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Brief communication

Parotid hypersalivation after inferior salivatory nucleus glutamate/NMDA receptor excitation in the rat

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ABSTRACT

Although salivation is essential during eating behavior, little is known about the brainstem centers that directly control the salivary glands. With regard to the inferior salivatory nucleus (ISN), the site of origin of the parasympathetic preganglionic cell bodies that innervate the parotid glands, previous anatomical studies have located it within the rostrodorsal medullary reticular formation. However, to date there is no functional data that shows the secretory nature of the somas grouped in this region. To activate only the somas and rule out the activation of the efferent fibers from and the afferent fibers to the ISN, in exp. 1, NMDA neurotoxin was administered to the rostrodorsal medullary region and the secretion of saliva was recorded during the following hour. Results showed an increased secretion of parotid saliva but a total absence of submandibular-sublingual secretion. In exp. 2, results showed that the hypersecretion of parotid saliva after NMDA microinjection was completely blocked by the administration of atropine (a cholinergic blocker) but not after administration of dihydroergotamine plus propranolol (α and β-adrenergic blockers, respectively). These findings suggest that the somata of the rostrodorsal medulla are secretory in nature, controlling parotid secretion via a cholinergic pathway. The data thus functionally supports the idea that these cells constitute the ISN.

1. Introduction

Salivation and the other cephalic secretions are essential during eating behavior and for the subsequent digestion and absorption of nutrients [1–3]. However, little is known about the cerebral control of the salivary glands. With regard to the parotid glands, their secretory activity depends directly on the inferior salivatory nucleus (ISN). This nucleus houses parasympathetic preganglionic cell bodies whose axons travel in the tympanic branch of the glossopharyngeal nerve to the otic ganglion, thus mediating parotid secretion [4].

Early anatomical studies on the location of the ISN within the medulla produced conflicting results due to the limitations of available techniques [5–7]. However, more modern studies using the horseradish peroxidase tracing method in the rat [8–10] or in the cat [11,12] and electrophysiological methods [13–15] have allowed for a precise localization of the ISN. All of the above studies have found preganglionic inferior salivatory somata to be scattered mainly in the dorsal border zone of the rostral medullary reticular formation. At this level, inferior salivatory neurons reached their highest density just ventrally to the most rostral portion of the nucleus of the solitary tract [8,10,11]. However, to the best of our knowledge, there has been no functional demonstration to date showing that this medullary region constitutes the inferior salivatory center. Previous studies in rats [16], cats [17–19] and monkeys [20] have observed salivary secretion after electrical stimulation in numerous points located in the rostral medulla oblongata and at pontine level, inside and outside the reticular formation. One critique of these studies is that electrical stimulation cannot differentiate between the activation of axons and dendrites and the activation of somata [21–23]. Consequently, the salivary secretion observed in these classical studies may be due to the stimulation of the preganglionic efferent fibers to the otic ganglion or to the activation of afferent fibers to the salivatory center, causing, in the latter, reflex salivation [13,14]. For a true functional demonstration it would be necessary to activate only the cell bodies of the ISN and rule out the activation of the efferent fibers from or the afferent axons to the ISN.

Based on the foregoing, the present study aimed to selectively activate the cell bodies of the rostrodorsal medullary reticular formation, the brainstem region in which anatomical studies have identified the greatest density of inferior salivatory somata. To do so we took into account previous studies in rats that have shown that over 80 % of retrogradely labeled ISN neurons innervating the parotid glands express glutamate/NMDA receptor subunits (NR1, NR2A and/or NR2B) [24]. Thus, in exp. 1 we microinjected N-methyl-D-aspartic acid (NMDA) into the medullary reticular formation and registered the immediate secre-

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tory effect produced exclusively by the activation of the salivatory cell bodies [23]. Exp. 2 was designed to examine the parasympathetic nature of the salivary secretion. To do so we blocked the cholinergic or adrenergic receptors of parotid salivary glands prior to the microinjection of NMDA neurotoxin.

2. Experiment 1

2.1. Methods

2.1.1. Subjects

The subjects were 26 male Wistar rats from Charles River Laboratories (France). In half the animals the submandibular-sublingual salivary glands were removed and in the other half the duct of the parotid glands was ligated. This made it posible to examine which salivary glands were under the control of the medullary reticular formation. The rats, weighing 280–300 g, were randomly assigned to one of the following four groups: NMDA microinjection + duct-ligated parotid glands (NMDA + duct-ligation, $n = 7$), NMDA microinjection + submandibular-sublingual extirpation (NMDA + subm./subl., $n = 7$), sham-lesioned + duct-ligated parotid glands (Control + duct-ligation, $n = 6$) and sham-lesioned + submandibular-sublingual extirpation (Control + subm./subl., $n = 6$). Table 1 describes the groups used in exp. 1. All experimental procedures were performed in conformity with European and Spanish legislation (2010/63 EEC and BOE 53/2013, respectively) and were approved by the Ethics Committee for Animal Research of the University of Granada.

2.1.2. Surgery

2.1.2.1. Duct-ligated parotid glands. Twenty minutes prior to the stereotaxic surgery the parotid ducts were ligated bilaterally in all the rats assigned to groups NMDA + duct-ligation and Control + duct-ligation. This was done to allow us to measure exclusively the amount of submandibular-sublingual saliva secreted into the oral cavity after NMDA administration. All animals received an analgesic opioid (buprenorphine, 0.1 mg/kg, i.p., Bupaq®, Richter Pharma AG, Austria) at least 30 min before the anesthesia. The rats were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (15 mg/kg). The surgical procedure used involved making a midline incision approximately 2 cm long on the ventral throat, through which the parotid ducts were ligated and transected at the level of the lateral region of the masseter muscle [25,26].

2.1.2.2. Submandibular-sublingual salivary glands extirpation. In the rats assigned to groups NMDA + subm./subl. and Control + subm./subl., the bilateral extirpation of the submandibular-sublingual glands also took place immediately prior to the stereotaxic surgery, using a surgical procedure described elsewhere [25]. This made it possible to measure exclusively the amount of parotid saliva secreted into the oral cavity following NMDA microinjection.

2.1.2.3. Stereotaxic surgery. Immediately after the surgery on the salivary glands, the rats were placed in a David Kopf stereotaxic apparatus (mod. 900, David Kopf Instruments, Tujunga, California) with the incisor bar adjusted so that lambda and bregma were level. The anatomical location at which the inferior salivatory neurons were found to be at their highest density in the rat [10] was transferred to a stereotaxic atlas of the rat brain [27]. The experimental subjects received bilateral injections of NMDA (Sigma Chemical, PBS, pH 7.4, 0.07 M) through the insertion of a 30-gauge stainless steel cannula. The anteroposterior (AP) stereotaxic coordinate was calculated relative to the coronal interaural plane, the lateral (L) relative to the midline and the dorsoventral (V) relative to the horizontal interaural plane: AP = −2.8, $L = \pm 2.2$, $V = 0.8$. NMDA was administered in a volume of 0.5 μl to each hemisphere, through the cannula attached to a 5-μl Hamilton microsyringe (Teknokroma, Barcelona, Spain). The solution was delivered by a Harvard Apparatus pump set (model 22, Panlab-Harvard Apparatus, Barcelona, Spain) at an infusion rate of 0.1 μl/min. The cannula was left in situ for an additional 5 min before being withdrawn. In the control groups the surgical procedures were identical with one exception, equivalent volumes of phosphate-buffered saline (PBS) were infused into the ISN. After surgery, each rat was injected with buprenorphine to reduce post-operative pain (0.2 mg/kg, i.p., Bupaq®, Richter Pharma AG, Austria). Following the surgery the animals were left in their boxes for 12–14 days. After this period their brains were obtained for histology.

Table 1
Description of the groups used in Experiment 1.

Group	Peripheral surgery	Stereotaxic microinjection of	Saliva collected in oral cavity from
NMDA + duct-ligation ($n = 7$)	duct-ligated parotid glands	NMDA	submandibular-sublingual glands
NMDA + subm./subl. ($n = 7$)	submandibular-sublingual glands extirpation	NMDA	parotid glands
Control + duct-ligation ($n = 6$)	duct-ligated parotid glands	buffer	submandibular-sublingual glands
Control + subm./subl. ($n = 6$)	submandibular-sublingual glands extirpation	buffer	parotid glands

2.1.3. Procedure

Parotid or submandibular-sublingual saliva secretion was measured in experimental and control rats during the hour following bilateral NMDA or buffer microinjection (1, 20, 40 and 60 min after surgery). The saliva secreted was collected directly from the oral cavity, using a slight modification of a technique developed by others [28–30]. Briefly, 3 pieces of absorbent cotton weighing approximately 10 mg each were used. Two were placed in the lateral zones of the oral cavity while the third was placed under the tongue. After 2 min the pieces of cotton were removed and weighed to the nearest 0.1 mg on a precision balance (Cobos, Barcelona, Spain), the amount of saliva secreted being taken as the difference between the initial weight of the cotton and its weight after 2 min in the rat's mouth. Baseline parotid and submandibular-sublingual saliva secretion had also been measured in each rat immediately before lowering the cannula into the brainstem.

2.1.4. Data analyses

To analyze the data ANOVAs and post-hoc Tukey tests were used. All analyses were conducted with the Statistica software 10.0 (StatSoft, Tulsa, Oklahoma).

2.1.5. Histology

In order to obtain the brain for histology, the rats were given an analgesic opioid (buprenorphine, 0.1 mg/kg, i.p., Bupaq®, Richter Pharma AG, Austria) and 30 min later they were injected with a lethal dose of a euthanasia solution (sodium pentobarbital, 200 mg/kg, i.p., Euthoxin®, Fatro Ibérica, S. L., Spain). Animals were perfused intracardially with 0.9 % saline, followed by 10 % formalin. After extraction from the skull, the brains were post-fixed in 10 % formalin for several days and then in 10 % formalin-30 % sucrose until sectioning. Coronal sections (40 μm) were cut on a cryostat (Leica CM 1850, Leica Microsystems, Germany) and stained with cresyl violet, a Nissl stain.

2.2. Results

2.2.1. Histological findings

Histological results are shown in Fig. 1. In experimental rats, the small lesions around the tip of the infusion cannula caused by the NMDA neurotoxin microinjection made it possible to determine the affected area. In all cases these small lesions were observed in the dorsal region of the medullary reticular formation, ventrally to the rostral-

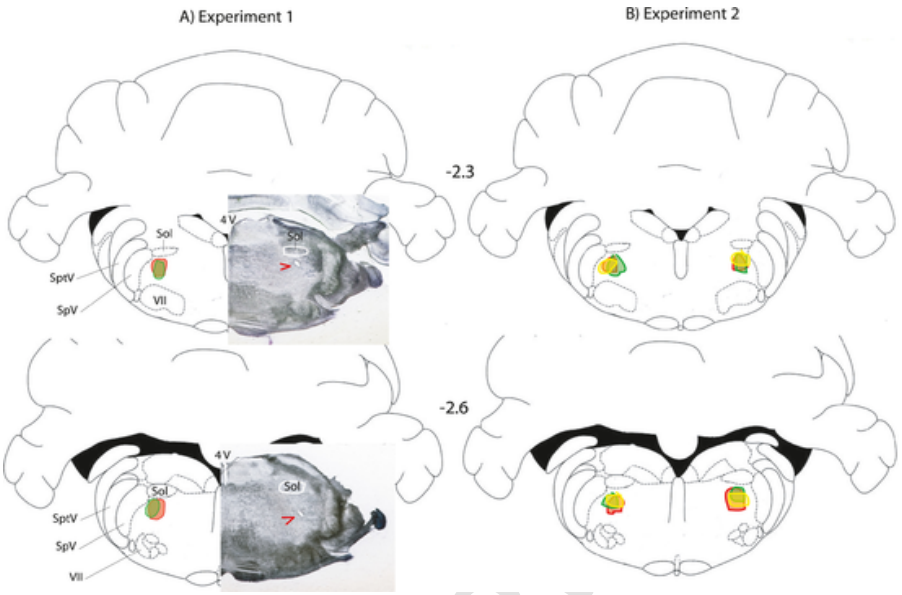

Fig. 1. A) Experiment 1. Right: Photomicrographs showing a small excitotoxic lesion (red arrow) within the rostrodorsal medullary reticular formation. The lesion was made by the NMDA neurotoxin microinjection in this area. Left: Coronal sections showing the localization of the small excitotoxic lesions caused by NMDA neurotoxin microinjection into the medullary reticular formation in rats of the group NMDA + duct-ligation (green) and the group NMDA + subm./subl. (red). B) Experiment 2: Coronal sections showing the localization of the small excitotoxic lesions caused by NMDA neurotoxin microinjection in rats of the group NMDA + atropine (green), the group NMDA + dihydro/propranolol (red) and the group NMDA + buffer (yellow). Abbreviations: 4 V, ventricle; Sol, nucleus of the solitary tract; SpV, spinal nucleus of the trigeminal nerve; SptV, spinal trigeminal tract; VII, nucleus of the facial nerve. AP coordinates with reference to the auditory meatus [27]. The target coordinates used in both experiments during stereotaxic surgery were: AP = −2.8, $L = \pm 2.2$, $V = 0.8$, in relation to the interaural zero point according to the atlas of Paxinos and Watson [27]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

most portion of the nucleus of the solitary tract and medially to the spinal nucleus of the trigeminal nerve. Anteroposteriorly these small lesions were located between −2.3 mm and −2.6 mm posterior to the interaural point, dorsally at the most caudal level of the nucleus of the facial nerve [27].

2.2.2. Salivary secretion

Results on salivary secretion indicated that during the baseline period the four groups secreted similar amounts of saliva ($F_{3, 22} = 0.55$, $p = 0.64$, range 3.1 to 5.5 mg). Fig. 2 shows submandibular-sublingual salivary secretion in the groups with duct-ligated parotid glands (NMDA + duct-ligation and Control + duct-ligation) and parotid salivary secretion in the groups with extirpation of submandibular-sublingual glands (NMDA + subm./subl. and Control + subm./subl.) during the hour following intracerebral microinjection. A 2-way mixed ANOVA (4 group x 4 time) found a significant effect in the group factor ($F_{3, 22} = 11.94$, $p < 0.0001$, $\eta^{2}_{p} = 0.61$), time factor ($F_{3, 66} = 9.79$, $p < 0.0001$, $\eta^{2}_{p} = 0.30$) and group x time interaction ($F_{9, 66} = 8.72$, $p < 0.0001$, $\eta^{2}_{p} = 0.54$). Analysis of the interaction using Tukey tests revealed that these effects were due to a significant increase in parotid saliva secretion and not to an increase in submandibular-sublingual secretion. Thus, although 1 min after the microinjection of the neurotoxin the NMDA + subm./subl. group showed no significant differences with its control group in the secretion of parotid saliva ($p = 0.62$), the two groups did differ significantly 20 min (NMDA + subm./subl. vs. Control + subm./subl., $p < 0.002$), 40 min (NMDA + subm./subl. vs. Control + subm./subl, $p < 0.0001$) and 60 min (NMDA + subm./subl. vs. Control + subm./subl, $p < 0.02$) after the microinjection. In contrast, no significant differences were detected in submandibular-sublingual secretion when comparing the NMDA + duct-ligation group to the Control + duct-ligation group, in any of the four time periods following the microinjection ($p = 1$).

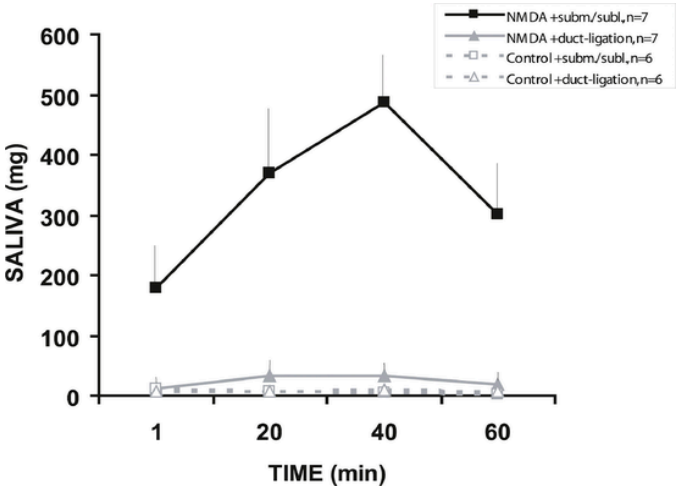

Fig. 2. Experiment 1: Mean amount (±SEM) of submandibular-sublingual and parotid saliva secreted in experimental and control groups during the hour following NMDA or buffer microinjection into the rostrodorsal medullary reticular formation. NMDA + duct-ligation (NMDA microinjection + duct-ligated parotid glands), $n = 7$; NMDA + subm./subl. (NMDA microinjection + submandibular-sublingual glands extirpated), $n = 7$; Control + duct-ligation (buffer microinjection + duct-ligated parotid glands), $n = 6$; Control + subm./subl. (buffer microinjection + submandibular-sublingual glands extirpated), $n = 6$.

To further examine these data we used Tukey tests to analyse the group factor. In this case we compared the total saliva secreted by the four groups during the hour following the microinjection of NMDA or buffer. Once again, the results revealed that the NMDA + subm./subl.

group secreted a larger quantity of parotid saliva than its Control ($p < 0.0005$). In clear contrast, however, the NMDA + duct-ligation group secreted a similar quantity of submandibular-sublingual saliva as its Control ($p = 0.99$).

These data clearly indicate that the cell bodies activated during the stereotaxic surgery control exclusively the parotid salivary glands' secretory activity. These results support the idea that the region activated corresponds functionally to the ISN. Previous anatomical [8,10] and physiological [31] data observed a certain overlap between the superior and inferior salivatory nucleus but only in the most rostral region of the ISN, at the level of the pontine reticular formation. That is why the observation of exclusively parotid secretion in exp. 1 suggests that we are activating an intermediate/caudal region of the ISN.

3. Experiment 2

In exp. 2 we investigated the parasympathetic versus sympathetic nature of parotid salivary secretion induced by medullary reticular formation activation. In rats stimulation of efferent preganglionic parasympathetic fibers produces a potent hypersecretory effect that is blocked by muscarinic antagonists such as atropine but not by adrenergic antagonists [4,32,33]. On the other hand, sympathetic secretion observed following stimulation of the superior cervical ganglion is mediated in rats by both α- and β-adrenergic receptors [4,32–34]. Based on the foregoing, we hypothesized that the blockade of the cholinergic receptors of the parotid salivary glands would abolish the saliva hypersecretion observed following microinjection of NMDA into the ISN. The blockade of the adrenergic receptors, however, should not affect the hypersecretion at all.

3.1. Methods

3.1.1. Subjects

The subjects were 17 male Wistar rats from Charles River Laboratories (France). The animals were divided randomly into three groups. In the first group, prior to the microinjection of NMDA into the ISN, buffer was administered i.p. to the animals (NMDA + buffer, $n = 5$). In the second group, prior to the NMDA microinjection, α- and β-adrenergic receptor antagonists were administered i.p. to the animals (NMDA + dihydroergotamine/propranolol, $n = 6$). Finally, in the third group, an antagonist of the cholinergic receptors was administered i.p. prior to the NMDA microinjection (NMDA + atropine, $n = 6$). All procedures were identical to those described in exp. 1 with the following exception. In this experiment, twenty minutes before the stereotaxic surgery the submandibular-sublingal glands were extirpated bilaterally to ensure that in all the rats the saliva collected in the mouth following the NMDA microinjection came exclusively from the parotid glands.

3.1.2. Surgery

The conditions of analgesia and anesthesia were identical to those described in exp. 1, as were the rest of the surgical procedures.

3.1.3. Procedure

During stereotaxic surgery, specifically 15 min before NMDA microinjection into the ISN, the rats in each group received the following drugs, i.p.:

Group NMDA + atropine: atropine sulphate (Sigma Aldrich, dissolved in PBS, pH 7.4, 1.2 mg/kg).

Group NMDA + dihydroergotamine/propranolol: dihydroergotamine mesylate (α-adrenergic receptor antagonist, Sigma Aldrich/European Pharmacopoeia Reference, PBS, pH 7.4, 0.6 mg/kg) plus propranolol hydrochoride (β-adrenergic receptor antagonist, Sigma Aldrich, PBS, pH 7.4, 2.2 mg/kg).

Group NMDA + buffer: only buffer was injected i.p., in a volume similar to that used in preceding groups (Sigma Aldrich, PBS, pH 7.4).

Previous studies in our lab and others have shown that the doses used are appropriate for blocking parasympathetic or sympathetic saliva secretion [16,33–35].

3.1.4. Histology

The procedures were the same as the ones followed in exp.1. The data analysis performed was also identical.

3.2. Results

Histological findings were similar to those of exp. 1 (Fig. 1). With respect to salivary secretion, during the baseline period the three groups secreted similar amounts of saliva ($F_{2, 14} = 0.11$, $p = 0.88$, range 4.1–5.9 mg). Fig. 3 shows the amount of parotid saliva secreted by each group during the hour after the intracerebral microinjection of NMDA. A 2-way mixed ANOVA (3 group x 4 time) found a significant effect in the group factor ($F_{2, 14} = 54.97$, $p < 0.0001$, $\eta^{2}_{p} = 0.88$), time factor ($F_{3, 42} = 17.16$, $p < 0.0001$, $\eta^{2}_{p} = 0.55$) and group x time interaction ($F_{6, 42} = 4.94$, $p < 0.0001$, $\eta^{2}_{p} = 0.41$). Analysis of the interaction revealed that only atropine administration blocked parotid hypersecretion. Specifically, upon comparing the NMDA + atropine group to the NMDA + buffer group, the latter secreted significantly more saliva 20 min ($p < 0.0004$), 40 min ($p < 0.0001$) and 60 min ($p < 0.0001$) after NMDA microinjection, but not 1 min ($p = 0.80$) after it. Similarly, the NMDA + atropine group secreted significantly less saliva than the NMDA + dihydro/propranolol group 20 min ($p < 0.005$), 40 min ($p < 0.0001$) and 60 min ($p < 0.0001$) after NMDA microinjection, but not 1 min ($p = 0.77$) after it. Finally, no significant differences were detected between NMDA + buffer vs. NMDA + dihydro/propranolol groups in any of the time periods measured (p from 0.30 to 1.0). These results support the parasympathetic nature of the parotid salivary secretion observed following medullary activation.

4. Discussion

The present study provides functional evidence that the dorsal border region of the rostral medullary reticular formation is secretory in

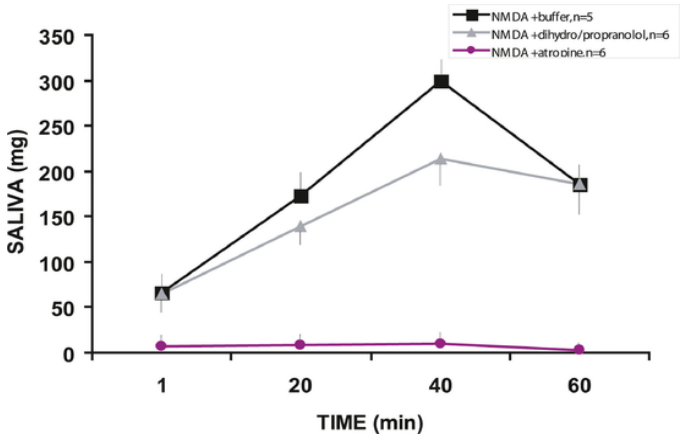

Fig. 3. Experiment 2: In all animals the submandibular-sublingual glands were extirpated prior to the stereotaxic surgery. The data indicate the mean amount (±SEM) of parotid saliva secreted in the three groups used in exp. 2 during the hour following NMDA microinjection into the medullary reticular formation. NMDA + buffer (NMDA microinjection + buffer i.p.), $n = 5$; NMDA + dihydro/propranolol (NMDA microinjection + dihydroergotamine plus propranolol i.p.), $n = 6$; NMDA + atropine (NMDA microinjection + atropine i.p.), $n = 6$.

nature. Experiment 1 found a high secretion of parotid saliva but a complete absence of submandibular-sublingual secretion during the hour after the microinjection of NMDA. In the second experiment, the blockade, with atropine, of the cholinergic receptors of the parotid glands, but not the blockade of the adrenergic receptors, completely eliminates the secretion of saliva induced by NMDA microinjection, thus confirming the cholinergic/parasympathetic nature of hypersalivation.

Taken together these findings provide physiological evidence that the somata of the rostrodorsal medullary reticular formation correspond to and are part of the ISN. Indeed, the area of the medulla activated in our experiments corresponds closely to the region that modern anatomical and electrophysiological methods have identified as the ISN in the rat and cat [8–11]. Specifically, the area activated in our experiments is the same area that anatomical studies have described as having the highest density of labelled inferior salivatory neurons [10,11]. Furthermore, in this region anatomical investigations have not observed overlap of inferior salivatory neurons controlling the parotid glands (inferior salivatory nucleus) and superior salivatory neurons participating in the innervation of the submandibular-sublingual glands (superior salivatory nucleus) [8,10]. This observation is coherent with the results of exp. 1 that show exclusively parotid secretion following activation of the somas of this region, which suggests that in our experiment NMDA microinjection is probably affecting an intermediate/caudal region of the ISN. Indeed, rostrally the ISN extends into the ventral pontine lateral reticular formation, just dorsal to the facial nucleus [8–10]. At this rostral level, however, anatomical studies have shown that inferior salivatory somata coexist with superior salivatory neurons [8,10]. Confirming, on physiological grounds, an overlap of inferior and superior salivatory cells, a previous study in our lab showed that NMDA-receptor activation of this pontine area caused both submandibular-sublingual and parotid secretion, although on this occasion the submandibular-sublingual secretion was four times greater than the parotid secretion [31].

Another important point of discussion is that the procedure used in our experiments to produce hypersecretion of saliva is completely different from the procedure used in classical physiological studies, which was not able to functionally identify the precise location of the salivatory centers [16–20]. In the classical studies salivary secretion was achieved by electrical stimulation of numerous points located within the medullary and pontine reticular formation, as well as locations lateral and dorsal to the reticular formation. The problem with these studies is that the results cannot be considered conclusive, given that salivary secretion can be provoked by direct stimulation of the efferent fibers from the salivatory nuclei and/or the afferent fibers connected to the salivatory centers [21,22]. Also, the very small size of the inferior salivatory cell bodies, between 15 and 25 μm diameter [11], in relation to the size of the tip of the stimulation electrodes, further reinforces the idea that efferent fibers, dendrites or afferent axons of the bodies are probably what were stimulated in the classical studies. As for the possibility that reflex salivation is evoked following electrical stimulation of afferent fibers to the ISN, some studies have demonstrated reflex salivation of parotid saliva after stimulation of gustatory afferents [13]. Additionally, other authors have found that in cats inferior salivatory neurons respond with spike potentials to the stimulation of trigeminal sensory branches [15]. Last of all, another study, also using electrophysiological methods, showed that excitatory inputs from the chorda tympani, glossopharyngeal and vagus nerves activated inferior salivatory neurons in the cat [14]. Thus, the salivation occurring after electrical stimulation of fibers afferent to a salivatory center can be confused with the stimulation of the salivatory nucleus itself, making it difficult to arrive at a precise functional location [4,31]. In the present study, however, we have applied an agonist of NMDA receptors to the medullary reticular formation to ensure that only the cell bodies are affected, thus excluding a possible activation of fibers [23,24].

In summary, the present series of experiments has specifically activated, for the first time, the cell bodies of the brainstem region proposed as the ISN based on anatomical studies. So, from a functional perspective, our data suggest that the somata of the rostrodorsal medullary reticular formation control the secretory activity of the parotid salivary glands via a cholinergic pathway and they therefore constitute the ISN.

CRediT authorship contribution statement

JuanM.J. Ramos: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

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