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Histamine H₃ receptors expressed in ventral horns modulate spinal motor output

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Abstract (135 words)

Motoneuron activity is modulated by histamine receptors. While H₁ and H₂ receptors have been widely explored, H₃ histamine receptors (H₃Rs) have not been sufficiently characterized. This paper targets the effects of the selective activation of H₃Rs and their expression on the membranes of large ventral horn cells. The application of selective pharmacological agents to spinal cords isolated from neonatal rats was used to identify the presence of functional H₃Rs on the membrane of physiologically identified lumbar motoneurons. Intra and extracellular recordings revealed that H₃R agonist, α -methylhistamine, depolarized both single motoneurons and VRs, even in the presence of tetrodotoxin (TTX), an effect prevented by H₃R antagonist, thioperamide. Finally, immunohistochemistry located the expression of H₃Rs on a subpopulation of large cells in lamina IX. This study identifies H₃Rs as a new exploitable pharmacological target against motor disturbances.

Keywords: motoneurons, motor pools, motor reflexes, spontaneous activity

Abbreviations: CV, cresyl violet; DRG, dorsal root ganglion; H₃Rs, H₃ histamine receptors; P, postnatal; VR, ventral root

Histamine H₃ receptors expressed in ventral horns modulate spinal motor output

(2005 words)

Histamine in the spinal cord is released by fibers descending from the tuberomammillary nucleus of the posterior hypothalamus, the exclusive location for histaminergic neurons (Haas et al., 2008). Histamine-immunoreactive spinal fibers are located around the central canal (Inagaki et al., 1988) and scattered in the anterior horn of the lumbar cord, mainly condensed in lamina X (Seybold, 1985).

In the mammalian spinal cord, the rhythmic patterns generated by neural circuits are modulated by histamine (Coslovich et al., 2018). Four metabotropic histamine receptors have been reported in the CNS (H₁₋₄; Haas et al., 2008) and are also identified in the spinal cord (Taylor et al., 1982 for H₁ subtype; Murakami et al., 1999 for H₂; Cannon et al., 2007 for H₃; Strakhova et al., 2009 for H₄). So far, the effects of histamine on spinal motoneurons are thought to mainly rely on H₁ and H₂ subtypes (Constanti and Nistri, 1976; Taylor et al., 1982; Saito et al., 1984; Wu et al., 2012). Nevertheless, H₃ histamine receptors (H₃Rs) also play a functional role in the spinal cord (Harasawa, 2000; Hough and Rice, 2011) and may thus modulate ventral spinal neuron activity, as well. We explored this hypothesis using *in vitro* neonatal rat spinal cords, which allow to examine spinal and locomotor functions at both circuit and cellular levels (Brumley et al., 2017). H₃Rs were studied on functionally-identified motoneurons using the selective pharmacological agonist for H₃Rs, α -methylhistamine, at a concentration similar to what previously reported on *in vitro* CNS preparations (Brown et al., 1996; Takei et al., 2012; 2017). Histological tools and selective staining assessed the presence of H₃Rs on large-diameter (>15 μ m) cells in the ventral horns, while electrophysiological recordings from lumbar ventral roots (VRs) explored the involvement of H₃Rs in modulating the output of lumbo-sacral networks.

Experiments were performed on 39 isolated spinal cords of postnatal day (P) 0 - 4 rats, as previously reported (Dose et al., 2014; 2016). Procedures were approved by the International School for Advanced Studies (SISSA) ethics committee and are in accordance with the guidelines of the Italian Animal Welfare Act 24/3/2014 n. 26 implementing the European Union directive on animal experimentation (2010/63/EU). All efforts were made to minimize number and suffering of animals used. Cords were isolated from the midthoracic region to the *cauda equina* and placed in a small recording chamber at room temperature to be continuously superfused (5 mL/min) with a Krebs solution, composed as follows (in mM): 113 NaCl, 4.5 KCl, 1 MgCl₂·7H₂O, 2 CaCl₂, 1 NaH₂PO₄, 25 NaHCO₃, and 11 glucose, gassed with 95 % O₂-

5 % CO₂, pH 7.4. Tight-fitting suction electrodes allowed DC-coupled extracellular recordings from lumbar ventral roots (VRs; Dose et al., 2016). Intracellular recordings with sharp electrodes (electrode resistance = $38.82 \pm 9.98 \text{ M}\Omega$) were obtained from 34 motoneurons total, impaled from both left (l) and right (r) L3 - L5 segments (Dose et al., 2014). In control conditions, cells' overall average resting potential was $-66.63 \pm 9.22 \text{ mV}$, with membrane resistance of $32.03 \pm 14.38 \text{ M}\Omega$ and antidromic spike amplitude of $65.50 \pm 9.32 \text{ mV}$. Selective histaminergic agents were added to the superfusing medium as needed. Histamine dihydrochloride (Murakoshi et al., 1985) and R- α -methylhistamine dihydrobromide (Dai et al., 2006) were purchased from Tocris (Bristol, UK). Thioperamide maleate (Dai et al., 2006), and tetrodotoxin (TTX) were bought from Ascent Scientific (Bristol, UK).

Histology and immunohistochemistry were performed on freshly isolated spinal cords fixed in 4% paraformaldehyde (Fluka, Buchs, Switzerland) for 3 days. Samples were soaked overnight in cryoprotecting 30% sucrose (Fluka) water solution and embedded in OCT (Kalttek, Padova, Italy). From L4 level, serial 15 μm thick cross cryosections were cut rostrocaudally with a 2800 Frigocut N cryostat microtome (Reichert-Jung GmbH, Nussloch, Germany) and mounted on polylysine (Sigma-Aldrich, St. Louis, MO, USA) precoated slides. Serial sections were processed alternatively for staining with 0.1% cresyl violet acetate (Sigma) water solution or H₃R selective immunostaining (Suppl. Fig. 1). For immunohistochemical analysis, slices were rinsed in PBS 0.1 M and then sections were incubated in 0.3% H₂O₂ (BDH Laboratory Supplies, Poole, UK) solution in PBS, at room temperature, in order to block endogenous peroxidase activity. After washing, the sections were blocked with 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.25 % Triton X-100 (Bio-Optica, Milano, Italy) PBS solution for 2 hours and then incubated overnight with polyclonal antibody NLS476, RRID:AB_2264153 (Cricco et al., 2008; Novus Biologicals, Littleton, CO, USA) at a dilution of 1:200, in a humidified chamber, at room temperature. After washing, sections were incubated 2 hours in biotinylated secondary antibody (Vector Laboratories) at a dilution of 1:200, and then rinsed. The slices were incubated in ABC solution (Vector Laboratories) for 1 hour at room temperature. Bound antibodies were detected using DAB solution (Vector Laboratories). After immunohistochemical labelling, slices were then counterstained with haematoxylin to visualize cell nuclei (Suppl. Fig. 2). Control experiments included omission of the primary antibody. All slices were subjected to ethanol/xylene (Carlo Erba, Milan, Italy) cleansing and then examined via photomicroscopy (Zeiss, Göttingen, Germany). Large cells in lamina IX (putatively motoneurons) were morphologically identified as cells with a diameter greater than 15 μm . The number of H₃R-positive large cells in ventral horns was calculated

based on immunostained sections ($n = 84$), whereas total number of large cells in ventral horns was calculated based on cresyl violet stained sections ($n = 84$), in a $350 \times 400 \mu\text{m}$ area, using ImageJ software.

Mean values from each spinal cord (P2-P4; $n = 5$) were then obtained. Since no differences were found between right and left spinal cord sides, mean values of those data were calculated and normality was assessed by means of Shapiro-Wilk test ($p > 0.05$).

As for statistical analysis, all data are indicated as a mean \pm SD, while n indicates the number of cells or spinal cords analyzed. Data were distinguished between parametric and non-parametric, using a normality test. Then, all parametric values were analysed with either Student's t-test (paired or unpaired) to compare two groups of data, or with ANOVA when groups were more than two. Non-parametric data was analysed with a Mann-Whitney test for two groups or, for multiple comparisons, with one-way ANOVA on ranks (Kruskal-Wallis) first, followed by a post hoc test (Dunnett's Method, Tukey Test). Results were considered significant when $p < 0.05$.

To verify the presence of functional H₃Rs in the spinal cord, we added the endogenous agonist, histamine, to the superfusing medium. An immediate depolarization (about 15 mV) was observed, associated with the superimposed firing activity (Figure 1A, left) previously reported (Coslovich et al., 2018). After an extensive washout from histamine (at least 15 min), the application of α -methylhistamine (20 μM) induced an appreciable depolarization (about 4 mV), accompanied by action potentials (Figure 1A, right). Serial applications of histamine and α -methylhistamine revealed that depolarizations induced by the first were significantly higher than the ones elicited by α -methylhistamine on the same cells (paired t-test, $p = 0.03$, $n = 3$).

A depolarization of 4.40 ± 1.34 mV was recorded in 10 out of 34 recorded motoneurons, after 154.03 ± 91.31 s from α -methylhistamine application. As data were collected from animals very close in age (P1-P3), the observed sensitivity to the agent in the 29% of recorded motoneurons did not appear to depend upon developmental stage, but rather upon different cell properties. In control conditions, the two groups of cells (sensitive and non-sensitive) showed similar membrane resistance (33.34 ± 16.91 m Ω , sensitive vs. 30.70 ± 13.85 m Ω non-sensitive), but different resting membrane potentials (-59.73 ± 7.53 , sensitive vs. -68.68 ± 9.38 , non-sensitive; t-test; $p = 0.010$).

To verify the presence of functional H₃Rs on the motoneuron membrane, we blocked the action potential-mediated transmission with TTX (0.5-1 μM) before and during α -methylhistamine application (20 μM). In control conditions, TTX suppressed spontaneous tonic activity, abolished action potentials, and hyperpolarized cells. Addition of α -methylhistamine (20 μM ;

Figure 1B) significantly depolarized 60% of motoneurons (mean depolarization 1.23 ± 0.79 mV, paired t-test, $p = 0.012$, $n = 6$). In the presence of TTX, the extent of depolarization induced by α -methylhistamine was significantly smaller than the one induced by histamine (Figure 1C, paired t-test, $p = 0.019$, $n = 5, 6$).

Since only a subgroup of cells showed to possess H₃Rs, we examined their selective contribution to the overall output of motoneuronal pools. Thus, VR activity was extracellularly recorded during bath-application of H₃R agonist, α -methylhistamine. In a sample cord in Fig. 1D, VRs were depolarized by 367 μ V, in accordance with the mean depolarization of 415 ± 64 μ V obtained from 11 experiments, where the selective activation of H₃Rs did not elicit any alternating activity from VRs.

In the presence of TTX, histamine ($n = 8$) and α -methylhistamine ($n = 6$) were always able to depolarize VRs (Figure 1E). This provides evidence that H₃Rs, although functionally expressed only by a third of cells, once activated, play an important role in modulating overall spinal motor output. In additional five preparations, co-application of histamine, TTX and the selective antagonist for the H₃R subtype, thioperamide, statistically reduced the depolarization induced by histamine (Figure 1E; one-way ANOVA followed by all pair-wise multiple comparison procedures with Tukey test; $p = 0.005$). The observation showed that H₃Rs mediate part of the effects of histamine application.

In summary, data indicate that, although H₃Rs were functionally involved in modulating the 29% of intracellularly-recorded motoneurons, their role is predominant as the overall VR motor output was indeed depolarized by H₃R selective agents.

Because electrophysiological experiments with TTX strongly indicated the presence of H₃Rs on motoneuron membranes, we performed immunohistochemical labelling to visualize the distribution of H₃Rs in the ventral spinal cord. Interestingly, H₃Rs were expressed on the membrane of 35% of the large diameter (>15 μ m) cells in lamina IX, as morphologically confirmed using cresyl violet staining (Figure 2, $n = 84$ cryosections from 5 spinal cords).

Indeed, α -methylhistamine in TTX affected the activity of only a portion of functionally-identified motoneurons, in line with the expression of H₃Rs on the membranes of one third of large cells in the ventral horn, immunohistochemically labelled.

The identification of H₃Rs on motoneurons explains part of the motor behavior following pharmacological manipulation with selective histamine agents (Chiavegatto et al., 1998; Toyota et al., 2002) and opens a new path in exploring therapeutics to alleviate spinal cord pathologies. Histamine is well known to be involved in nociceptive and antinociceptive processing (Wei et al., 2016) and itch sensations (Lee et al., 2016; Luo et al., 2015). In

particular, H₃Rs, acting on sensory afferents and sensory-responsive neurons in the spinal cord (Cannon et al., 2007), attenuate mechanically-induced nociception (Cannon et al., 2003). Moreover, H₃R antagonism has been suggested as a potential mechanism for the treatment of neuropathic pain in preclinical rat models (Coward et al., 2012; Hsieh et al., 2010). Our findings suggest that H₃R agents also act on motoneurons in the ventral spinal cord to influence motor behavior and can represent promising pharmacological targets in the CNS, because of their characteristic of being constitutively active *in vivo* (Passani and Blandina, 2011). A direct effect of H₃Rs on interneuronal spinal networks for locomotion should be excluded, since no locomotor-like alternating patterns (Taccola and Nistri, 2005) were elicited by α -methylhistamine alone. The inverse agonism of these receptors unveils their ability to modulate neuronal excitability, even in the absence of any endogenous histamine, as shown by innovative molecules that underwent preclinical testing for the treatment of cognitive disorders and feeding behavior (Arrang et al., 2007).

Recently, histamine has been reported to improve long-term recovery in a rat model of spinal injury, by decreasing the lesion area, inhibiting astrogliosis and glial scar formation, and improving locomotor recovery (Zhao et al., 2015). Although many of the effects were reversed by a treatment with a H₁ receptor antagonist, the authors suggested that the overall protective role of histamine may be due to different histamine receptors in different cell types. Then, the modulation of H₃Rs expressed in a subpopulation of large ventral horn cells could represent a potential neuroprotective strategy to rescue spinal tissue after damage (Kukko-Lukjanov et al., 2006).

Compliance with Ethical Standards

Ethical Approval: Procedures were approved by the International School for Advanced Studies (SISSA) ethics committee and are in accordance with the guidelines of the Italian Animal Welfare Act 24/3/2014 n. 26 implementing the European Union directive on animal experimentation (2010/63/EU). All procedures performed in studies involving animals were in accordance with the ethical standards of the SISSA Laboratory Animal Center and the protocol was approved by the local Animal Committee (Organismo preposto al benessere degli animali, OPBA) of SISSA, Italy.

Conflict of interest: no conflicts of interest to declare

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Authors' contributions: All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, G.T.; Methodology, G.T.; Investigation, T.C., G.D'A., A.DM. and G.T.; Formal Analysis, T.C., G.D'A., A.DM. and G.T.; Writing - Original Draft, T.C., G.D'A., A.DM. and G.T.; Writing - Review & Editing, A.DM. and G.T.; Visualization, G.D'A., A.DdM. and G.T.; Supervision, F.O. and G.T.; Funding Acquisition, G.T.

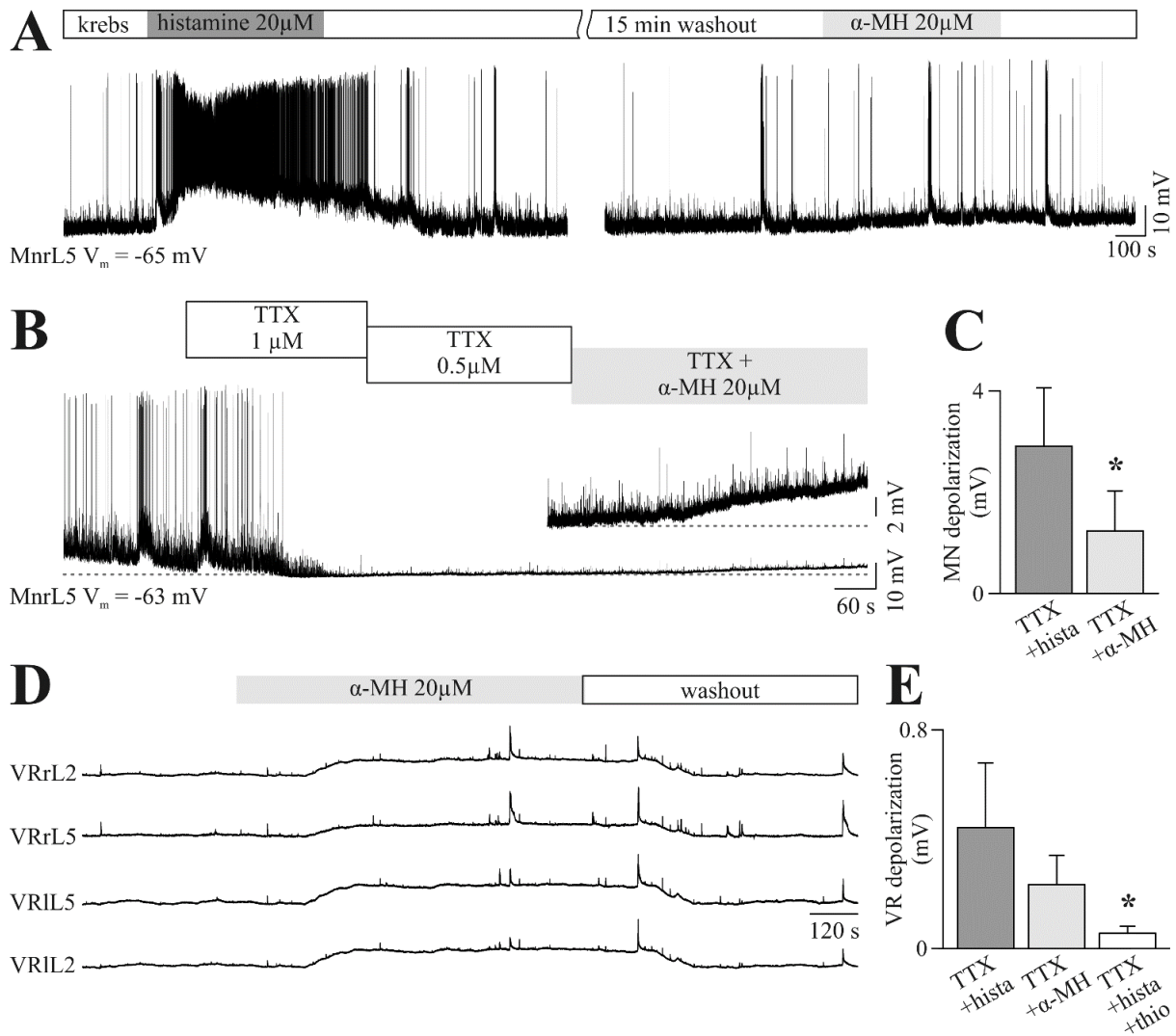


Figure 1. α -methylhistamine directly depolarizes a subset of recorded motoneurons and affects the motor output recorded from VRs.

A.) After 84 s from the application of histamine (upper gray bar; 20 μ M), a sustained depolarization (15.32 mV) with superimposed intense firing (3.97 Hz) is recorded from a rL5 single motoneuron (resting potential = -65 mV). After 15 min of washout, the baseline returns to control level and only sporadic action potentials can be derived (trace break = 2 min). Application of α -methylhistamine (α -MH) slightly depolarizes the same cell (4.15 mV) with the occurrence of faster spiking activity. Based on the extent of depolarization induced by 20 μ M α -methylhistamine (α -MH), single motoneurons are *a posteriori* divided in sensitive and non-sensitive. B.) A different rL5 motoneuron (- 63 mV initial resting potential, V_m) is hyperpolarized (- 4.94 mV) by TTX (1 and then 0.5 μ M) with the suppression of both spontaneous firing and tonic activity, as well. α -methylhistamine (α -MH, 20 μ M) slightly depolarizes the motoneuron (2.56 mV) as indicated at a higher magnification in the insert

above. C.) Analysis of pooled data from different experiments during TTX perfusion indicates that the mean depolarization elicited by histamine (20 μ M; dark gray bar) is significantly higher than the one induced by α -methylhistamine (20 μ M; light gray bar; *, t test; $p = 0.019$; $n = 5, 6$). D.) Application of α -methylhistamine (20 μ M, 15 min application) induces comparable stable depolarizations with sporadic synchronous events among all VRs (VRrL2 = 349 μ V; VRrL5 = 452 μ V; VRlL5 = 321 μ V; VRlL2 = 347 μ V). After 5 min of the following washout, traces return to baseline and discharges are progressively attenuated, until mostly suppressed. E.) Histamine (20 μ M) and α -methylhistamine (α -MH; 20 μ M) depolarize VRs in the presence of TTX, while the selective H₃R antagonist thioperamide (20 μ M) abolishes the depolarization induced by histamine (*, one-way ANOVA followed by all pair-wise multiple comparison procedures with Tukey test; $p = 0.005$; $n = 8, 6, 5$). Note that A and B traces come from different motoneurons.

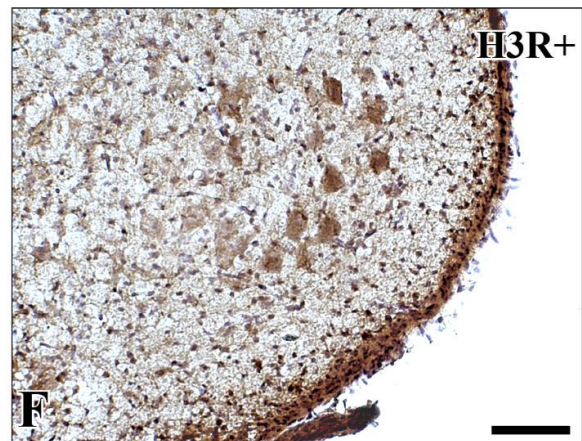
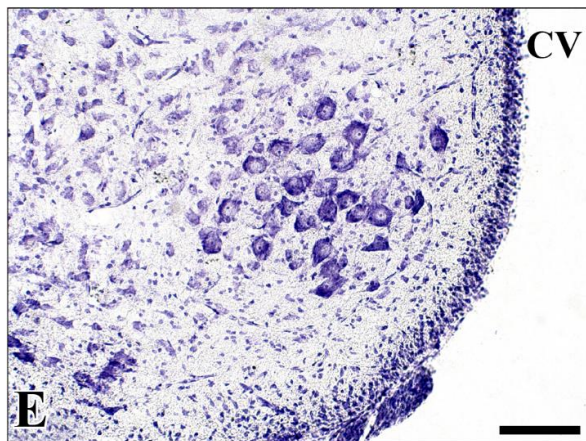
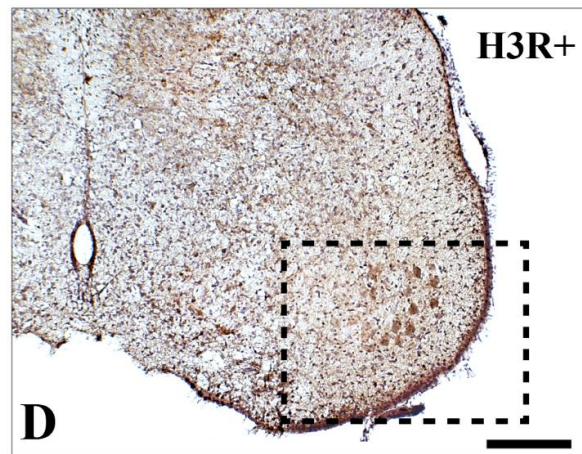
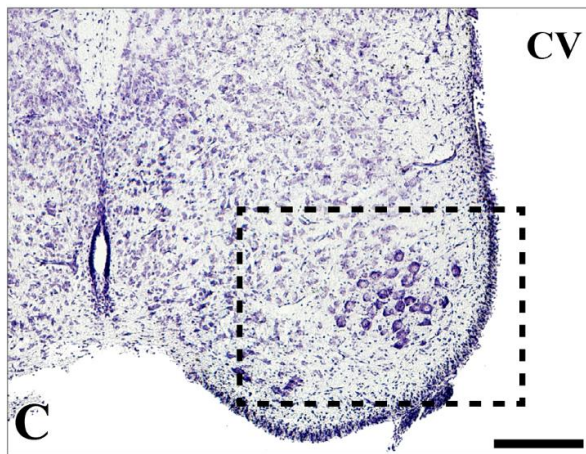
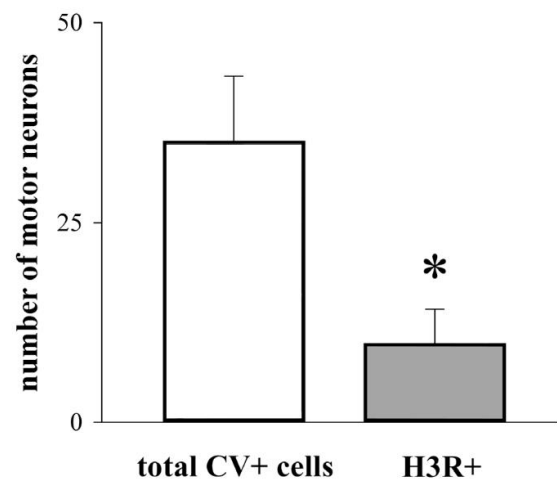
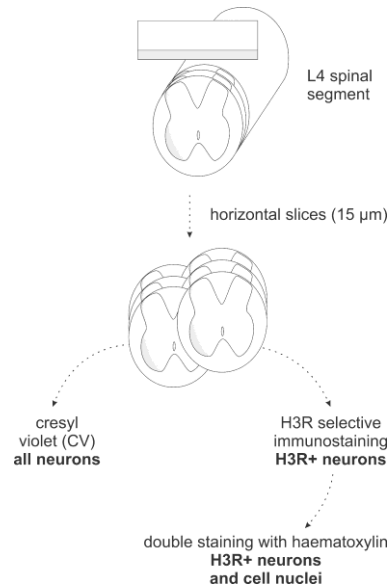


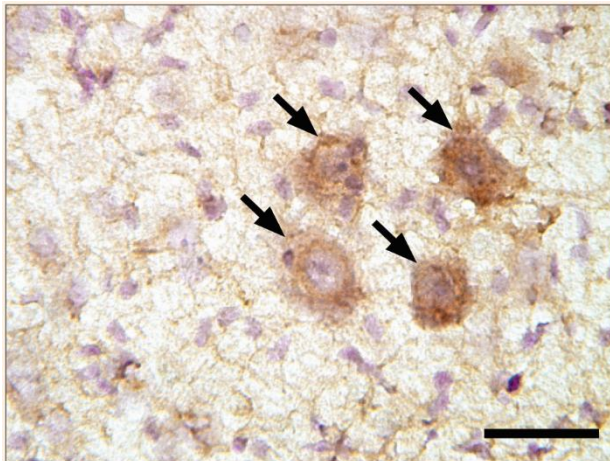
Figure 2. Immunostaining confirms that a subgroup of large cells in lamina IX contains H₃Rs. A.) Cresyl violet staining of 15 µm thick cross cryosection of L4 spinal cord. Dotted rectangle delimiting lamina IX, in which large diameter (>15 µm) ventral horn cells are morphologically identified, is shown in C.) and E.) at a higher magnification. Scale bars: 250 µm A.), 200 µm C.) and 100 µm E.), respectively.

D.) Immunohistochemical detection by H₃R marker on a serial slice from the same cord reveals diffuse labelling concentrated around a set of large ventral horn cells. Dotted rectangle delimiting lamina IX, is shown in F.) at a higher magnification. Scale bars: 200 µm D.) and 100 µm. F.)

B.) Quantification of mean number of H₃R-positive cells among the total number of large ventral horn cells in lamina IX (motor neurons). Large cells in lamina IX were morphologically identified as cells with a diameter greater than 15 µm. Number of H₃R-positive cells is significantly different from the total number of cells, morphologically identified in cresyl violet stained (CV+) sections (*; Student's t-test, $p < 0.001$, $n = 84$ sections from five different cords).



Supplementary Figure 1. The cartoon schematizes the histological processing. Spinal cords, fixed in PFA, were cryosected rostrocaudally from L4 level. Serial 15 µm thick sections were processed alternatively for staining with cresyl violet or H₃R selective immunostaining. After immunohistochemical labelling, slices were then counterstained with haematoxylin to visualize cell nuclei.



Supplementary Figure 2. Double staining by H₃R antibody and haematoxylin shows the expression of H₃R on four large lamina IX cells (black arrows). Scale bar: 50 μ m.

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