamillary transection walking spontaneously on a treadmill (Garcia Rill et al., 1983a), decorticate (Perret, 1976) and thalamic cats (Shimamura and Kogure, 1983; Shimamura et al., 1982). In several studies (Drew et al., 1986; Garcia Rill et al., 1983a; Shimamura et al., 1982) the activity of reticulospinal cells was correlated with the activation of specific muscle groups. Something similar occurs with the VLF. In the latter, descending volleys can be correlated with the activity of limb muscles during spontaneous forelimb locomotion, according to studies on thalamic cats (Drew and Rossignol, 1984; Shimamura et al., 1984).

When stimulated, the ventral portion of the MRF produces hindlimb or quadrupedal locomotion, or facilitates locomotion evoked by the MLR (Garcia-Rill and Skinner, 1987a). Garcia-Rill and colleagues (Garcia-Rill and Skinner, 1987b) also showed that stimulation of the MLR may elicit activation of reticulospinal cells, which descend unilaterally or bilaterally via the VLF. In general, the finding that the MRF is able to produce locomotion when electrically stimulated has been reported in several species: cat (Garcia-Rill and Skinner, 1987a; Noga et al., 1988), rat (Kinjo et al., 1990; Ross and Sinnamon, 1984), bird (Steeves et al., 1987), stingray (Livingstone, 1986) and lamprey (McClellan and Grillner, 1984). Drew and colleagues demonstrated that stimulation of MRF is capable of resetting the locomotor rhythm (Drew and Rossignol, 1984). It has

been shown that metabolic activity in the MRF increased during MLR-evoked fictive locomotion (Shimamura etal, 1987; Kettler and Jordan, 1984).

More recently, Garcia-Rill and colleagues reported the induction of long-lasting depolarizations in neurons in the medioventral medulla following stimulation to the pedunculopontine nucleus (Mamiya et al., 2005). Activity in medullary neurons during fictive locomotion has been studied (Perreault et al., 1993; Perreault et al., 1994), showing that "the majority of reticular neurons projecting as far as the lumbar spinal cord are phasically modulated during locomotion, even in the absence of phasic peripheral afferent inputs" (Perreault et al., 1993).

Pharmacological

Studies exploring the pharmacology of the MRF have shown that activation of the MRF through application of substance P or cholinergic agonists (Garcia-Rill and Skinner, 1987a) or through injections of excitatory amino-acids or picrotoxin (Noga et al., 1988) can elicit locomotion or facilitate MLR-evoked locomotion. On the other hand, inhibition of the MRF (through application of GABA) abolishes MLR-evoked locomotion (Noga et al., 1988).

It is clear that evidence implicating the reticulospinal system (originating in the MRF) in the initiation of locomotion is vast. However, the precise neurons of origin of the reticulospinal pathways involved in the initiation of locomotion in mammals have not been clearly identified (Jordan et al., 2008).

Evidence supporting the involvement of the serotonergic system in locomotion

Spinal projections of the serotonergic system

The reticulospinal system involved in the initiation of locomotion contains cells expressing different neurotransmitters, including serotonin (Jordan et al., 2008). There is extensive evidence pointing to the role of serotonin in locomotion in mammals (reviewed in Jordan and Schmidt, 2002; Schmidt and Jordan, 2000). Several studies showed that serotonergic cells in the raphe nuclei as well as some laterally located cells project to the spinal cord using combined retrograde transport and immunocytochemical techniques to label serotonergic neurons in the rat (Bowker and Abbott, 1990; Bowker et al., 1981b; Dahlstrom, 1964; Holstege, 1987b; Jones and Light, 1992; Skagerberg and Bjorklund, 1985; Toth et al., 2006; Helke et al., 1989; Hökfelt et al., 2000; Holstege and Kuypers, 1987b; Schmidt and Jordan, 2000) as well as in the cat (Basbaum et al., 1978; Martin et al., 1978) and the monkey (Bowker et al., 1982). In fact, almost all of the serotonergic terminals in the rat spinal cord are projections from serotonergic cells located in the brainstem (reviewed in Lakke, 1997). The evidence shows that fibers from the raphe and more lateral regions innervate different neuron types: autonomic preganglionic neurons (Allen and Cechetto, 1994; Loewy and McKellar, 1981) as well as somatic neurons (Allen and Cechetto, 1994; Liu and Jordan, 2005; Ross et al., 1981) or both (Allen and Cechetto, 1994).

Physiological evidence for the involvement of the serotonergic system in locomotion

Studies with freely moving cats have strongly suggested that serotonergic cells play a role during locomotion (Fornal et al., 1985; Jacobs and Azmitia, 1992; Veasey et al., 1995). Cowley and Schmidt (1994) demonstrated that in the isolated neonatal rat spinal cord, serotonin was the most reliable substance (among NMDA, dopamine, norepinephrine and acetylcholine) in evoking a locomotor-like pattern of hindlimb nerve activity (see also Atsuta et al., 1991; Cazalets et al., 1992; Cowley and Schmidt, 1997; Kiehn and Kjærulff, 1996; Kiehn et al., 1999; Kudo and Yamada, 1987; Pearlstein et al., 2005; Smith et al., 1988). Application of monoamines also modulated the timing, amplitude, and pattern of CPG activity in the mouse (Gordon and Whelan, 2006, 2008; Madriaga et al., 2004; Whelan et al., 2000). Jordan and colleagues stimulated the brainstem in neonatal rat brainstem-spinal cord preparation and were able to evoke locomotor-like activity (Jordan and Schmidt, 2002). They demonstrated that this effect was mediated by serotonergic receptors. 5-HT agonists can also evoke locomotion in rabbits (Viala and Buser, 1969) and spinal rats (Feraboli-Lohnherr et al., 1999). Serotonin enhanced the locomotor activity in marmoset monkeys (Fedirchuk et al., 1998). 5-HT contributes to locomotion in the neonate mice (Nishimaru et al., 2000; Whelan et al., 2000) as well as in those animals "motor functionally mature", meaning mice that can bear weight and walk, approximately P10-P12 (Jiang et al., 1999). It was also shown that pharmacological blockage of serotonin impaired the ability of several neurochemicals to evoke locomotor-like activity (MacLean et al., 1998).

Studies on serotonergic receptors in the spinal cord, through which putative serotonergic reticulospinal neurons may have an effect on spinal cells, are also abundant (for a review see Hochman et al., 2001; Schmidt and Jordan, 2000).

Importantly, when adult spinal rats are transplanted with embryonic serotonergic neurons in the lower thoracic region, locomotion can be activated (Giménez Y Ribotta et al., 2000; Majczynski et al., 2005; Slawinska et al., 2000). Recently, it has been demonstrated that transplanting embryonic serotonergic cells destined to belong to groups B1, B2 and B3 (caudal groups) to spinal cord injured rats can restore coordinated locomotion and that this effect is mediated by 5-HT_2 and 5-HT_7 receptors, strengthening the idea that specific groups of serotonergic neurons play an important role in to locomotion (Sławińska et al., 2013; Sławińska et al., 2014). Administration of serotonin or serotonergic agonists can induce locomotion in spinal rodents (Antri et al., 2002; Feraboli-Lohnherr et al., 1999; Feraboli-Lohnherr et al., 1997; Landry et al., 2006; Orsal et al., 2002). It has been recently reported that serotonergic cells in the parapyramidal region (PPR) at the level of the seventh cranial nucleus can be antidromically activated from the thoracic cord (31/47) in the rat (Takakura and Moreira, 2013).

In spite of all the evidence pointing to the involvement of the serotonergic medullary system in locomotion, until 2005 there were no reports on the specific 5-HT cells responsible for this effect (Liu and Jordan, 2005). Liu and Jordan identified for the first time a serotonergic group of cells that when stimulated evoked locomotion. They reported that “electrical and chemical stimulation of a specific group of 5-HT neurons in the brain stem of the isolated neonatal rat brain stem and spinal cord preparation is sufficient for evoking locomotor-like activity. These cells are localized in the PPR of the

mid-medulla" (Liu and Jordan, 2005; reviewed in Jordan and Sławińska, 2014). The effect was mediated by 5-HT_2 and 5-HT_7 receptors. In adult rats, Light and colleagues found that, even though a small percentage of cells in this region (no more than 25%) project to the spinal cord, a large percentage of them produce serotonin (over 65% in average) (Jones and Light, 1992). More recently, VanderHorst and collaborators performed similar studies on the mouse. They injected wheat germ agglutinin-horseradish peroxidase (WGA-HRP) and cholera toxin subunit B (CTB) in the spinal cord (VanderHorst and Ulfhake, 2006). They found that serotonergic cells in the raphe pallidus (Rpa), raphe obscures (Rob), raphe magnus (Rmg) and *especially* lateral paragigantocellular nucleus (LPGi) project to the spinal cord (LPGi corresponds to the PPR described by Liu and Jordan, 2005). Physiologically, it was shown that cells in the PPR region were active during treadmill exercise, as revealed by the expression of c-fos (Iwamoto et al., 1996; Ohiwa et al., 2006). In these studies, however, the neurotransmitter profile was not assessed. Work in the Jordan lab has revealed that when the PPR is stimulated, serotonin, dopamine and noradrenalin are released in the spinal cord (Fyda et al., 1997; Jordan and Schmidt, 2002).

In this study we intended to localize the serotonergic neurons in the PPR that are active during locomotion on a treadmill in the adult mouse, revealed by the expression of c-fos. We consider it important to study the adult mouse because the neonatal preparation, even though very useful to elucidate some characteristics of the system, lacks some others that appear as the animal develops (for example, neonatal mice are not able to support their weight). We made use of a particular mouse line developed by the Deneris lab (Scott et al., 2005) in which serotonergic neurons are genetically marked with enhanced Yellow

Fluorescent Protein (eYFP). Deneris and colleagues used a region that, in their own words, "can direct highly reproducible 5HT neuron-specific transgene expression with little or no ectopic expression among independent founder lines" (Scott et al., 2005). Finally, we used the c-fos technique because previous reports have successfully used this technique to label active cells in the brainstem and spinal cord; particularly, many have used this technique to study cell groups involved in the motor system (Barajon et al., 1992; Carr et al., 1995; Dai et al., 2005; Huang et al., 2000; Jasmin et al., 1994; Ohiwa et al., 2006; Todd et al., 2010; Wilson et al., 2010; Wilson et al., 2005). We understand, however, that every technique has its own pitfalls, and in the case of c-fos, a negative result must be very carefully analyzed because it has been proven that certain neurons do not express c-fos when activated (Dragunow and Faull, 1989).

Hypothesis

A specific group of serotonergic cells located in the parapyramidal region (PPR) in the mouse medulla is active during treadmill locomotion, as revealed by the expression of c-fos.

Methods

Experimental Animals

All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and were approved by the University of Manitoba Animal Protocol Committee. Experiments were performed on 14 adult (2 – 6 months old) ePet-EYFP

transgenic mice in which eYFP (enhanced yellow fluorescent protein) expression is driven by Pet-1 expression (Hendricks et al., 1999; Hendricks et al., 2003; Liu et al., 2010; Scott et al., 2005). Therefore, serotonergic neurons express eYFP in this model.

Treadmill locomotion

Animals were separated in 2 groups: Control and treatment (locomotion on a treadmill). Mice in the control group were maintained in a quiet and illuminated environment for at least 3 hours. In all cases mice fell asleep for at least 2 hours before perfusion. This time period was chosen because the half-life of c-fos protein is 2 hrs (Kovacs, 1998), see their table 1. Animals in the treatment group were subjected to a locomotor task on a treadmill consisting of: 10 minutes warm up at 0.1 m/s, and then a progressive increase of velocity up to 0.2 m/s (Kemi et al., 2002; Ohiwa et al., 2006). Animals ran for 1 hour at this velocity (see attached video 1). The average run distance was 750 m. The maximal level of c-Fos protein expression occurs between 1 and 3 hrs after acute challenge (Kovacs, 1998); therefore, animals were allowed to rest for 2 hours before perfusion.

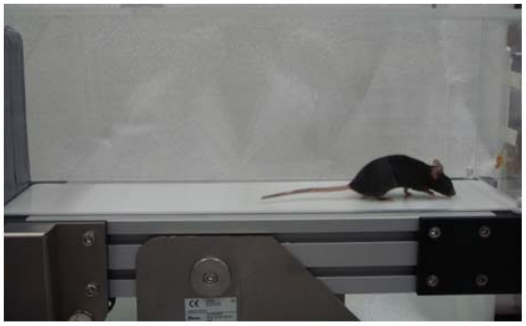


Figure 1 Mouse running on a treadmill

Transcardial perfusion

Mice were anesthetized with equithasine. The proper depth of anesthesia was tested by pinching the limbs of the animal without response and by the lack of a pupillary reflex. Under deep anesthesia, and cold conditions on ice, the heart was exposed through a dissection of the ventral side of the thorax and abdomen. A small incision in the right atrium was performed, and 3 ml of ice-cold pre-fixative solution were applied to the left ventricle, followed by 40 ml of ice-cold fixative solution. The brainstem was removed

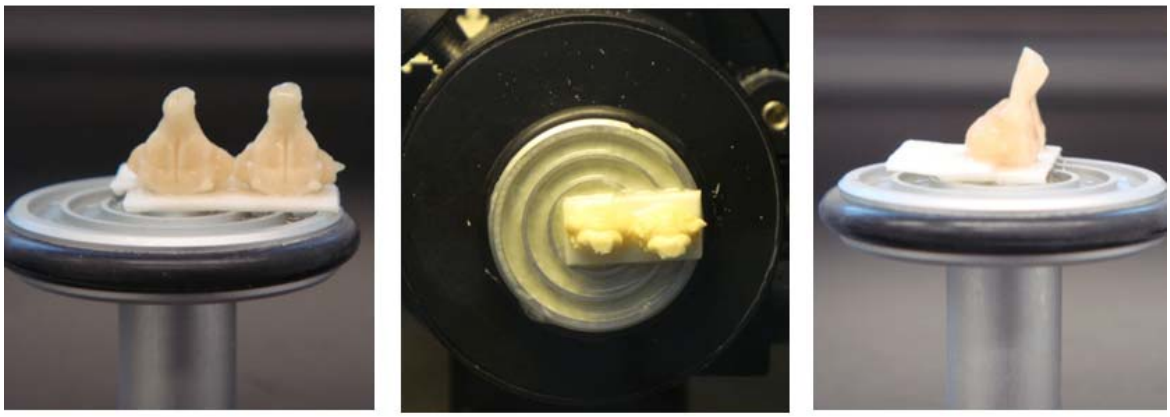


Figure 2. Tissue mounted on a cryostat's chock. Control and locomoted tissue were mounted alongside

and immersed in 10 ml of fixative solution for 2 hrs. Finally the tissue was kept in cryoprotectant solution until cut. The cryoprotectant solution, consisting of 30% sucrose in sodium phosphate buffer (25mM) , pH 7.4, was changed overnight to remove remaining fixative solution (for more information about the solutions used, see Appendix 1).

Inmunohistochemistry

Coronal tissue sections were cut on a cryostat at either 16 µm or 20 µm thickness and mounted on gelatinized slides. Control and treatment sections were mounted on the same chuck (see figure 2). Sample sections were selected following a "systematic-random sampling" procedure (Schmitz and Hof, 2005), with every third section encompassing the entire region of interest mounted on glass slides. Adjacent sections were used to test antibodies. The approximate rostro-caudal extent of our region extended from bregma -8.0 to bregma -5.5.

Premounted tissue was washed during 20 minutes in 1.5M TBST solution. Then, it was incubated overnight at 4°C in primary antibody solution consisting of anti-c-fos antibody (Calbiochem, rabbit polyclonal, 1:5000, or Santa Cruz Biotechnology,1:1000), 10% normal donkey serum and 1.5M TBST.

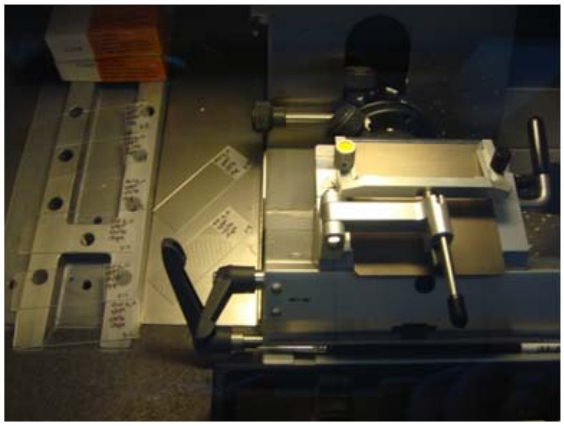


Figure 3. Cryostat

Three 20-minute rinses in 1.5 TBST were performed on the tissue. Then, the slides were incubated during 1 ½ hr in secondary antibody solution consisting of donkey anti rabbit Cy5 (1:500) or donkey anti goat Cy3 (1:300), 10% normal donkey serum and 1.5 TBST. The tissue was washed in 1.5 TBST during 20 minutes, and then two 15-minutes washes in 50 mM Tris solution were applied. In this work we were interested in the number of c-fos -positive and -negative eYFP neurons in the medulla. C-fos is localized in the cell nucleus. It is possible that cell nuclei

of neurons can be lost during processing (we found several examples, data not shown). In order to corroborate that cell nuclei were intact in the eYFP cells we counted, we applied DAPI. DAPI is a fluorescent stain that binds to DNA. It has been widely used for DNA visualization and quantification (Kapuscinski, 1995). Finally the slides were coverslipped with a glycerol-based antifade medium (Valnes and Brandtzaeg, 1985) or fluoromount-G (Southern Biotech).

Imaging

Conventional immunofluorescence images were acquired on a Zeiss Axioskop2 fluorescence microscope using Axiovision 4.0 software (Carl Zeiss Canada, Toronto, ON, Canada), and assembled using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA) (Nagy et al., 2011). Alternatively, cells were counted and plotted using software from MicroBrightField (USA): Stereo Investigator v11 (counting) and Neurolucida v11 (plotting). Images were superimposed in tracings/3D model based on the Paxinos and Franklin's mouse brain atlas (Paxinos and Franklin, 2007).

Cell population estimations

Design-based stereology was used to estimate the number of cells in this project (West, 1993, 2002). Stereo Investigator is software commonly used to apply the principles of design-based stereology to brain tissue (Schmitz and Hof, 2005). We used Stereo Investigator's "Optical Fractionator Method" for cell counting.

Results

Inmunohistochemistry

The mouse model: eYFP is expressed in serotonergic cells

The mouse model used in this work was designed using a Pet-1 enhancer region that is capable of direct 5-HT neuron-specific transgene expression in the Central Nervous System (Scott et al., 2005). Serotonergic cells expressed the enhanced Yellow Fluorescent Protein (eYFP) in the soma and proximal processes.

The localization of eYFP positive cells corresponded well with the localization of serotonergic cells in the rat (Dahlstrom and Fuxe, 1964), particularly with the localization reported recently in mice (VanderHorst and Ulfhake, 2006). Compare our figure number 4 with their figure 6.

eYFP positive cells were found throughout the rostrocaudal levels from bregma -5.5 to bregma -8.0. The caudal serotonergic nuclei (Rpa, Rob, Rmg, LPGi) are located in this region in the mouse (VanderHorst and Ulfhake, 2006). At the level of bregma -8.0, scattered eYFP cells were observed in the ventral and ventrolateral medulla; at this level the pyramidal decussation (Pyx) was visible in the tissue, see figure 4A. More rostrally, at the level of bregma -7.0, Rpa, Rob and laterally located cells in the LPGi were clearly identified, and the inferior olive was also detected under the microscope (see figure 4C and 4B). This pattern was observed until about bregma -6.75, where laterally located cells were more sparse and cells more ventrally located appeared, see figure 4D. At bregma -6.0 no more laterally located cells were found, see figure 4E.

Localization of eYFP/c-fos positive cells

C-fos inmunohistochemistry was used to determine cells that were active during a locomotor task on a treadmill. Previous reports have successfully used this technique to label active cells in the brainstem and spinal cord. Many have used this technique to study cell groups involved in the motor system (Barajon et al., 1992; Carr et al., 1995; Dai et al., 2005; Huang et al., 2000; Jasmin et al., 1994; Ohiwa et al., 2006; Todd et al., 2010; Wilson et al., 2010; Wilson et al., 2005). C-fos and DAPI co-labelling were used to determine c-fos positive cells (see figure 5). Figure 6 shows examples of an eYFP/c-fos positive cell (arrow) and an eYFP-only positive cell (asterisk).

Localization of eYFP/c-fos positive cells in a control animal is shown in figure 7, and a representative example of a locomoted animal is shown in figure 8. eYFP-only positive cells are illustrated in green, eYFP/c-fos positive cells are illustrated in red, and c-fos-only positive cells are illustrated in blue. The most pronounced increase in eYFP/c-fos positive cells in the locomoted animals compared to the control ones was observed between bregma -6.84 and bregma – 6.48 (see figures 7 and 8, panels C-E). This region is located between the rostral end of the inferior olive and the caudal end of the facial nucleus. Notice, however, that eYFP/c-fos positive cells located in the midline raphe area increased mainly at the level of bregma -6.84 (figures 7 and 8, panel D).

Neuron population estimations

In order to quantify the number of eYFP/c-fos-positive cells in the PPR and midline raphe area, a design-based stereological approach was taken (Schmitz and Hof, 2005). Several shortcomings were encountered, and will be analyzed in the discussion section.

Figure 9 shows the number of eYFP/c-fos positive (darker aqua) and eYFP-only cells (lighter aqua) in one control (panel A) and one animal subjected to a locomotor task on a treadmill (panel B). Sections with no counts in them are sections that were lost during tissue processing (during cryocutting and inmunostaining) in both control (sections 7, 13, 16, 19, 37, 55, 58, 61) and locomoted (sections 13, 40, 43 and 55) animals. Table 1 illustrates the number of eYFP cells counted (including those expressing and not expressing c-fos) and c-fos/eYFP positive cells in each sampled section. Section number 1 corresponds with bregma level -7.2, and section 61 corresponds with level bregma level -6.2 (this data would roughly correspond to panels B to F in the diagrams shown in figures 7 and 8). Lost sections are shown in gray.

The total count of eYFP positive cells in the control animal was of 86; from this result the total population calculated for the sampled region by stereo investigator was 434.51, with a coefficient of error of 0.15. Among these 86 eYFP-positive cells, only 1 cell expressed c-fos. The total count of eYFP positive cells in the animal subjected to treadmill locomotion was 253; the total population estimation calculated by stereo investigator was 1033.97, with a coefficient of error of 0.07. Among these 253 eYFP-positive cells, 47 expressed c-fos. The population estimation for locomotor-related serotonergic neurons calculated by stereo investigator was 235.91, with a coefficient of error of 0.19. The

localization of eYFP-only positive cells and eYFP/c-fos positive cells of this animal was superimposed on a 3D model of adult mouse brain constructed on Neurolucida from images in the Paxinos and Watson's atlas (Franklin and Paxinos, 2007) for adult mouse brain (see video 2).

In the control animal, the only eYFP/c-fos positive cell found was located in section 52, which corresponds with bregma level -6.3, in between panel E and F in figures 7 and 8. In the case of the animal subjected to treadmill locomotion, a peak in the percentage of c-fos expressing eYFP cells was found at section 19, where 35% of eYFP cells expressed c-fos (8 out of 23) . This section corresponds with bregma level -6.9, panel C in figures 7 and 8. A second peak was found at section 46, where 92% of eYFP cells expressed c-fos (12 out of 13). This section corresponds with bregma level -6.5, in between panel D and E in figures 7 and 8. Sections 4, 13, 16, 31, 43, 49, 52 and 61 were lost during tissue processing

Figure 10 shows data from one animal subjected to a locomotor task on a treadmill. Cells in the midline raphe area are depicted in panel A, whereas panel B corresponds with data from the PPR. Darker green and aqua represent the number of eYFP cells that expressed c-fos; lighter green and aqua represent the number of eYFP cells that did not express c-fos. Sections with no counts in them are sections that were lost during tissue processing (4, 13, 16, 31, 43, 49, 52 and 61). Table 2 illustrates the number of eYFP cells counted (including those expressing and not expressing c-fos) and c-fos/eYFP positive cells in each sampled section. Section number 1 corresponds with bregma level -6.9, and section 64 corresponds with level bregma level -6.1 (this data would roughly correspond to panels C to F in the diagrams shown in pictures 7 and 8). Cells in gray show lost sections.

The total count of eYFP positive cells in the raphe region was of 594, and the total population estimation calculated by stereo investigator was of 2573.09, with a coefficient of error of 0.06. Among these 594 eYFP-positive cells, 24 expressed c-fos; the population estimation calculated by stereo investigator was 103.96, with a coefficient of error of 0.20. The total count of eYFP positive cells in the PPR was of 219, and the total population estimation calculated by stereo investigator was of 947.26, with a coefficient of error of 0.09. Among these 219 eYFP-positive cells, 16 expressed c-fos; the population estimation calculated by stereo investigator was 69.21, with a coefficient of error of 0.24.

In the raphe area, a modest increase in the percentage of c-fos expressing eYFP cells can be found at sections 55 and 58, where 14% and 21% of eYFP cells expressed c-fos, respectively (5 out of 36, and 5 out of 24, respectively). These sections correspond with bregma level -6.2, panel F in figures 7 and 8. In the PPR, a modest increase in the percentage of c-fos expressing eYFP cells can be found at sections 7 and 10, where 17% and 14% of eYFP cells expressed c-fos, respectively (1 out of 6, and 2 out of 14, respectively). These sections correspond with bregma level -6.8, panel C in figures 7 and 8. A second peak was found at sections 55 and 58, where 27% and 57% of eYFP cells expressed c-fos, respectively (3 out of 11, and 4 out of 7, respectively). These sections correspond with bregma level -6.2, panel F in figures 7 and 8.

Data from two animals (number 11 and 13) subjected to treadmill locomotion are shown in figure 11; cells included were those located between bregma -6.2 to -7.2 (approximately corresponding to panels B to F in figures 7 and 8), where the majority of eYFP-positive cells were located. The percentage of c-fos expressing eYFP cells in the raphe area was 4% (data from only one animal, 13), whereas in the PPR 13.3% of eYFP

cells expressed c-fos (standard deviation of 7.9). Note that the total number of neurons expressing c-fos in the raphe, however, was higher than in the PPR.

Discussion

The use of a design-based method of cell counting

At the beginning of this work we counted cells using photomicrographs taken with a 10x objective, we call this approach "manual counting" from now on. However, later we realized this approach presented several setbacks (Schmitz and Hof, 2005; West, 1993, 1999, 2002; West and Slomanka, 2001). In particular, West and colleagues have postulated that counting cell profiles seen on a tissue section introduces error because the number of cell profiles does not correspond with the number of cells. Furthermore, there is no simple relation among these two values (Coggeshall, 1992). According to Coggeshall and colleagues " this is because profile "number" depends not only on the actual number of particles, but also on the size, shape, orientation and many other variables of the particles being counted" (Coggeshall, 1992). Finally, studies analysing the bias introduced by manual counting indicate that results can be biased by as much as 40% (Coggeshall, 1992; West, 1993)

On the other hand, it is well known that the serotonergic system in rodents consists of a total of only a few tens of thousands of serotonergic neurons, segregated into a rostral and a caudal group (Hornung, 2010). Furthermore, as Hornung indicates, "the proportion of serotonin-containing neurons in a given cytoarchitectonic subdivision varies from

between 80-100% in regions of the dorsal raphe, to 10% in the lateral reticular formation".

In this work, we are interested in serotonergic neurons in the caudal groups, particularly in the proportion of cells that expressed c-fos after a locomotor task in the PPR (located in the reticular formation) compared to those in the midline raphe area. Therefore, whereas in other studies the error introduced by counting cell profiles might be acceptable, that is not the case for us. This is because the reduced number of cells implies that our population of interest is very susceptible to such an error.

Of course, data obtained using the manual counting technique can still be useful because it indicates the localization of eYFP-only positive cells and c-fos/eYFP positive cells (figure 7 and 8). Moreover, localization of cells using the manual method corresponded well with that obtained using a design-based stereological approach (compare figures 7 and 8 with the 3D model in video 2).

Issues preventing us from obtaining valid neuron population estimations

The method known as the "optical fractionator method" was used in this study to estimate numbers of cells. This method has been reviewed extensively (West, 1993, 1999, 2002). Several conditions must be met when applying it to histological sections:

1. The correct use of an optical dissector probe, following the appropriate rules. First, the user must choose a particular point in the cell to be counted (we chose the cell top) and count the cell only if that particular point is located inside the

dissector probe. The probe can be defined as a 3-D space flanked by green (inclusion) and red (exclusion) planes (counting frame), for more details, review (West, 2002). The objects are counted only if they lie inside the counting frame or if they are touched by a green (inclusion) line but not a red (exclusion) line.

2. The sections selected to sample must be chosen in a statistically unbiased manner.
3. The dissector probe must be placed in a statistically unbiased manner in the sections chosen. This is achieved through an in-built mechanism in the Stereo Investigator software.

During the present study we had difficulties following criterion number 2. Therefore, we will discuss it in more depth. In order to achieve meaningful (unbiased) sampling, a random selection of sections to be sampled must be performed. There are two approaches to meet this requirement: "independent random" and "systematic random" sampling selection (West, 2002). We used systematic random sampling because it is more efficient and easily applied to histological preparations, but most of all, because the software we are using to estimate number of cells (Stereo Investigator v11) required us to do so. The goal of the "systematic random sampling technique" (as for any other procedure that intends to obtain unbiased sampling) is to guarantee that every area of the region has an equal probability of being sampled. It is systematic because sections will be chosen at every determined by the section sampling interval; the first section being chosen randomly. Figure 12 shows the type of sampling we used in this study. We chose every third section in our region. We randomly chose the first section and (for the sake of simplicity) we called it number one (even though it is not the first in our region of interest). Unfortunately, during the processing of the tissue (specifically during sectioning

and staining) we lost sections belonging to the sample group. Sections lost are represented in figures 9 and 10 as those showing zero cells counted, and are shaded in gray in tables 1 and 2. The estimations obtained, therefore, are not valid. This fact is also revealed by the high coefficients of error of such estimations (see tables 1 and 2 caption and the results section). Even though high coefficients of error are expected because the cells we are interested in are very sparsely located (especially those located in the PPR) and the population is relatively small, we believe that a sample with no sections lost would render lower coefficients of error. Supporting this view, the coefficient of error for the estimation of the total population of eYFP cells in animal number 11, which is the one with the least tissue lost (only 4 sections lost), was of 0.07 (see figure 9 panel B, and the last two columns of table 1).

A second observation revealed another technical issue that prevented us from obtaining valid cell population estimations. Notice that the total number of eYFP cells counted in the PPR of animals number 11 (two last columns in table 1) and 13 (last two columns in table 2), 253 and 219 respectively, are very similar as it would be expected. However, notice that the total number of eYFP cells counted in the PPR of animal number 12 (first two columns in table 1) is substantially lower, 86 cells. Animals 11 and 13 were animals subjected to a locomotor task on a treadmill, whereas animal 12 belonged to the control group. In the case of the manual counting, the total sample in the PPR in one animal subjected to a locomotor task (animal number 3) was of 607 eYFP cell profiles; whereas in the control mouse (animal number 4), it was of only 349. Therefore, the number of eYFP cells/cell profiles sampled was consistently lower in control animals compared to those subjected to a locomotor task. At least two hypotheses could explain this

observation. Number one, the locomotor task causes an overexpression of eYFP and this is reflected in more eYFP cells/cell profiles counted. Inmunostaining to reveal serotonergic cells in control and locomoted animals could be used to decipher if this is in fact the case, but a thorough strategy to test this hypothesis is out of the scope of this discussion. A simpler explanation could be that eYFP cells in control animals faded while cells were being counted in the tissue of animals subjected to a locomotor task. This hypothesis is possible because, as detailed in the methods section, sections from control and locomoted animals were mounted on the same glass. On the other hand, experimental animals were consistently (and without any specific reason) counted first. Mounting control and locomoted tissue on separate glass and redoing the counting could be a simple way to evaluate this hypothesis.

Taking into account the restrictions previously discussed, it can be said that data obtained manually and the sampling obtained with the designed-based approach suggest that there is in fact an increase in the number of serotonergic cells that express c-fos after a locomotor task in the PPR (figure 7 and 8, panels C and E; figure 9). Quantification of these populations remains to be studied.

A very restricted region with high c-fos expression

An interesting observation in this study is the high c-fos level found at bregma -6.64 (figure 8, panel D). This observation was not quantified (because it was not related to our main objective) but was consistently observed in all the animals subjected to locomotion. The area shown in the figure represents the sampled area; we do not know the

dorsoventral extent of this high-c-fos expression region. In general, this region has been suggested to contain putative chemoreceptors, which are "cells that are sensitive to changes in PCO_2 or pH and contribute to the stimulation breathing elicited by hypercapnia or metabolic acidosis" (Guyenet et al., 2010). It has been shown that glutamatergic cells located in the rat retrotrapezoid nucleus (Abbott et al., 2011; Abbott et al., 2009), which in mouse roughly corresponds with the ventrolateral medulla at the level of bregma -6.4 to bregma -6.6, regulate ventilation. Moreover, Abbott and colleagues showed that the tonic activation of these cells "produces far greater breathing stimulation" than their intermittent activation. This region has also been involved in vasomotor, cardiovagal autonomic functions (Arango et al., 1988; Benarroch, 1993; Dergacheva et al., 2010; Gaytán and Pásaro, 1998; Goutagny et al., 2008; Guyenet, 2006; Guyenet et al., 1989; Ishikawa et al., 2001; Iwamoto et al., 1996; Kuwaki et al., 1991; McAllen and Dampney, 1990; Nattie, 2000; Okada et al., 2001; Ross et al., 1981; Yang et al., 1999; Zagon and Bacon, 1991). Therefore, it is suggested that further analysis of this finding, a very restricted region with high c-fos expression, may render interesting insights in the regulation and integration of autonomic functions during locomotion.

Finally, regarding the use of c-fos as a neuronal activity marker, previous studies have demonstrated that certain neurons do not show c-fos elevation in spite of being activated (Dragunow and Faull, 1989). Therefore, it is possible that some serotonergic neurons in the medulla do not express c-fos even when activated. Future exploration of this issue is also recommended.

Higher proportion of c-fos/eYFP neurons in the PPR in comparison with the raphe area

We found that a greater percentage of eYFP cells expressed c-fos after a locomotor task on a treadmill in the PPR in comparison with the raphe area (figure 11). Our findings are in agreement with Iwamoto and colleagues (Iwamoto et al., 1996). This group reported that in rats subjected to a locomotor task on a treadmill, c-fos is expressed in the PPR in greater extent than in the raphe area, see their figure 6. Our results are also in agreement with the report by Liu and Jordan regarding the ability of a specific area to evoke locomotion when stimulated in the neonatal in vitro preparation (Liu and Jordan, 2005). These authors described such region as follows: "The effective area [to evoke locomotor-like activity] was 0.5-1.2 mm caudal to the junction between the pons and medulla (just rostral to the inferior olivary nucleus at the level of the nucleus of the VIIth cranial nerve), 0.5-1.0 mm lateral to the midline, and 0.2-0.5 mm beneath the ventral surface". Furthermore, they found that stimulation in the raphe area did not evoke locomotion-like activity (see their figure 1, panels A and B). Moreover, they demonstrated that this effect was mediated by 5-HT_7 and 5-HT_{2A} receptors. As discussed in the introduction, serotonergic cells in the PPR (as well as those located in the raphe area) project to the ventral horn of the lumbar spinal cord (Basbaum et al., 1978; Bowker et al., 1981a; Bowker et al., 1983; Dahlstrom and Fuxe, 1964; Helke et al., 1989; Hökfelt et al., 2000; Holstege, 1987a; Holstege and Kuypers, 1987a, b; Jones and Light, 1992; Martin et al., 1978; Schmidt and Jordan, 2000; Skagerberg and Bjorklund, 1985; Steinbusch, 1981).

Among these studies, the one authored by Light and Jones (Jones and Light, 1992) has particular relevance for our findings because they found that a higher proportion of serotonergic neurons located in the PPR (in rat) compared to those located in the raphe project to the lumbar spinal cord at different rostrocaudal levels, see their figure 2E. It is interesting to note that they found a very high percentage of serotonergic cells in the PPR projecting to the lumbar cord at bregma levels -9.8 (75%), -11.6 (75%) and -12.8 (91.3%). On the other hand, the proportions of serotonergic cells in the raphe area that project to the lumbar cord were less than 70% at all levels studied (bregma -9.8-60.5%, -10.8-54.3% , -11.3-52.4%, -11.6-53.3%, -12.8-69.0%). More recently, VanderHorst and colleagues found spinally projecting serotonergic cells throughout the medulla oblongata; they reported that "All of these regions [Rmg, Rpa, Rob, LPGi, and the ventrolateral medulla at the level of xCST], ***but specially the LPGi,*** contained neurons double labeled for CTB and 5-HT", emphasis added (VanderHorst and Ulfhake, 2006).

Conclusion

Our results show that the adult mouse has serotonergic cells in regions able to evoke locomotion in the neonate - PPR - (Liu and Jordan, 2005). Furthermore, our results suggest that there is an increase in c-fos labelling in such serotonergic cells after a locomotor task. Our data indicates that this population of serotonergic neurons is located in between bregma -6.8 and bregma -6.48 (figure 8, panels C to E) in the mouse, as revealed by the expression of c-fos after a locomotor task on a treadmill. The rostral limit of this region corresponds to the caudal end of the seventh cranial nerve nucleus, whereas the caudal limit corresponds to the rostral end of the inferior olive. Our observations

support the idea that serotonergic cells in both the PPR and the midline raphe play a role in locomotion. A higher proportion of PPR serotonergic cells appear to be locomotion-related.

Images

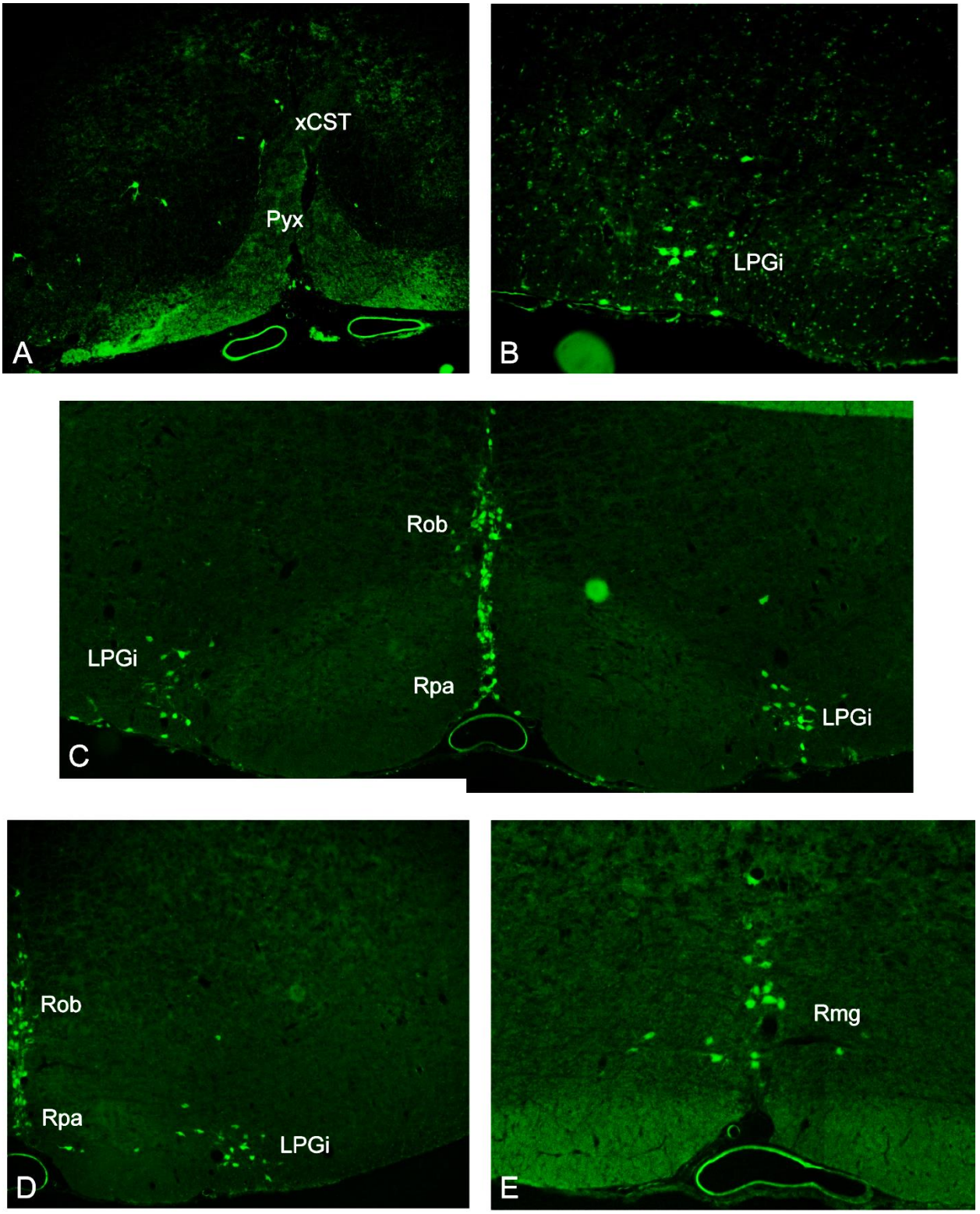


Figure 4. Photomicrographs of eYFP positive cells in the brainstem (5x objective). A: Bregma -8.00, B: Bregma: -7.02, C: Bregma -7.0, D: Bregma -6.75, E: Bregma -6.0.

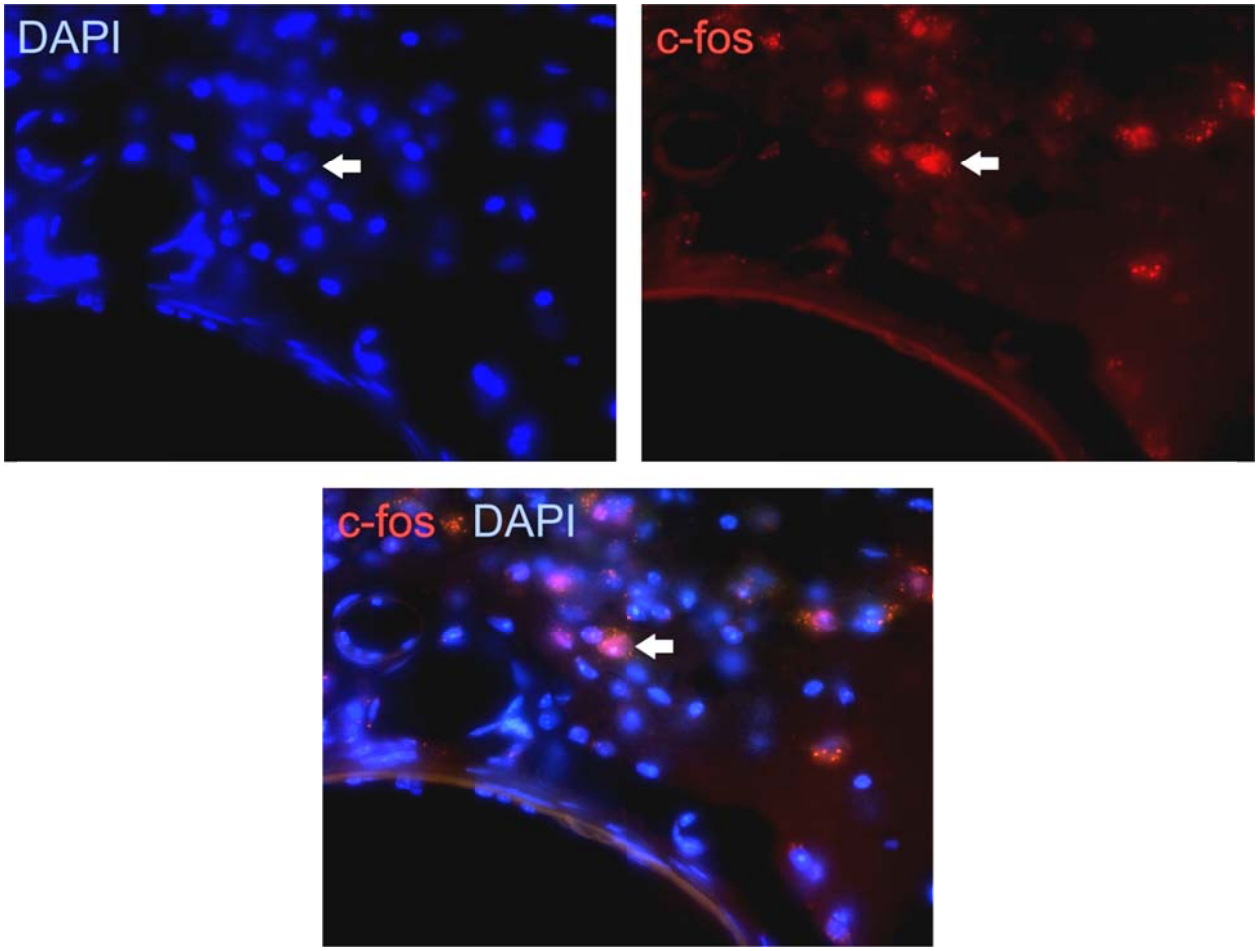


Figure 5. Photomicrograph illustrating DAPI and c-fos co-labeling (63x oil objective). DAPI was used to identify with higher certainty c-fos positive (arrow) and negative cells.

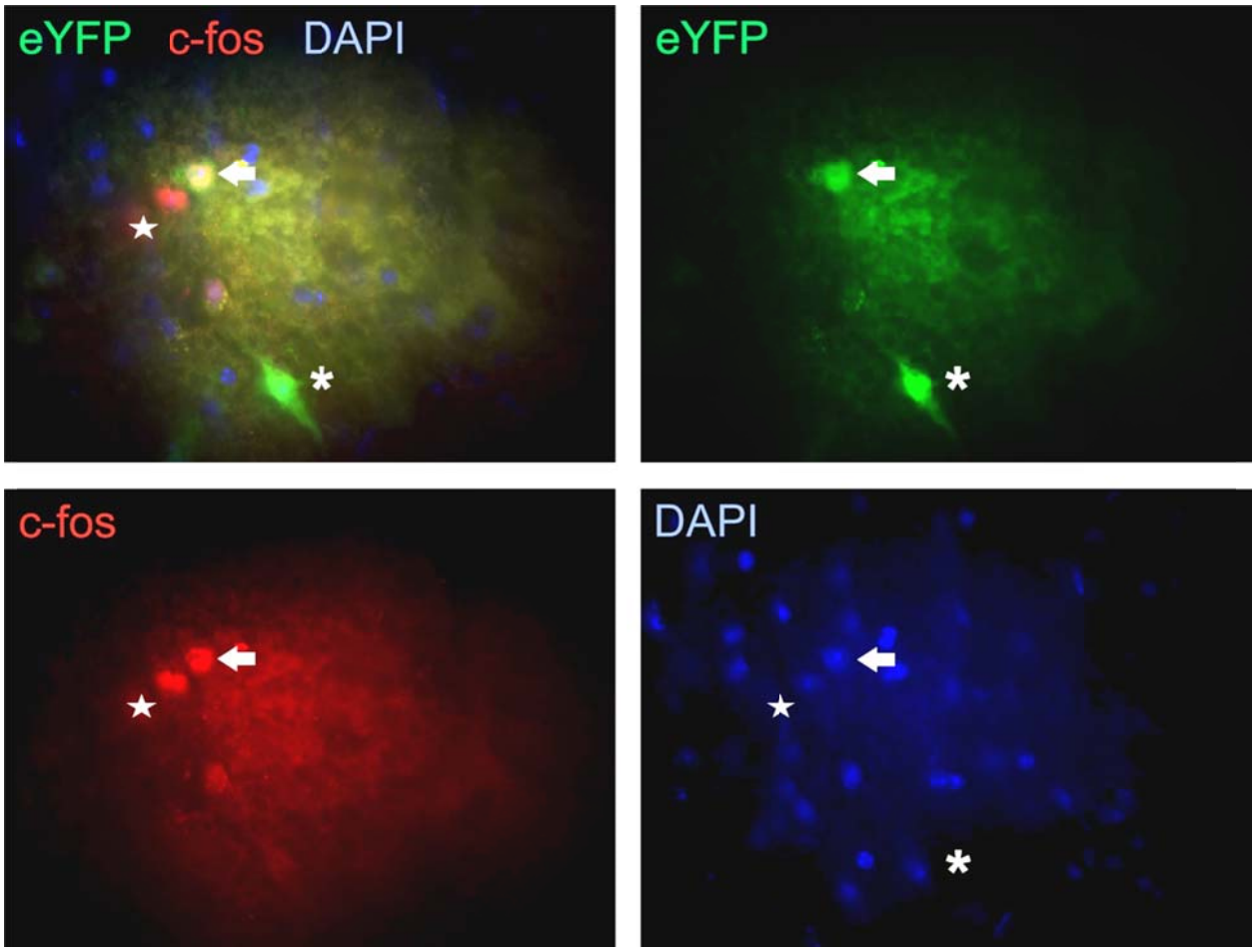


Figure 6. Photomicrograph illustrating the appearance of one eYFP/c-fos positive cell (arrow) one eYFP-only positive cell (asterisk) and one c-fos-only positive cell (star). 63x oil objective.

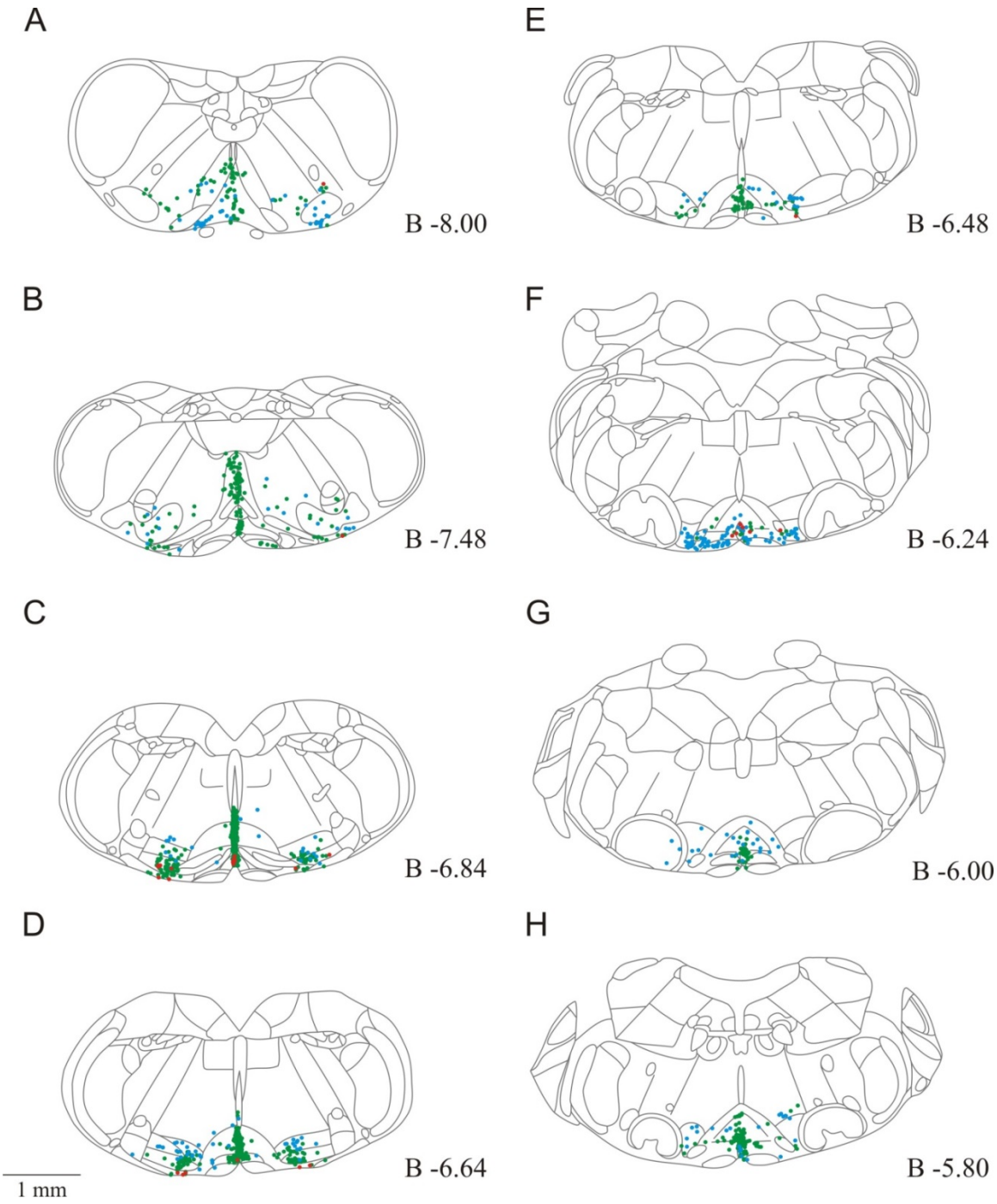


Figure 7. Drawings of coronal sections through the medulla oblongata (bregma levels are indicated) that show the distribution of eYFP-only positive cells (green), eYFP/c-fos positive cells (red), and c-fos-only positive cells (blue) in one animal kept at rest (animal number 3). For more details on the anatomical structures drawn, refer to the Paxinos and Watson adult mouse brain atlas (Franklin and Paxinos, 2007).

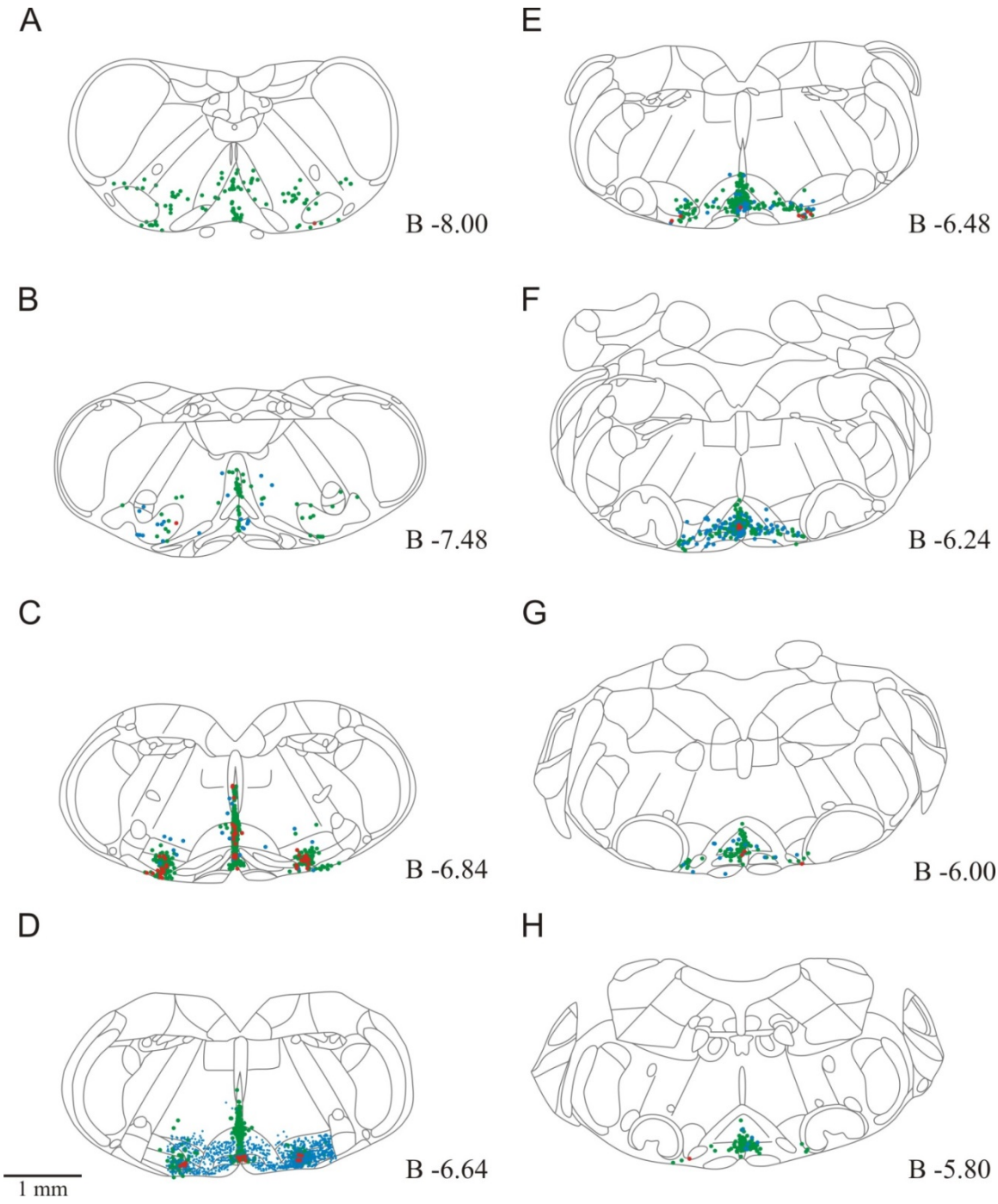
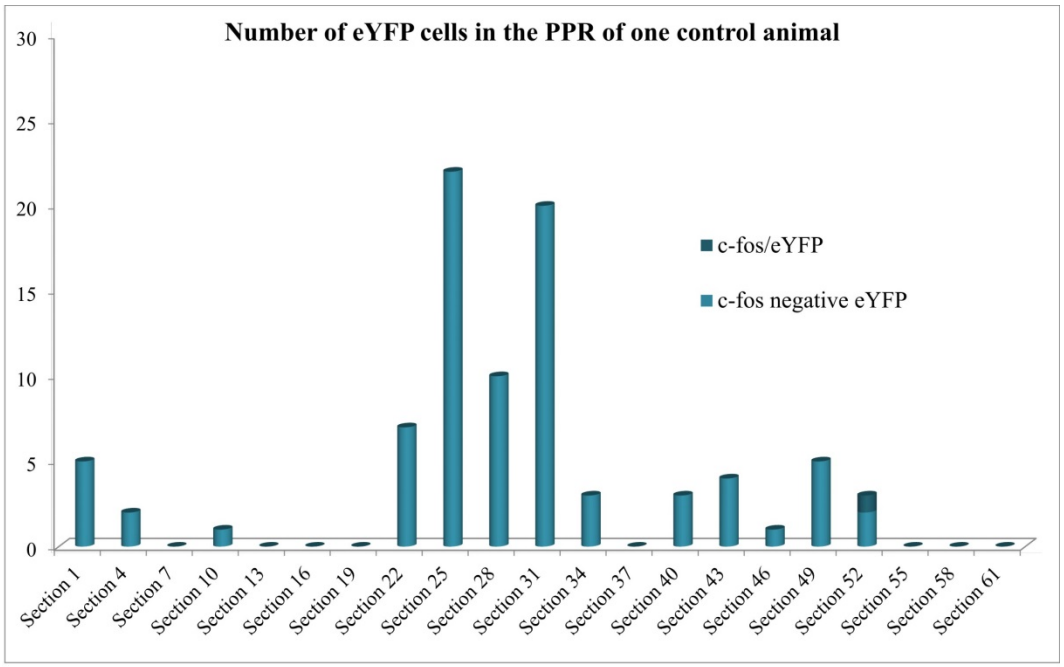


Figure 8. Drawings of coronal sections through the medulla oblongata (bregma levels are indicated) that show the distribution of eYFP-only positive cells (green), eYFP/c-fos positive cells (red), and c-fos-only positive cells (blue) in one animal subjected to treadmill locomotion (animal number 4). For more details on the anatomical structures drawn, refer to the Paxinos and Watson adult mouse brain atlas (Franklin and Paxinos, 2007).

A



B

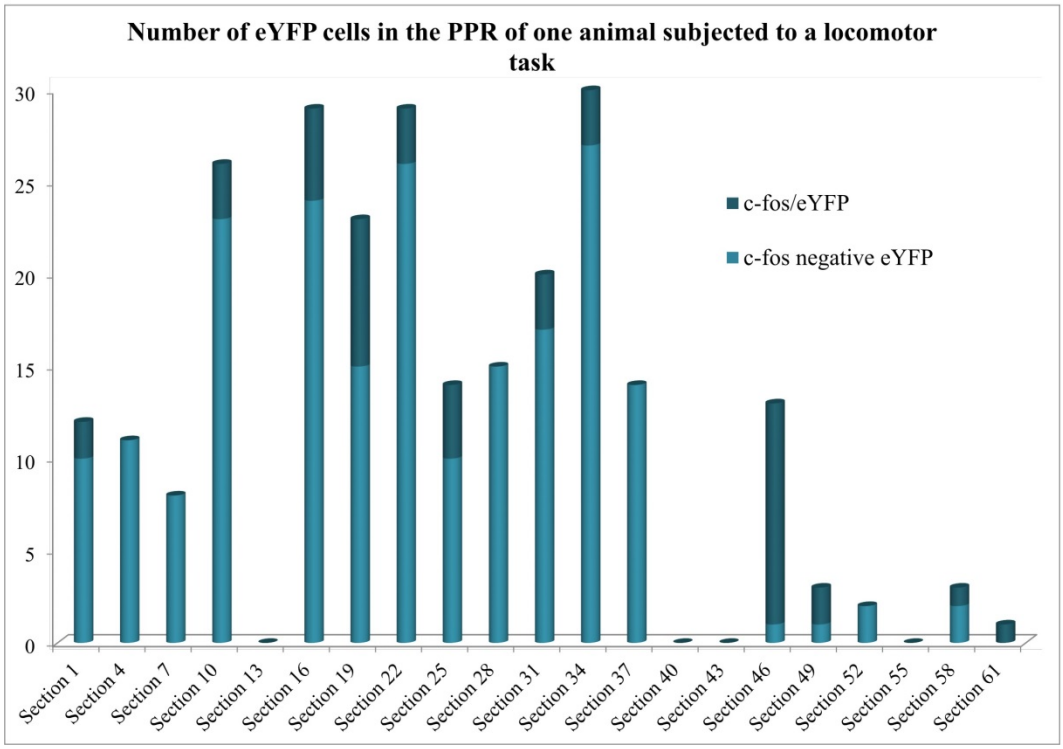
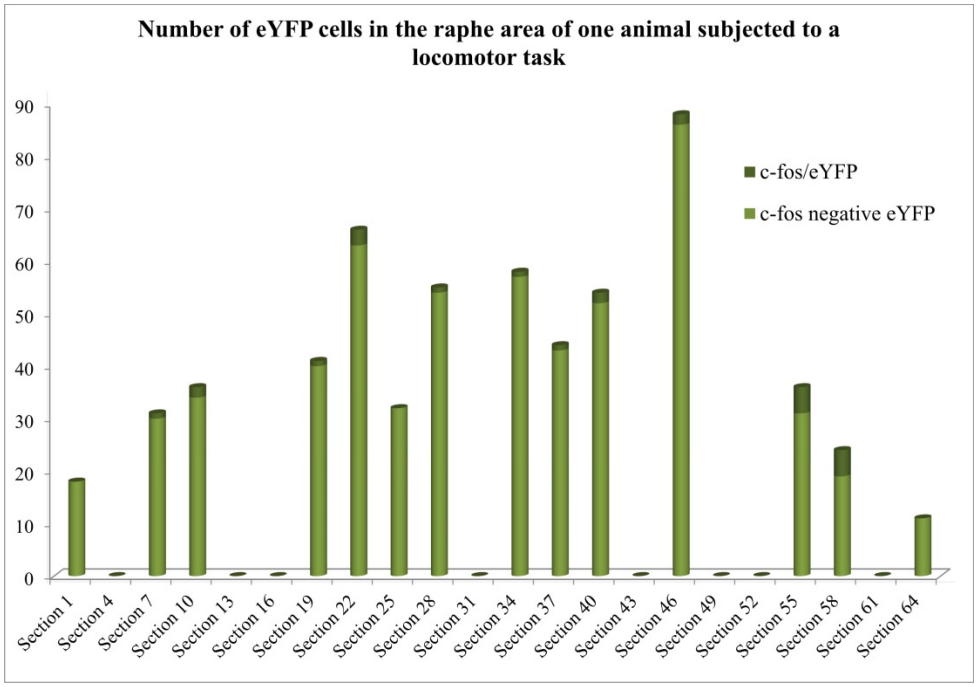


Figure 9. Number of eYFP neurons in the PPR expressing or not c-fos. Panel A shows one mouse kept at rest (animal number 12). Panel B shows one animal subjected to treadmill locomotion (animal number 11), along the rostrocaudal medulla. Sections cover bregma levels from -7.2 (section 1) to -6.2 (section 61), approximately. Sections lost during the processing of the tissue are those that have no cell counting (cero).

Table 1. Number of eYFP-only positive cells and eYFP/c-fos positive cells counted in one mouse kept at rest (animal number 12) and one mouse subjected to treadmill locomotion (mouse number 11). Data corresponds with figure 9. Tissue lost during processing is shown in gray. Total population estimation is the number of cells calculated by the stereological software (Stereo Investigator, MBF) using "mean section thickness with counts". The coefficients of error (Gundersen, m=0) were as follows: Control eYFP = 0.15, control c-fos/eYFP = 1.00, locomoted eYFP = 0.07, locomoted c-fos/eYFP = 0.19.

	CONTROL		LOCOMOTION	
Section	eYFP	c-fos/eYFP	eYFP	c-fos/eYFP
Section 61	0	0	1	1
Section 58	0	0	3	1
Section 55	0	0	0	0
Section 52	3	1	2	0
Section 49	5	0	3	2
Section 46	1	0	13	12
Section 43	4	0	0	0
Section 40	3	0	0	0
Section 37	0	0	14	0
Section 34	3	0	30	3
Section 31	20	0	20	3
Section 28	10	0	15	0
Section 25	22	0	14	4
Section 22	7	0	29	3
Section 19	0	0	23	8
Section 16	0	0	29	5
Section 13	0	0	0	0
Section 10	1	0	26	3
Section 7	0	0	8	0
Section 4	2	0	11	0
Section 1	5	0	12	2
Total Sampling	**86**	**1**	**253**	**47**
Total population estimation	**439.62**	**5.11**	**1269.88**	**235.91**

A



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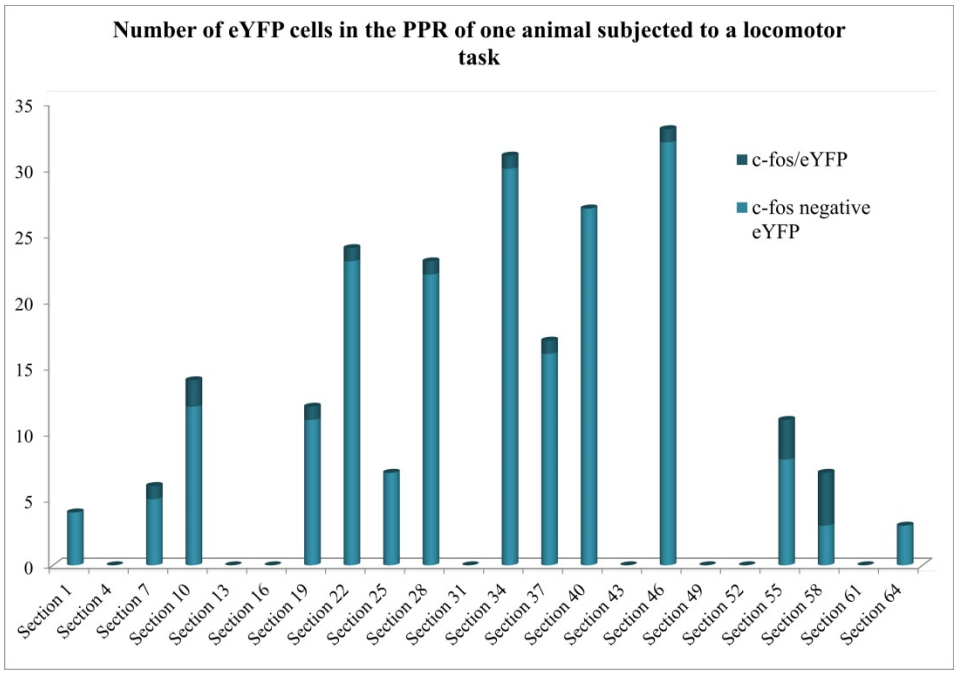

Figure 10. Number of eYFP neurons expressing or not c-fos in one mouse subjected to treadmill locomotion (animal number 13) in the raphe (panel A) and the PPR (panel B). Sections cover bregma levels from -7.2 (section 1) to -6.2 (section 64), approximately. Sections lost during the processing of the tissue are those that have no cell counting (cero). Notice y axis are different.

Table 2. Number of eYFP-only positive cells and eYFP/c-fos positive cells counted in one mouse subjected to treadmill locomotion (mouse number 13), in the raphe area and PPR. Data corresponds with figure 10. Tissue lost during processing is shown in gray. Total population estimation is the number of cells calculated by the stereological software (Stereo Investigator, MBF) using "mean section thickness with counts". The coefficients of error (Gundersen, m=0) were as follows: Raphe eYFP = 0.06, raphe c-fos/eYFP = 0.20, PPR eYFP = 0.09, PPR c-fos/eYFP = 0.24.

	RAPHE		PPR	
Section	eYFP	c-fos/eYFP	eYFP	c-fos/eYFP
Section 64	11	0	3	0
Section 61	0	0	0	0
Section 58	24	5	7	4
Section 55	36	5	11	3
Section 52	0	0	0	0
Section 49	0	0	0	0
Section 46	88	2	33	1
Section 43	0	0	0	0
Section 40	54	2	27	0
Section 37	44	1	17	1
Section 34	58	1	31	1
Section 31	0	0	0	0
Section 28	55	1	23	1
Section 25	32	0	7	0
Section 22	66	3	24	1
Section 19	41	1	12	1
Section 16	0	0	0	0
Section 13	0	0	0	0
Section 10	36	2	14	2
Section 7	31	1	6	1
Section 4	0	0	0	0
Section 1	18	0	4	0
Total Sampling	**594**	**24**	**219**	**16**
Total population estimation	**2573.09**	**103.96**	**947.26**	**69.21**

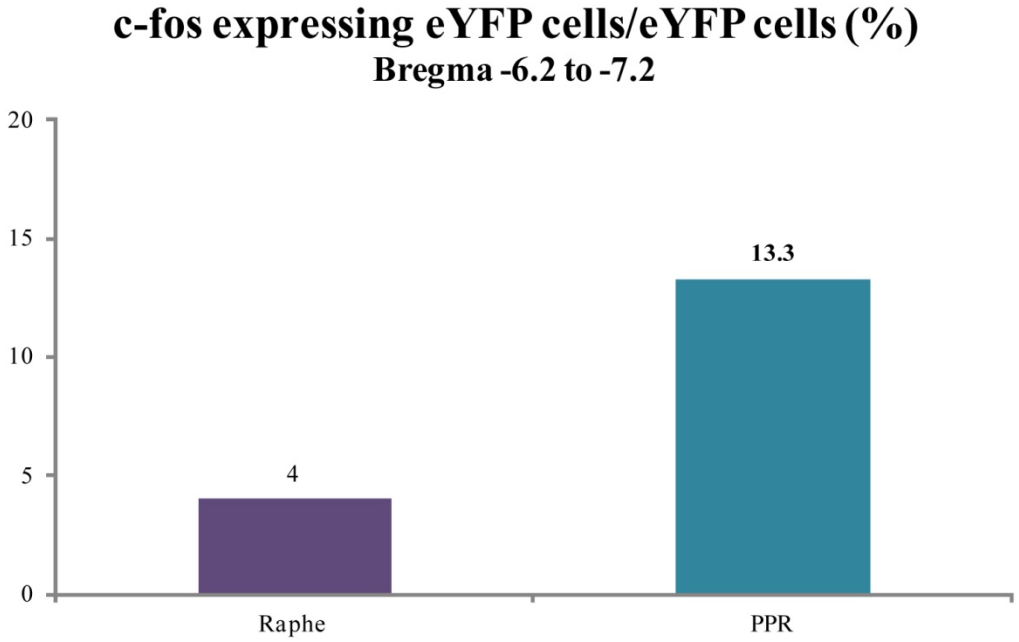

Figure 11. Proportions of c-fos expressing eYFP neurons in the raphe (purple, one animal-13) and the PPR (aqua, two animals- 11 and 13, standard deviation of 7.9) of two animals subjected to treadmill locomotion. The figure includes sampled data from level bregma -6.2 to -7.2, approximately corresponding to cells in panel B to F in figures 7 and 8.

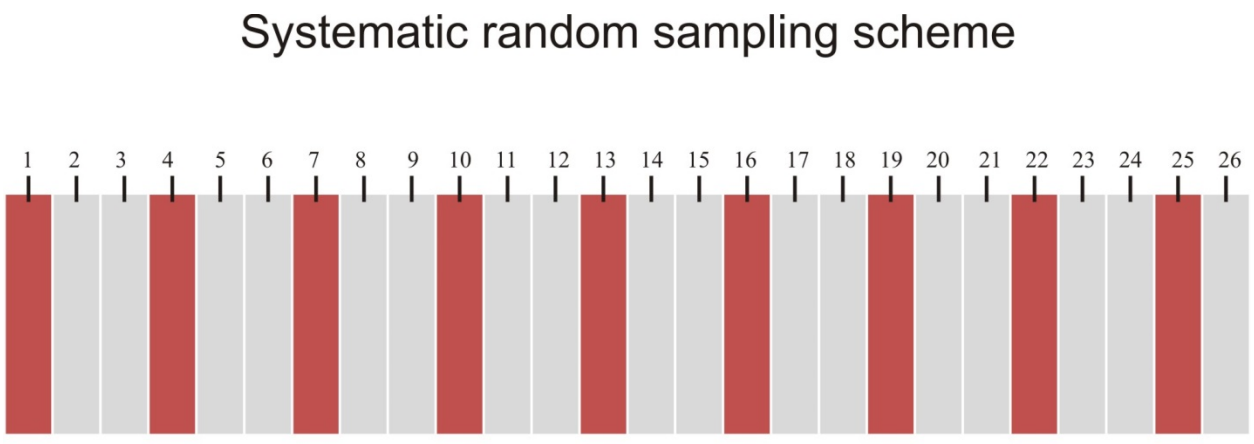

Figure 12. Sampling scheme used in this study. Every third section was included. The first section was randomly chosen (and named section 1 for the sake of simplicity).

Appendix 1. Solutions

Pre-fixative solution (50 ml)

Materials

5 ml	0.5 M Phosphate buffer
5 ml	**9% Na Cl**
0.05 ml	**Heparin**
0.05 gr	Sodium nitrite
	Nanopure water

Procedure

1. To mix all the components at room temperature (bring the volume to 50 ml with nanopure water).
2. To put the new pre-fixative solution on the fridge to keep it cold

Fixative solution: 4% Para-picric Fixative (50 ml)

Materials

2 gr	Paraformaldehyde
1 drop	**Sodium Hydroxide solution**
16 ml	**$NaPO_4$ solution**

7 ml Picric acid

Nanopure water

Procedure

1. To dissolve paraformaldehyde in approximately 16 ml of nanopure water.
2. To heat this solution up to 50 °C.
3. To add 1 drop of sodium hydroxide solution and immediately turn the heat off.
4. To pour 16 ml of $NaPO_4$ solution into a separate beaker and bring pH to 7.1.
5. To pour 7 ml of picric acid into a separate graduate cylinder.
6. To pour both, the $NaPO_4$ solution (with pH=7.1) and the picric acid, into the dissolving paraformaldehyde beaker.
7. To add up to 50 ml with nanopure water.
8. To filter the solution.
9. To put the new fixative solution on the fridge to keep it cold.

1.5 M TBST solution (1 L)

Materials

6.05 gr Trizma Base

15 gr NaCl

3 ml` 0.3% Triton X10

Nanopure water

Procedure

1. To weight Trizma and NaCl
2. To add approximately 800 ml of nanopure water and mix the solution
3. While mixing, to add triton
4. To bring pH to 7.6
5. To add up to 1 L (with nanopure water)

Appendix 2. Description of videos

Video 1. This video shows one mouse (animal 11) running at 20 cm/s.

Video 2. This video shows a 3D reconstruction of the mouse brain based on Paxinos and Watson mouse brain atlas (Franklin and Paxinos, 2007) and the tissue obtained in this project. Cell nuclei are shown as follows: Raphe nuclei (obscurus, pallidus and magnus) are green, seventh cranial nerve nuclei are pink, pyramids are depicted in orange, ambiguous nucleus in purple, parapyramidal region according to the atlas is depicted in red. Notice that the position of the PPR described in this study does not correspond with the position described in the atlas; it corresponds with the lateral paragigantocellular nucleus (LPGi). Circles in green represent the location of eYFP-only positive cells, whereas circles in purple represent the location of eYFP/c-fos positive cells in one animal (animal no. 11).

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