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INTERACTION BETWEEN NESFATIN-1 AND OXYTOCIN IN THE MODULATION OF THE SWALLOWING REFLEX

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Running title: Inhibition of the swallowing reflex by nesfatin-1

Abstract

Nesfatin-1, an 82-amino acid peptide encoded by the secreted precursor nucleobinin-2 (NUCB2), exerts potent anorexigenic action independently of leptin signaling. This propensity has propelled this peptide and its analogues as potential anti-obesity drug candidates. However, a more extensive comprehension of its biological actions is needed prior to envisaging its potential use in the treatment of metabolic diseases. Swallowing is an essential motor component of ingestive behavior, which induces the propulsion of the alimentary bolus from the mouth to the esophagus. The dorsal swallowing group (DSG) which constitutes a part of the central pattern generator of swallowing (SwCPG) is located within the solitary tract nucleus (STN), a region reported to contain nesfatin-1/NUCB2 expressing neurons. In this context, we investigate here the possible effects of nesfatin-1 on swallowing discharge. Nesfatin-1 dose-dependently inhibited swallowing reflex and activated neurons located in the DSG region. In addition, we provide evidences that strongly suggest that this nesfatin-1 inhibitory effect involved an oxytocinergic relay. Indeed, oxytocin (OT) injection at the brainstem level inhibited swallowing reflex and OT receptor antagonist prevented nesfatin-1 inhibitory action. Altogether, these data constitute the first demonstration that nesfatin-1 modulates swallowing reflex by acting at the brainstem level via an oxytocinergic relay.

Keywords: Superior laryngeal nerve, electrophysiology, solitary tract nucleus, central pattern generator of swallowing, vasotocin.

1. Introduction

Nesfatin-1 is an 82-amino acid peptide encoded by the secreted precursor nucleobinin-2 (NUCB2), reported to inhibit food intake via modulation of neuropeptides in the feeding centers of rodent brain (Oh-I et al., 2006). Nesfatin-1 quickly aroused community interest as it was shown that its action was independent of leptin signaling (Goebel et al., 2011; Oh-I et al., 2006; Shimizu et al., 2009a). Indeed, given the well-known leptin resistance that occurs in obese individuals, this propensity has propelled this peptide and its analogues as potential anti-obesity drug candidates. In addition, in humans, the modulation of nesfatin-1 expression was reported in various metabolic conditions, so that it could be considered as a potential biomarker for obesity (Dogan et al., 2016; St-Pierre et al., 2016; Tsuchiya et al., 2010). For instance, a significant negative correlation between plasma concentrations of nesfatin-1 and BMI, body fat percentage, and body fat weight was described in healthy non-obese individuals ($BMI < 25 \text{ kg/m}^2$) (Tsuchiya et al., 2010). Furthermore, these authors reported significantly lower fasting concentrations of plasma nesfatin-1 in a group of high BMI ($BMI > 28 \text{ kg/m}^2$) subjects compared to non-obese subjects (Tsuchiya et al., 2010). This negative correlation between nesfatin-1 and BMI also suggests that overweight or obesity could result from a deficiency of nesfatin-1 and thus that increasing the plasma nesfatin-1 concentration in the body could result in reduced body fat mass. Like-minded, it was shown that morbidly obese patients who had undergone laparoscopic sleeve gastrectomy exhibited significant increases in nesfatin-1 hormone levels in parallel to weight loss (Dogan et al., 2016). Accordingly, the therapeutic use of nesfatin-1 was envisaged for the treatment of obesity and associated co-morbidities (Ayada et al., 2015; Shimizu et al., 2009b). However, a deeper and comprehensive understanding of specific effects of nesfatin-1 is critical prior to envisaging its potential use in the detection and treatment of metabolic diseases. Indeed, nesfatin-1 was reported to exert multiple and diverse biological actions. Thus, nesfatin-1 regulates insulin secretion, adipocyte differentiation, gastric motility and arterial pressure (see (Wang et al., 2016); (Ramesh et al., 2017) for review). In accordance, nesfatin-

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96 1/NUCB2 expression was described in diverse peripheral tissue including pancreas (Mohan and
97 Unniappan, 2012), adipocyte (Ramanjaneya et al., 2010), stomach (Chung et al., 2013), intestine
98 (Zhang et al., 2010) and heart (Feijoo-Bandin et al., 2013).

99 Swallowing is an essential motor component of ingestive behavior, which induces the propulsion
100 of the alimentary bolus from the mouth to the esophagus and involves various muscles localized in
101 the mouth, larynx, pharynx and esophagus. The superior laryngeal nerve (SLN) contains the sensory
102 afferent fibers involved in the swallowing reflex. The afferent fibers contact interneurons located
103 rostrocaudally within the medial part of the lateral NTS defined as the intermediate-subpostremal
104 portion of this nucleus. This region overlaps with the interstitial, intermediate, ventral, and, to some
105 extent, the ventrolateral subdivision of the NTS (Barraco et al., 1992). This neuronal population
106 referred as the dorsal swallowing group (DSG) constitutes, with a ventral swallowing group located
107 within the ventrolateral medulla above the nucleus ambiguous, the central pattern generator of
108 swallowing (SwCPG) (Jean, 2001). Interestingly, previous works have shown that the swallowing
109 reflex could be modulated by anorexigenic and orexigenic compounds (Abysique et al., 2015;
110 Bariohay et al., 2008; Felix et al., 2006; Kobashi et al., 2010; Kobashi et al., 2014; Kobashi et al.,
111 2017; Mostafeezur et al., 2012) suggesting that this motor component and the regulation of energy
112 balance could be simultaneously targeted by the same effectors. For instance, leptin (Felix et al.,
113 2006), ghrelin (Kobashi et al., 2010), brain derived neurotrophic factor (BDNF) (Bariohay et al.,
114 2008) or glucagon like peptide-1 (Kobashi et al., 2017) were reported to inhibit the swallowing reflex
115 after their central administration while cannabinoids facilitate the swallowing reflex elicited by the
116 superior laryngeal nerve stimulation in rats (Mostafeezur et al., 2012).

117 In this context, the purpose of the present study was to perform the first investigation
118 regarding the possible effects of nesfatin-1 on swallowing discharge. Since the anorexigenic action of
119 central nesfatin-1 requires the recruitment of oxytocin neurons (Maejima et al., 2009; Nakata et al.,

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3120 2016; Saito et al., 2017; Yosten and Samson, 2010), we also tested a possible interaction between
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5121 oxytocin and nesfatin-1 signaling in the control of swallowing.
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10123 **2. Results**

11 12124 **2.1. Inhibition of swallowing by nesfatin-1 administered into the DSG**

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14125 Effects of nesfatin-1 were studied after central microinjections on 32 rats from 44 trials. The present
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16126 results showed that nesfatin-1 induced a significant dose-dependent decrease in the number of
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19127 swallows recorded during SLN stimulation. At 100 nM (22 rats, 22 trials), nesfatin-1 decreased the
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21128 number of swallows with a short latency of 42.27 ± 4.70 s and this effect lasted for 12.11 ± 0.63 min
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24129 (Fig. 1 and 2A). This effect was maximal for 10 minutes after nesfatin-1 injection ($P < 0.0001$) (Fig.
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26130 2A). At 50 nM (10 rats, 10 trials), nesfatin-1 also induced after a short latency of 51 ± 11.88 s a
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29131 significant decrease in the number of swallows and this effect persisted for 13.35 ± 0.68 min (Fig.
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31132 2A). Compared with 100 nM, the maximal inhibitory effect presented similar amplitude ($P < 0.0001$)
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34133 but lasted less time, only 4 minutes after microinjection. The effect of nesfatin-1 at 10 nM was
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36134 investigated on the same rats used to study the effect of nesfatin-1 at 50 nM. In contrast, at this low
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38135 dose (10 nM), nesfatin-1 did not modify the pattern of swallowing during SLN stimulation (10 rats,
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41136 12 trials). Therefore this dose could be considered as ineffective in our experimental conditions (Fig.
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43137 2A). Statistical comparison of the inhibitory effects of nesfatin-1 doses showed that inhibition time
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46138 course was significantly different only between 100 nM and 10 nM and the difference between these
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48139 two doses was significant between 2 and 12 minutes after microinjection (Fig. 2A). Nesfatin-1
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51140 administered within the DSG inhibited swallowing, without any variation of either cardiac frequency
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53141 or respiratory activity (Fig. 1 and 2B-C).

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3 145 **2.2. Effects of 4th ventricle nesfatin-1 injection on c-Fos expression in the brainstem**
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6 146 Central structures activated in response to i.c.v. (4th ventricle) nesfatin-1 injection were identified
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9 147 using the immune detection of the c-Fos protein. A low basal level of c-Fos positive nuclei was
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11 148 observed in the brainstem of control (NaCl) rats (Fig. 3A-C). We firstly evaluated the effect of 100
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14 149 nM nesfatin-1 on c-Fos expression since this dose induced a robust inhibition of swallowing. At this
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16 150 concentration, the ventricular injection of nesfatin-1 resulted in a modest but non-significant
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19 151 induction of c-Fos expression (data not shown). This negative result could be explained by a different
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21 152 route of administration used here i.e. intraventricular vs intraparenchymal. We next tested an higher
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24 153 nesfatin-1 dose i.e. 300 nM. This concentration was chosen since it was lower than concentrations
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26 154 reported to induce reduction of food intake after their intracerebroventricular administration (Oh-I et
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28 155 al., 2006; Stengel et al., 2009). Interestingly, at this concentration, treated rats exhibited an elevated
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31 156 number of c-Fos positive nuclei throughout the dorsal vagal complex (DVC, Fig. 3D-F) with a
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33 157 particular strong c-Fos labelling observed in STN regions surrounding the solitary tract (Fig.3G-H).
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36 158 Counts of positive nuclei in the STN revealed significant increases in the number of c-Fos labeled
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38 159 nuclei in treated animals compared with control animals whatever the sub-region of the STN
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41 160 considered i.e. rostral (NaCl : 8.3 +/- 3.2 vs nesfatin-1 : 18.0 +/- 4.3; p < 0.001), subpostremal (NaCl
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43 161 : 5.1 +/- 2.3 vs nesfatin-1 : 26.1 +/- 7.3; p < 0.001) and caudal (NaCl : 2.5 +/- 1.2 vs nesfatin-1 : 5.8
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45 162 +/- 2.4 ; p < 0.001) parts (Fig. 3I). No labelling was observed outside the DVC including other
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48 163 brainstem nuclei and hypothalamus (data not shown).
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53 165 **2.3. Oxytocinergic terminals place alongside brainstem nesfatin-1/NUCB2 expressing**
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55 166 **neurons**
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58 167 We next performed oxytocin (OT) and nesfatin-1/NUCB2 double labelling on brainstem sections to
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60 168 evaluate the possible neuronal co-expression of these two peptides and/or the juxtaposition of
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3 169 neuronal elements expressing OT and nesfatin-1/NUCB2. In contrary to hypothalamic nuclei where
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5 170 OT and nesfatin-1/NUCB2 were reported to co-localize in neuronal sub-populations (Goebel-Stengel
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8 171 and Wang, 2013), no localization was observed within the STN (Fig. 4A). Nevertheless, throughout
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10 172 the STN, including its lateral region comprising the DSG, OT positive fibers and varicosities were
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13 173 found in close apposition to nesfatin-1/NUCB2 expressing neurons (Fig. 4B and C). This closeness
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15 174 led us to consider a possible interaction between these two peptides at the brainstem level and in the
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18 175 context of swallowing control.

22 177 **2.4. Oxytocin signaling and nesfatin-1-induced inhibition of the swallowing reflex**

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25 178 We investigated the effects of oxytocin (10 nM) on 11 rats from 13 trials. Microinjection of oxytocin
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27 179 in the DSG induced a significant decrease in the number of swallows recorded during SLN
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30 180 stimulation with a latency of 41.5 ± 5.41 s. This inhibitory effect was maximal for 4 minutes after
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32 181 oxytocin microinjection ($p < 0.0001$) and persisted for 7.3 ± 1.44 min (Fig. 5 and 6A). Oxytocin
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35 182 administered within the DSG inhibited swallowing, without any variation of either cardiac frequency
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37 183 or respiratory activity (Fig. 5 and 6B-C). To investigate the possible involvement of oxytocin in the
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40 184 mechanism of the nesfatinergic inhibitory effect on swallowing reflex, we have studied the effects of
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42 185 the highest dose of nesfatin-1 after pre-treatment by vasotocin (OVT), an oxytocin receptor
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44 186 antagonist. Before OVT microinjection, nesfatin-1 (100 nM) induced a significant decrease in the
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47 187 number of swallows with a latency of 33 ± 3 s and a duration of 13.7 ± 0.92 min (10 rats, 10 trials,
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49 188 Fig. 7A and C). OVT (1 μ M) was administered in the DSG 5 minutes before microinjection of
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52 189 nesfatin-1 (100 nM). In these conditions, nesfatin-1 did not modify the swallowing pattern discharge
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54 190 (10 rats, 10 trials, Fig. 7B and C). Moreover, OVT microinjected alone did not affect swallowing.
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3. Discussion

In the present study, we observed for the first time that nesfatin-1 injected into the DSG is able to inhibit the swallowing reflex. Central administration of nesfatin-1 reduced the number of swallows in a dose-dependent and specific manner, without affecting other physiological functions (heart rate and respiratory activity). Whatever the dose of nesfatin-1 injected, the inhibitory action appeared with a short latency (<1 min) but this effect was relatively labile (~12-13 min). This duration was shorter when compared to other energy homeostasis-linked effectors reported to decrease swallowing i.e. leptin, BDNF (Bariohay et al., 2008; Felix et al., 2006). Interestingly, the nesfatin-1 doses able to modify swallowing frequency (50-100 nM) are consistent with endogenous nesfatin-1 levels reported within the rat hypothalamus and LCR (Oh-I et al., 2006). The pioneer work of Oh-I and colleagues (2006) reported a concentration of ~240 and 200 ng/ml within the hypothalamus and LCR respectively. Based on these values, we can approximate a nesfatin-1 concentration of ~ 24 and 20 nM within the hypothalamus and LCR respectively. Moreover, we also showed that 4th ventricle nesfatin-1 injection resulted in a strong and significant increase in c-Fos expression within the STN including the DSG. This nesfatin-1-induced c-Fos expression was specific as it was absent in other brainstem or forebrain nuclei. Importantly, hypothalamic nuclei did not exhibit increased of c-Fos expression in response to 4th ventricle nesfatin-1 administration. When nesfatin-1 was injected into the 4th ventricle, its effects must have been larger than when injected in the DSG and in particular, it may have acted on the entire STN, involved in various autonomic functions. c-Fos data showed neuronal activation in various parts of the STN involved in autonomic functions including respiratory and cardiac functions. However, electrophysiological data did not present any variations of respiratory and cardiac activities. The data suggested that these functions were not affected by nesfatin-1 dose reaching the dedicated neurons. The subpostremal NTS level appears particularly sensible to icv nesfatin-1 injection. It's difficult to know why since the nesfatin-1 receptor or binding

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3 218 site(s) remain unknown. Nevertheless, we can speculate that this region contains a large part of
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5 219 interneurons regulating not only swallowing but also food intake. Indeed, peripheral administration
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8 220 of anorexigenic nesfatin-1 dose (0,25 nmol/g of body weight), was reported to specifically induced c-
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10 221 Fos expression at the subpostremal NTS level after its peripheral injection (Shimizu et al., 2009a).
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13 222 Swallowing is the first motor component of the ingestive behavior and is essential for normal food
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15 223 intake; our results suggest that inhibition of swallowing by nesfatin-1 may contribute, at least in part,
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17 224 to the well-known anorexigenic effects of this substance.
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22 226 To investigate the mechanism by which nesfatin-1 inhibits swallowing reflex, we aimed to
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25 227 establish the existence of interactions between nesfatin-1 and oxytocin signaling in this context of
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27 228 swallowing control. In addition to classical effects of oxytocin, numerous works have involved
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30 229 oxytocin in the regulation of feeding and energy expenditure. Evidences showed clearly that oxytocin
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32 230 acts as an anorexigenic hormone (Arletti et al., 1990; Olson et al., 1991; Yosten and Samson, 2010).
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35 231 Accordingly, animals deficient in oxytocin gene or genes related to the differentiation of oxytocin
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37 232 neurons exhibit hyperphagia and obesity (Kublaoui et al., 2008; Takayanagi et al., 2008). Moreover,
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39 233 several lines of evidences suggest that oxytocin may relay nesfatin-1 anorexigenic effects (Maejima
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42 234 et al., 2009; Price et al., 2008; Yosten and Samson, 2010). Nesfatin-1 exerts direct depolarizing
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44 235 action on oxytocinergic neurons and its central injection recruited both magnocellular and
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47 236 parvocellular oxytocin neurons which in turn stimulates oxytocin release (Price et al., 2008; Yosten
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49 237 and Samson, 2010). Altogether, these observations led to the conclusion that nesfatin-1 decreases
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52 238 food intake by activating oxytocinergic neurons. Here, we reported that the STN area corresponding
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54 239 to the DSG contains both oxytocin fibers and nesfatin-1 neurons which are often found in close
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56 240 apposition. In accordance, (Blevins et al., 2003) have clearly shown a high density of oxytocin
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59 241 projections in the medial subdivision of the STN, an area which includes the DSG. In parallel, we
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61 242 observed that oxytocin injection into the DSG inhibited significantly the swallowing reflex. Oxytocin
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3 243 inhibited the swallowing reflex with a similar latency to nesfatin-1 while the inhibition duration was
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5 244 shorter. In the brain, the main sources of oxytocin are the magnocellular and parvocellular neurons of
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8 245 the paraventricular nucleus (PVN) and the supraoptic nucleus (Armstrong, 2004; Rosen et al., 2008).
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10 246 Moreover, in the rat, oxytocin receptors are abundantly present in several brain regions such as the
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13 247 hypothalamus and the brainstem (Gimpl and Fahrenholz, 2001). As described by several authors,
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15 248 there is a significant anatomical link between PVN and STN since PVN oxytocinergic neurons,
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18 249 particularly neurons of the posterior parvocellular subdivision, convey descending inputs from the
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20 250 hypothalamus to the STN neurons involved in food intake control (Blevins et al., 2003; Uchoa et al.,
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22 251 2013). Moreover (Rinaman, 1998) demonstrated by a retrograde transport of cholera toxin that all
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25 252 oxytocinergic neurons projecting to the STN originate from the PVN. *In vivo* experiments also
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27 253 demonstrated that electrical stimulation of the ipsilateral PVN triggered a release of oxytocin in the
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30 254 STN (Landgraf et al., 1990). Therefore, in our study, in regard to the inhibition of rhythmic
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32 255 swallowing induced by brainstem oxytocin administration, we can hypothesized that PVN
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35 256 oxytocinergic neurons could project to the STN area corresponding to the DSG and modulate the
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37 257 activity of the interneurons involved in the coordination of motor sequences necessary for
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39 258 swallowing reflex.

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42 259 In our study, the inhibitory effect induced by the high nesfatin-1 dose was blocked by
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44 260 administration into the DSG of OVT, an oxytocin receptors antagonist. This suggests that the
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47 261 brainstem effects of nesfatin-1 on the swallowing reflex requires a release of oxytocin by the OT
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49 262 neurons endings. Remarkably, it was previously shown that intra-PVN nesfatin-1 injection induces c-
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52 263 Fos expression within the STN and that nesfatin-1 induced anorexia is blocked by 4th ventricle
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54 264 injection of oxytocin receptor antagonist (Maejima et al., 2009; Yosten and Samson, 2010). Hence,
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57 265 the blockade of the nesfatin-1 inhibitory effect by OVT pre-treatment strongly suggests that nesfatin-
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59 266 1 stimulates locally (i.e. within the DSG) OT release from PVN-originating OT fibers.
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In summary, our study demonstrates that nesfatin-1 inhibits the swallowing reflex in anesthetized rats. The nesfatin-1 mechanism of action appears to involve the oxytocin signaling. Nesfatinergic neurons, previously characterized in the STN by our team (Bonnet et al., 2009; Bonnet et al., 2013) and reported to be sensitive to inflammatory and glycemic related signals, may stimulate the release of oxytocin by the PVN neurons endings, and oxytocin may modulate swallowing by acting on the DSG.

4. Materials and methods

4.1. Ethical Statement

The experimental procedures were carried out in accordance with the directives of the French Ministry of Agriculture and Fisheries and the European Community Council (86/609/EEC). The protocol was approved by the committee on the ethics of animal experiments of Marseille/N°14 (authorization number: 01288.02).

4.2. Surgical and electrophysiological procedures

This study was performed on 43 adult male Wistar rats weighing 350 g (Charles River, l'Arbresle, France). The animals were anesthetized with 0.6 ml of a mixture of ketamine (100 mg/ml; Kétamine 1000 Virbac) and xylazine (20 mg/ml; Rompun, Bayer Santé, France), injected intraperitoneally in a proportion of 90% and 10%, respectively. The level of anaesthesia was maintained by perfusing the same mixture diluted at 10 % through a catheter inserted in the femoral vein, at a rate of 0.01-0.05 ml/h.

As previously described by (Abysique et al., 2015), the SLN was placed on bipolar electrodes included in a plexiglas gutter and, after craniotomy, the surface of the medulla was exposed in order to allow drugs microinjections in the intermediate STN which contains the DSG.

4.3. Drugs microinjections

As previously described by (Abysique et al., 2015), we used glutamate microinjection (1 fmol) as control to induce swallowing and to verify that the microelectrode was well positioned within the DSG. Then, these stereotaxic coordinates were conserved, and all drugs tested were microinjected in the DSG extending between 0.2-0.7 mm rostral to the caudal edge of the area postrema (taken as the 0), 0.5-0.7 mm laterally and 0.4-0.8 mm in depth.

The following drugs were used: nesfatin-1 (Phoenix Pharmaceuticals, France), oxytocin (Tocris, France) and oxytocin receptor antagonist: vasotocin/Compound IV/OVT (Phoenix Pharmaceuticals, France). Nesfatine-1, oxytocin and OVT were dissolved in NaCl 0.9% solution. As described by (Abysique et al., 2015), 100 nl of all the drugs were injected in the DSG by pressure ejections through glass micropipettes.

4.4. Stimulations and recordings

Swallowing was triggered by stimulation of the SLN. In this study, repetitive long trains of pulses (5 s duration at 15-30 Hz frequency every 30 s) were used and produced several swallows at a rhythm depending on stimulation frequency. The parameters of stimulation varied according to the animal (1.0-5.5 V; 0.02-0.8 ms) to induce a basal swallowing frequency comprised between 0.6 and 1.2 Hz.

As described by (Abysique et al., 2015): *i*) the electromyographical (EMG) activity of sublingual muscles was recorded to monitor swallowing, *ii*) the respiratory activity and the electrocardiogram (ECG) were recorded to evaluate the level of anaesthesia and to determine any variation induced by drugs injection. Rectal temperature was monitored and maintained around 37°C with a heating pad. The EMG, ECG and respiration activity were recorded on a computer using an analog-to-digital interface (PowerLab 8SP data acquisition software for Windows, ADInstruments, USA).

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4.5. Signal Analysis

A stable control sequence of swallowing involving three 5s trains of stimulations was recorded before each drug injection and the mean values calculated were used as control values. Stimulations and recordings were maintained until recovery. A specially designed computer program Chart5.5 software calculated: ∫EMG (electromyogram envelope signal normalized), Sw Number (number of swallows triggered by SLN stimulation), ECG and respiratory frequency. All swallows calculated values were normalized as percent of control values. ECG and respiratory frequencies were expressed as percent of control values recorded before each drug injection.

4.6. Surgery and intracerebroventricular injection of nesfatin-1

For cannula implantation, animals (n=6) were anesthetized by an intraperitoneal (ip) injection of ketamine (100 mg/ml, Ketamine 1000 Virbac, France) and xylazine (20 mg/ml; Rompun, Bayer Santé, France), and then placed in a digital stereotaxic apparatus (Model 502600, WPI) coupled to the neurostar software (Neurostar GmbH). A 26-gauge stainless steel cannula was implanted into the lateral ventricle at the following coordinates: 12.8 mm posterior to bregma, 0.2 mm lateral to the midline and 7.5 mm ventral to the skull surface. The cannula was secured to the skull with dental cement and sealed with removable obturators. The animals were sutured, placed in individual cages and allowed to recover for 7 days. During this resting period, animals were injected with physiological saline every other day for habituation. One week post-surgery, rats were administered 10 µl (2.5 µl/min) of either physiological saline (NaCl) or nesfatin-1 (300 nM) solution.

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4.7. Immunohistochemistry

Animals treated by intracerebroventricular (icv) 10 µl injection of nesfatin-1 (300 nM, n=3) or NaCl (0.9 %, n=3) were used for immunostaining. Ninety minutes after injections rats under deep anesthesia were firstly perfused intracardially with ice-cold 0.1M phosphate buffered saline (PBS, pH 7.4) and then with ice-cold freshly prepared solution of 4 % paraformaldehyde (PFA) in 0.1M PB. The brains were immediately removed, post-fixed 1h in 4 % PFA at room temperature, rinsed overnight in PBS and then cryoprotected for 24 to 48h in 30 % sucrose at 4°C. The brains were frozen in isopentane (-40°C), then coronal sections (40 µm thick) of the brainstem were made with a cryostat (Leica CM3050, France) and collected serially in 0.1M PBS.

For c-Fos immunohistochemistry, brainstem sections were incubated for 10 minutes in 0.1M PBS containing 1.5 % H₂O₂ for quenching of endogenous peroxidase activity. After one hour in saturation PBS buffer containing 3% normal goat serum and 0.3 % triton X-100, sections were incubated for 48h at 4°C with a rabbit anti-c-Fos antibody (1/5000 Ab-5; Calbiochem). A biotinylated goat anti-rabbit IgG (1/400, Vector Labs) was used as a secondary antibody (incubated for 1h30 at room temperature). Peroxidase activity was revealed using the avidin-biotin complex (1/200, Vector Labs) and diaminobenzidine as chromogen. Non-specific labelling was observed on adjacent sections that were treated identically but without the primary antibody. The reaction was closely monitored and terminated by washing the sections in distilled water when optimum intensity was obtained (3-5 min). For each animal, c-Fos immunostaining photomicrographs were acquired using a 10 fold lens with a DMX 1200 camera (Nikon) coupled to ACT-1 software. The microscope was set at a specific illumination level, as was the camera exposure time. c-Fos positive nuclei were then counted on these pictures by computer-assisted morphometry using the ImageJ software.

To investigate relationships between nesfatinergic neurons and oxytocin fibers located in the DVC, we performed a double immunohistochemistry. Brainstem sections were treated with PBS containing 5 % donkey serum to block non-specific binding sites and 0.3 % triton X-100. Sections were incubated

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3 367 overnight at room temperature with a primary antibody raised against nesfatin-1 (1/10000, Phoenix
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5 368 Pharmaceuticals), washed in PBS and then incubated for 2h at room temperature with a secondary
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7 369 antibody conjugated with Alexa 594 (1/400, Invitrogen). To identify oxytocin fibers, sections were
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10 370 then treated with PBS containing 3 % bovine serum albumin (BSA) to block non-specific binding
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13 371 sites and 0.3 % triton X-100. Sections were incubated overnight at room temperature with a primary
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15 372 antibody raised against oxytocin (1/1000, Millipore), washed in PBS and then incubated for 2h at
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17 373 room temperature with a secondary antibody conjugated with Alexa 488 (1/400, Life Technologies).
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20 374 Fluorescent images were acquired on a confocal microscope (Zeiss LSN 700) using the 488 nm band
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22 375 of an Ar-laser and the 543 nm band of an He/Ne-laser for excitation of Alexa-488 and Alexa-594,
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25 376 respectively. In double labeling experiments, images were sequentially acquired. All images were
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27 377 further processed in Adobe Photoshop 6.0, only contrast and brightness were adjusted.
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32 379 **4.8. Statistical analyses**

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35 380 For electrophysiological data, statistical analyses were performed using one- or two-way analysis of
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37 381 variance (ANOVA) followed by Fisher's protected least-significant difference post-hoc test
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39 382 (StatView for Windows 5.0.1; SAS Institute). For c-Fos immunostaining, comparisons between data
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42 383 from vehicle and nesfatin-1 treated rats were performed using unpaired 2-tailed Student's t-test. All
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44 384 data were expressed as mean \pm SEM. For all data, differences were considered significant when $P <$
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57 389 contribution to confocal images acquisition. The authors thank Jérôme Espejo for taking care of the
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59 390 animals.

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Authors contributions

GF, RG, FB, DM and AA performed experiments. AA, FB, DM and TJD designed the study and analyzed data. AA, DM and TJD wrote the paper.

Figure Legends

Figure 1: Effects of nesfatin-1 injection on sublingual muscle electromyographical activity (EMG) induced by SLN stimulation and on cardiorespiratory activity.

Nesfatin-1 (100 nM) injected within the DSG induced a rapid and powerful inhibition of the number of swallows. 90 s after nesfatin-1 microinjection, only one swallow was triggered by SLN stimulation. This effect was transient since the swallows recovered after 16 minutes. Patterns of heart (ECG) and respiration rates were illustrated before and after nesfatin-1 injection (basal heart rate: 254.27±11.32/min, basal respiration rate: 95.87±6.58/min).

Figure 2: Specific and dose-dependent inhibition of triggered swallowing by nesfatin-1.

A: Time course of nesfatin-1 effects on the number of swallows after its injection within the DSG (100 nM : 22 trials, 50 nM :10 trials and 10 nM : 12 trials). Recordings were performed over 2 minutes periods until recovery. Note that at 10 nM, nesfatin-1 did not modify swallowing triggered by SLN stimulation.

B-C: Quantification of heart (B) and respiration (C) rates during nesfatin-1 application.

Time 0 represent the mean control value recorded before nesfatin-1 microinjection (100 %). Data are represented as means ± SEM normalized to the control value. * P < 0.05, *** P < 0.001, **** P <

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3 416 0.0001 significantly different from control values. # P < 0.05, ## P < 0.01, ### P < 0.001, #### P <
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5 417 0.0001 significant differences between 100 nM and 10 nM treated rats.
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10 419 **Figure 3: Effects of central nesfatin-1 administration on brainstem c-Fos immunoreactivity.**
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13 420 **A-F:** Representative coronal sections illustrating c-Fos labeling observed at different levels of the
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15 421 NTS of rats treated with NaCl 0.9 % (**A-C**) or nesfatin-1 300 nM (**D-F**) and sacrificed 1h30 post-
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17 422 treatment. Scale bar: 100 µm.
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20 423 **G-H:** High magnification of images **B** and **E** illustrating the strong labelling observed in
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22 424 subpostremal STN regions surrounding the solitary tract. Scale bar: 50 µm.
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25 425 **I:** Quantification of immunoreactive c-Fos nuclei in the brainstem of rats treated either with NaCl
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27 426 (0.9 %, gray bars) or nesfatin-1 (300 nM, black bars). ** P < 0.01, *** P < 0.001 significantly
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30 427 different from NaCl-treated rats; AP, area postrema; cc, central canal; STN, solitary tract nucleus; ts,
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32 428 solitary tract; X, Dorsal motor nucleus of the vagus; 4V, 4th ventricle.
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37 430 **Figure 4: Illustration of the strong spatial association between OT positive processes and**
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39 431 **nesfatin-1/NUCB2 expressing neurons.**
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42 432 **A:** Immunohistochemical double-labelling of nesfatin-1/NUCB2 and oxytocin (OT) performed on
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44 433 coronal sections of STN. **Arrows:** OT+ fibers. Scale bar: 30 µm. **B:** Higher magnification of OT and
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47 434 nesfastin-1/NUCB2 labelling in the STN. The area where the image originates is indicated by a
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49 435 rectangle in the low-power photomicrograph in **A**. Scale bar: 5 µm. **C:** Serial confocal images
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52 436 illustrating the close apposition of OT fibers and nesfatin-1/NUCB2 neurons. Scale bar: 5 µm.
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54 437 AP, area postrema; STN, solitary tract nucleus; ts, solitary tract.
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Figure 5: Effects of oxytocin central administration on swallowing triggered by SLN stimulation and on cardiorespiratory activity.

Oxytocin (10 nM) inhibited sublingual muscle electromyographical activity. 2 minutes after oxytocin microinjection within the DSG, only three swallows were triggered by SLN stimulation. This effect was transient since the swallows recovered after 9 minutes. Patterns of heart (ECG) and respiration rates were illustrated before and after oxytocin injection (basal heart rate: 280.77 ± 19.20 /min, basal respiration rate: 70.99 ± 5.74 /min).

Figure 6: Specific inhibition by oxytocin on triggered swallowing.

A: Time course of the effects of oxytocin (10 nM, 13 trials) microinjected within the DSG on the number of swallows recorded over 2 minutes periods and until recovery.

B-C: Quantification of heart (**B**) and respiration (**C**) rates during oxytocin application.

Time 0 represent the mean control value recorded before oxytocin microinjection (100%; black bars).

Data are represented as means \pm SEM normalized to the control value. ** $P < 0.01$, **** $P < 0.0001$.

Figure 7: Effects of nesfatin-1 central administration on swallows before and after OVT microinjection.

A: Before OVT microinjection, nesfatin-1 (100 nM, 10 trials) always induced a rapid and powerful inhibition of the number of swallows. 49s after nesfatin-1 microinjection, only two swallows were triggered by SLN stimulation. This effect was transient since the swallows recovered after 16 minutes.

B: After pre-treatment by OVT (1 μ M), nesfatin-1 microinjection within the DSG no longer changed the number of swallows.

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3462 **C:** Time course of the effects of nesfatin-1 centrally administered (100 nM, 10 trials) on the
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5463 swallowing reflex before and after pre-treatment by OVT (1 μ M). The number of swallows was
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8464 recorded over 2 minutes periods and until recovery. Time 0 represent the mean control value
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10465 recorded before nesfatin-1 microinjection (100 %). Data are represented as means \pm SEM normalized
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13466 to the control value. ** P < 0.01, *** P < 0.001, **** P < 0.0001.

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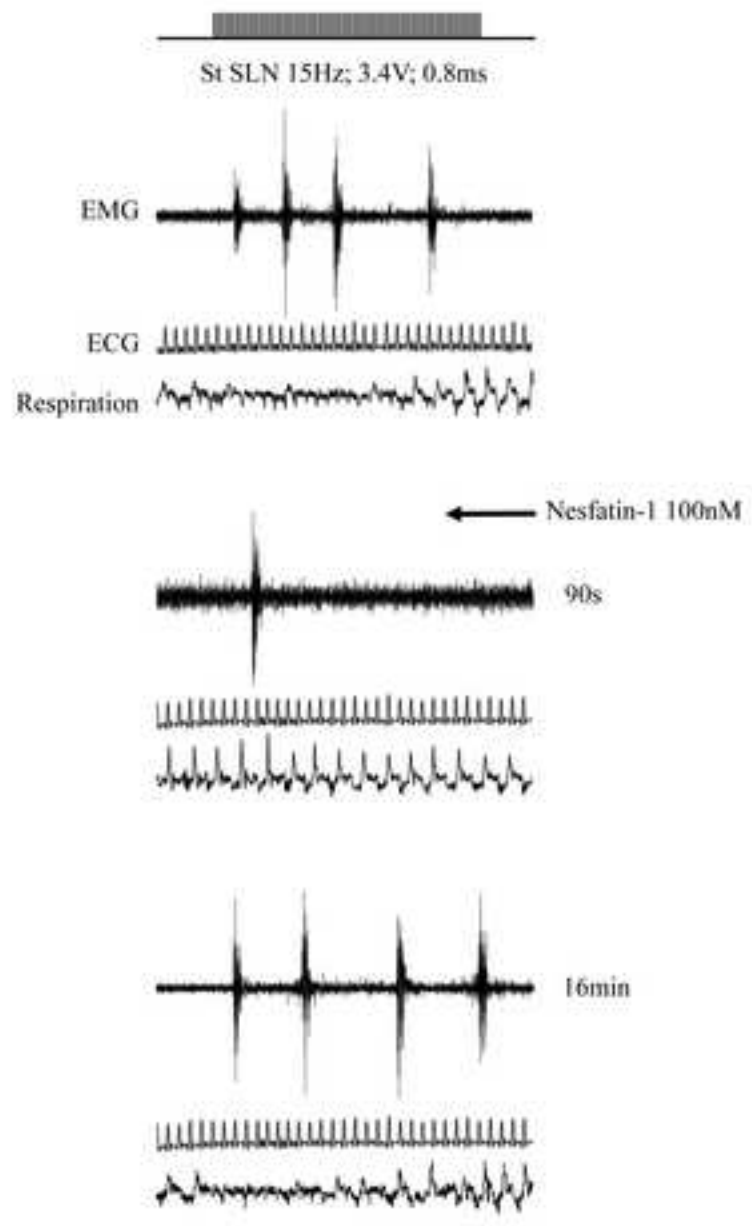


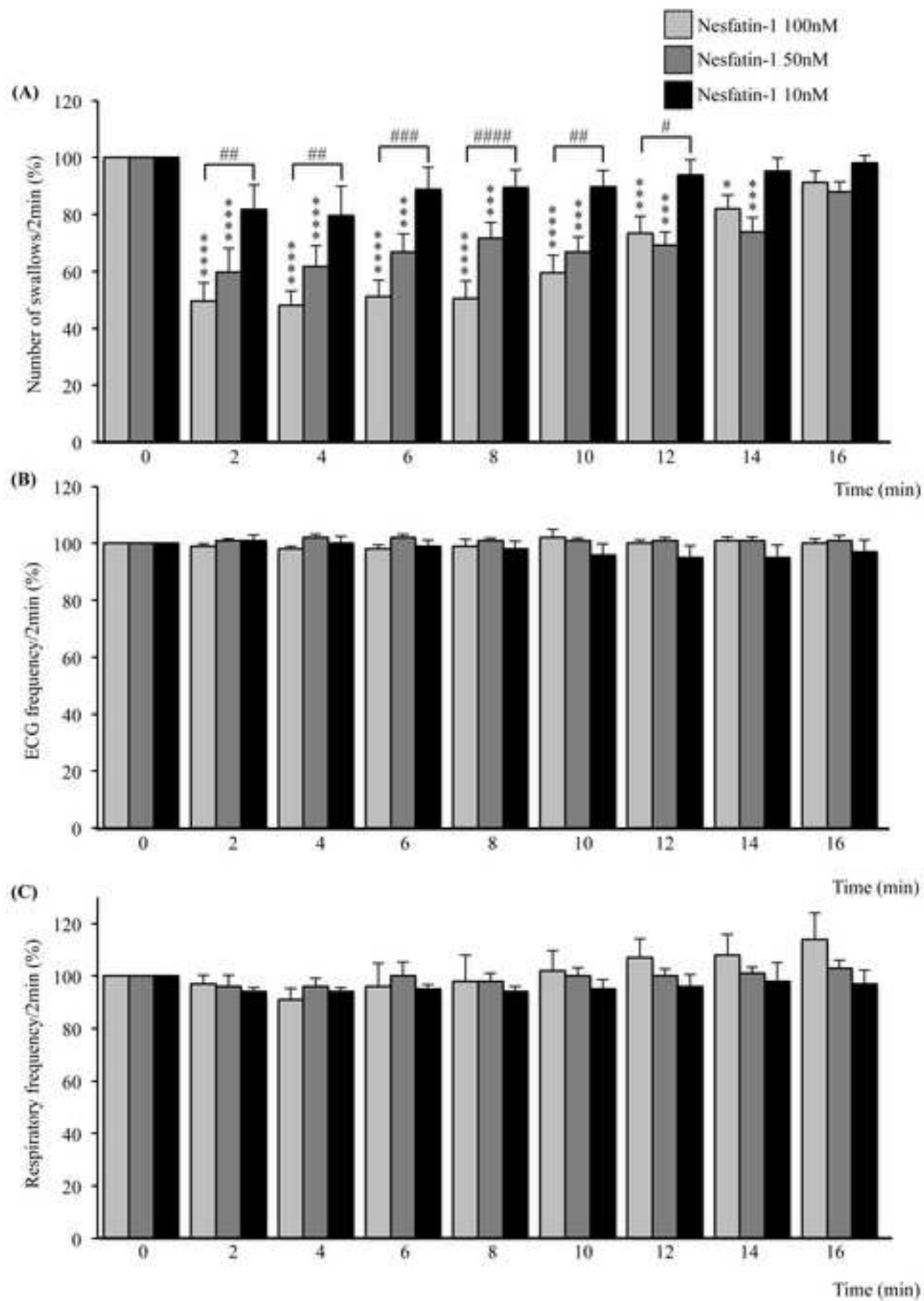
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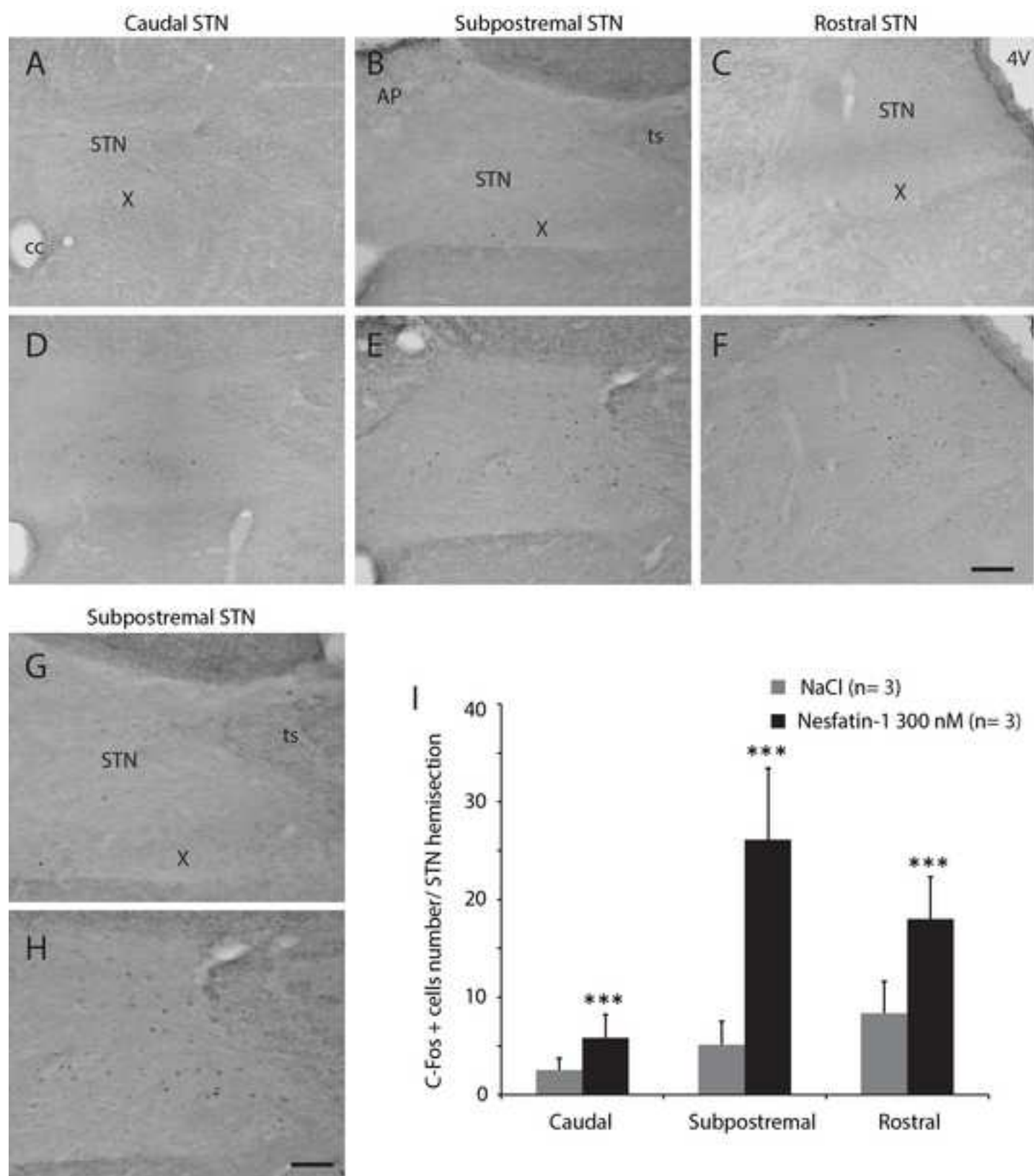


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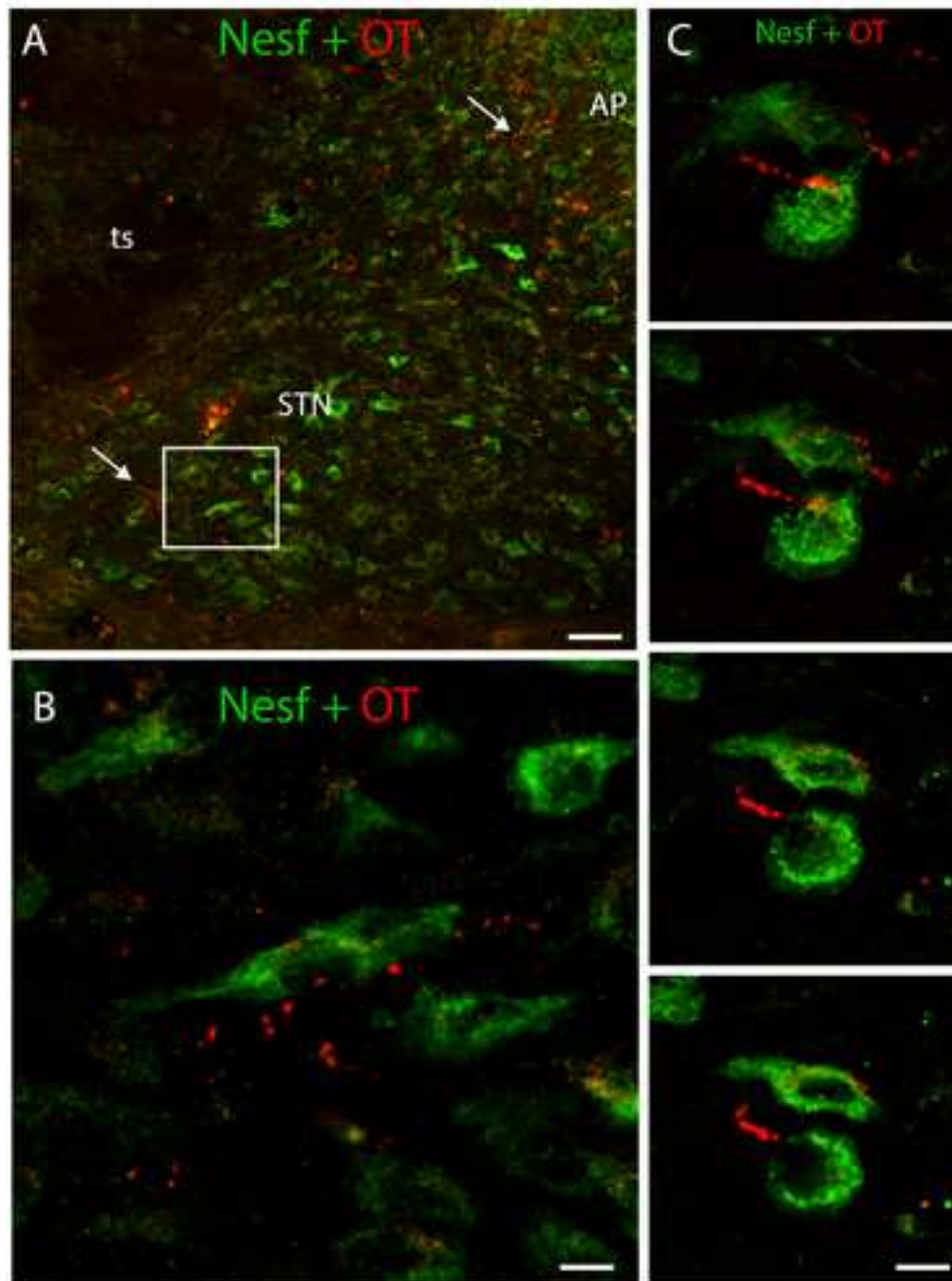


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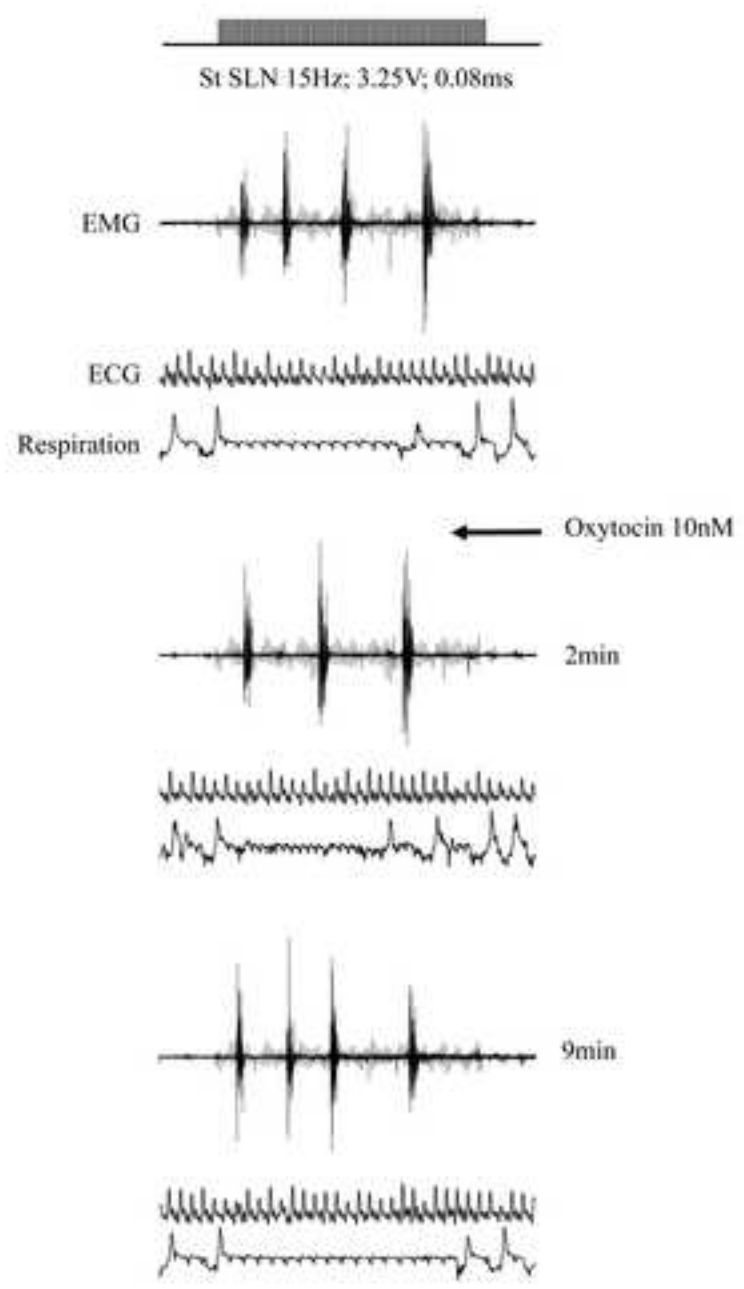


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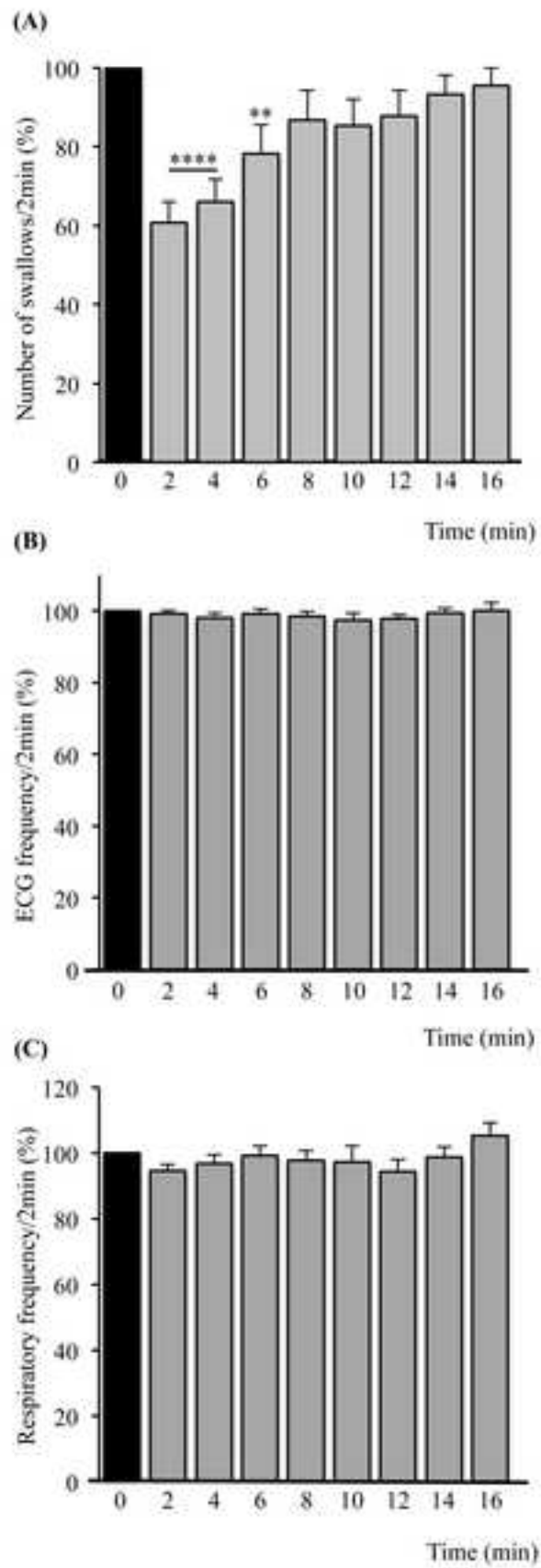


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