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Microinjection of NMDA-neurotoxin into the superior salivatory nucleus of the rat: Short-term secretory and long-term drinking behavior effects

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Abstract

The anatomical location of the superior salivatory nucleus (SSN), the site of origin of the parasympathetic preganglionic cell bodies that innervate the submandibular-sublingual salivary glands, is well established in rats. However, as of yet there is no functional data that convincingly shows the secretory nature of this region. Previous studies have not been able to differentiate between interventions on efferent or afferent fibers connected to the SSN versus interventions on the salivatory nucleus itself. Taking advantage of the fact that salivatory neurons express NMDA-receptors on their somas, in the present study SSN cell bodies were activated and lesioned sequentially by means of intracerebral application of NMDA-neurotoxin. In exp. 1 two effects, a short- and a long-term effect, were observed following NMDA administration. The first effect was high submandibular-sublingual saliva secretion during the hour following administration of the neurotoxin and the second was a profound change in drinking behavior once the animals recovered from the lesion. Thus, on post-surgery days 16, 17 and 18, the rats exhibited hyperdipsia in the presence of dry food but not in the presence of wet food. In expt. 2 results showed that saliva hypersecretion observed after NMDA-microinjection was completely blocked by the administration of atropine (a cholinergic blocker) but not after the administration of dihydroergotamine plus propranolol (α and β-adrenergic blockers, respectively). From a functional perspective, these data suggest that the somata of the parvocellular reticular formation control the secretory activity of the submandibular-sublingual salivary glands and thus constitute the SSN.

Keywords: Superior salivatory nucleus; Salivation; Salivary glands; Saliva; Prandial drinking

1. Introduction

Since the early research by Ivan P. Pavlov on the cephalic phase of digestive secretions, numerous studies have attempted to locate the brain centers that control the digestive glands [1-3]. With regard to cerebral control of saliva secretion, pioneering anatomical studies on the possible location of the superior salivatory nucleus (SSN), which houses the parasympathetic preganglionic cell bodies that innervate the submandibular-sublingual salivary glands, were based on observations of retrograde chromatolytic changes after transections of the chorda tympani. These studies produced conflicting results that suggested at least three different locations [4-6]. Subsequent studies using histochemical techniques for identifying the SSN in the rat also produced discrepancies, mainly due to the difficulty of differentiating between the reaction produced in cells and the one produced in axonal bundles [7, 8].

This entire panorama changed in the late 70s and early 80s and a consensus was reached regarding the precise anatomical location of the SSN. This progress was based on studies using the horseradish peroxidase tracing method [9-19], retrograde transneuronal labelling by pseudorabies virus [20, 21] and electrophysiological methods [22-26]. All the aforementioned studies found the preganglionic salivatory somata to be scattered in the parvocellularis lateral reticular formation of the brainstem, extending dorsolaterally to the facial motor nucleus. However, to the best of our knowledge, to date there has been no functional demonstration indicating that this region constitutes a salivatory center. With respect to the foregoing, first, previous studies in rats [27], cats [28-30] and monkeys [31] described salivary secretion after electrical stimulation around the facial genu and in nearby regions located inside and lateral to the reticular formation. One problem with these studies is that electrical stimulation cannot differentiate between the activation of axons and dendrites and the activation of somata [32-35]. For a true functional demonstration it would be necessary to activate only the cell bodies of the SSN and not the efferent axons from or the afferent axons to the SSN [36]. Second, previous studies in our lab have found a profound deficit in submandibular-sublingual salivary secretion in rats after electrolytic lesions to the lateral reticular formation [37-39]. However, again, this

approach cannot easily differentiate between damage to cell bodies and damage to axons, or a combination of both.

The aim of the present study was therefore to selectively activate/lesion the cell bodies of the lateral reticular formation, the brainstem region identified as SSN based on anatomical studies. To do so we made use of previous studies in rats that have shown the expression of ionotropic NMDA receptors in antidromically identified SSN neurons [40, 41]. In parallel with these data, other studies have shown that ionophoretic application of NMDA receptor agonists have an excitatory effect on about half of the SSN neurons [42, 43]. So, on the basis of these findings, in Exp. 1 we microinjected N-methyl-D-aspartic acid (NMDA) into the lateral reticular formation and registered the immediate short-term secretory effect produced by the activation of the salivatory cell bodies. After 16 days of recovery, we also examined in the same rats the long-term effect caused by the NMDA-excitotoxic lesions to the somata of lateral reticular formation. Specifically, previous studies have described in desalivated rats a prandial style of drinking and polydipsia associated with the intake of dry food, which has been interpreted as an attempt to facilitate the swallowing of dry food in the absence of saliva [39, 44-46]. Therefore, we hypothesize that SSN somata lesions will produce a deficit in submandibular-sublingual saliva secretion and consequently an alteration in drinking behavior when presented with dry food, but not when presented with a wet diet. Exp. 2 was designed to examine the parasympathetic nature of the salivary secretion observed in the short term. To do so we blocked the cholinergic or adrenergic receptors of submandibular-sublingual salivary glands prior to the microinjection of NMDA-neurotoxin.

2. <u>Experiment 1</u>: *Short-term secretory effects and long-term drinking behavior after NMDA-neurotoxin microinjection into the superior salivatory nucleus*.

The aim of this experiment was to produce a biphasic hyper/hypo-secretion of salivation due to overactivation of somas of the nucleus parvocellularis followed by underactivation/lesion of the same tissue. Specifically, after NMDA-neurotoxin injection into the SSN, the present experiment studied two effects in the same rats, first, the short-term secretory effect caused by

somata activation and, second, long-term drinking behavior effect due to permanent excitotoxic lesions of the cell bodies.

2.1. Methods

2.1.1. Subjects

The subjects were 32 male Wistar rats from Charles River Laboratories (France). Two rats were excluded because they died during the hour following the NMDA injection. Since the first objective of this experiment was to study saliva secretion following the activation of the cellular somas with NMDA microinjections, in half of the animals the submandibular-sublingual salivary glands were removed and in the other half the duct of the parotid glands was ligated. The latter generally produces atrophy in the glands [38, 47]. This made it possible to examine which salivary glands were under the control of the lateral reticular formation. The rats were randomly assigned to one of the following four groups: NMDA microinjection + duct-ligated parotid glands (NMDA + duct-ligation, n = 8), NMDA microinjection + submandibular-sublingual extirpation (NMDA + subm./subl., n = 8), sham-lesioned + duct-ligated parotid glands (Control + duct-ligation, n = 7) and sham-lesioned + submandibular-sublingual extirpation (Control + subm./subl., n = 7). The animals, initially weighing between 280-290 g, were individually housed in single polycarbonate cages (480 x 265 x 210 mm, Tecniplast, Italy), maintained at a constant temperature of 22 ± 1° C and under controlled lighting conditions (light on from 8:00 a.m. to 8:00 p.m.). All experimental procedures were performed during the light phase of the cycle. 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

Duct-ligated parotid glands. Two weeks before the stereotaxic surgery the parotid ducts were ligated bilaterally in all the rats assigned to groups NMDA + duct-ligation and Control + duct-ligation. The purpose of parotid desalivation was to allow us to measure exclusively the amount

of submandibular-sublingual saliva secreted into the oral cavity after NMDA administration in the lateral reticular formation. All animals received an analgesic opioid (buprenorphine, 0.1 mg/kg, i.p., Bupaq®, Richter Pharma AG, Austria) at least 30 min before the anaesthesia. The rats were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (15 mg/kg). The surgical procedure used involved 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 [39, 47]. Four hours after surgery each rat was injected with buprenorphine to reduce post-operative pain (0.2 mg/kg, i.p., Bupaq®, Richter Pharma AG, Austria).

Submandibular-sublingual salivary glands extirpation. Two weeks prior to the stereotaxic surgery the submandibular-sublingual salivary glands were removed bilaterally in all the rats assigned to groups NMDA + subm./subl. and Control + subm./subl. This allowed us to measure exclusively the amount of parotid saliva secreted into the oral cavity following NMDA microinjection. The analgesia and anesthesia were identical to those described above for the rats with duct-ligated parotid glands. The surgical procedure used involved a midline incision approximately 2 cm long on the ventral throat, through which the submandibular-sublingual glands were carefully separated from the surrounding tissue. The submandibular-sublingual ducts were then transected at the level of the glandular hilium and the glands themselves were removed [38, 46, 47].

Stereotaxic surgery. The analgesia and anesthesia procedures were identical to those described above. After anesthesia, the rats were placed in a David Kopf stereotaxic apparatus (model 900, Tujunga, CA, USA). The distance between the horizontal plane passing through the interaural line and the horizontal plane passing through the incisors was 5 mm. The anatomical location of the SSN in the rat was transferred to a stereotaxic atlas of the rat brain [48]. Because the dorsoventral extent of these cells is quite broad and in the vertical plane they are arranged in an oblique line, two contiguous excitotoxic lesions were made on either side of the brainstem, one

in the ventral part and one in the dorsal part of the parvocellular lateral reticular formation. This made it possible to affect as much of the salivatory center as possible.

The bilateral lesions aimed at the ventral part of the SSN were located 2.6 mm posterior to the auditory meatus, ±2.2 mm lateral to the sagittal sinus and 0.1 mm ventral to the horizontal interaural zero plane [48]. The lesioned subjects received bilateral injections of N-methyl-D-aspartic acid (NMDA, Sigma Chemical, PBS, pH 7.4, 0.06 M) through the insertion of a 30-gauge stainless steel cannula. The neurotoxin was administered in a 0.25 µl volume at each site 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 3 min before being withdrawn. Bilateral lesions aimed at the dorsal part of the SSN were located 2.6 mm posterior to the auditory meatus, ±1.8 mm lateral to the sagittal sinus and 0.3 mm above the horizontal interaural plane. The procedure used to damage the dorsal part of the lateral reticular formation was the same as above. The control groups underwent identical surgical procedures, the one exception being that equivalent volumes of phosphate-buffered saline (PBS) were infused into each of the four sites. Nontraumatic ear bars developed in our own laboratory were used to avoid possible damage to the salivatory fibers running through the middle ear.

2.1.3. Procedure

Saliva Collection Procedure

Parotid or submandibular-sublingual saliva secretion was measured in lesioned 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 [39, 49, 50]. 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 was also measured in each rat, immediately before lowering the cannula into the brainstem.

Drinking Behavior

After the stereotaxic surgery the animals had a recovery period of 10 days. Over the next five days the rats were habituated to eating their usual daily dry diet (Envigo, Global Diet 2914, Madison, Wisconsin) from 10.00 a.m. to 12.00 p.m. only, while water remained available 24 h *ad lib*. On the following two days (experimental days 16 and 17), the amounts of food ingested and water consumed by all subjects were recorded during the 2-h feeding period. The water consumed during the 22-h period of food restriction on experimental days 16 and 17 was also measured. On experimental day 18 the dry food was replaced by a wet mash consisting of 3 parts isotonic saline to 2 parts dry food, and again the amounts of water consumed and of food ingested were recorded throughout the 2-h feeding period.

2.1.4. Histology

When the behavioral testing was complete, the rats were given an analgesic opioid (buprenorphine, 0.1 mg/kg, i.p., Bupaq®, Richter Pharma AG, Austria) and 30 min later 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 subsequently 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. The sections obtained were studied under the microscope and the extension of the lesion was transferred to coronal sections of the

stereotaxic atlas by Pellegrino and associates [48]. Micrographs were obtained using an Olympus CH30 microscope and a Nikon FM2 analogic camera.

2.1.5. Data analyses

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

2.2. Results

2.2.1. Histological findings

Tissue damage was microscopically identified by the presence of pronounced necrosis or missing tissue (Figure 1). In all lesioned rats the central region of the lesions affected the lateral reticular formation just dorsal to the lateral half of the facial nucleus and just medial to the spinal nucleus of the trigeminal nerve, at -2.4/-2.5 mm anteroposteriorally in relation to the interaural zero point [48]. In some animals a small portion of the most dorsal area of the facial nucleus was also slightly affected. In contrast, no damage was observed in the most medial region of the spinal trigeminal nucleus. In the lateral plane the size of the lesion was 0.3-0.4 mm. In the dorsoventral plane, the lesions extended to the ventrolateral edge of the genu of the facial nerve, presenting a length of 0.4-0.7 mm. At the most rostral level, lesions were observed to be dorsolateral to the facial nucleus and medial to the descending root of the facial nerve, within the lateral reticular formation, between 2.0 and 2.2 mm posterior to the interaural coronal plane. Caudally, damage extended in the majority of animals up to 2.8/2.9 mm posterior to the auditory meatus, always within the lateral reticular formation. The entire damaged area thus corresponded very precisely to the location of salivatory cell bodies that have been identified as the SSN based on anatomical studies [see, for example, 10, 13, 20].

2.2.2. Short-term secretory effect

During the baseline period the four groups secreted similar amounts of saliva ($F_{3, 26} = 0.56$, $p = 0.64$, range 5.9 to 8.8 mg). Figure 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 injection. A 2-way mixed ANOVA (4 group x 4 time) found a significant effect in the group factor ($F_{3, 26} = 57.82$, $p<0.0001$, $\eta^2_p = 0.86$), time factor ($F_{3, 78} = 3.86$, $p<0.01$, $\eta^2_p = 0.12$) and group x time interaction ($F_{9, 78} = 3.43$, $p<0.001$, $\eta^2_p = 0.28$). Analysis of the interaction using Tukey tests revealed that these effects were due to a significant increase in submandibular-sublingual saliva secretion after the activation of the pontine reticular formation, but not to an increase in parotid secretion. This is why 1 min after the microinjection of NMDA the group with duct-ligated parotid glands secreted significantly more submandibular-sublingual saliva than its control group that was given buffer (Control + duct-ligation, $p<0.0001$). Similar results were observed when comparing the two groups 20 min ($p<0.0001$) and 60 min ($p<0.0001$) after microinjection, but not at 40 min after microinjection ($p = 0.13$). In contrast, no significant differences were detected in parotid salivary secretion when comparing the NMDA + subm./subl. group to the Control + subm./subl. group, in any of the four time periods following the microinjection (1 min, $p = 0.80$; 20 min, $p = 0.99$; 40 min, $p = 0.82$; 60 min, $p = 0.98$). Confirming the superiority of the submandibular-sublingual saliva secretion as compared to parotid saliva, Tukey tests also showed that the NMDA + duct-ligation group secreted a larger quantity of submandibular-sublingual saliva than the quantity of parotid saliva secreted by the NMDA + subm./subl. group 1 min ($p<0.0001$) and 20 min ($p<0.0001$) after activation, but not 40 ($p = 0.99$) or 60 min after ($p = 0.48$).

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. Results indicated once again that NMDA administration in the duct-ligated parotid glands group caused secretion of significantly more submandibular-

sublingual saliva than that observed in its control group, the Control + duct-ligation group ($p<0.0001$). However, in this case, the analysis showed that the NMDA + subm./subl. group secreted significantly more parotid saliva than did its control, the Control + subm./subl. group ($p<0.01$). On the other hand, when comparing the two experimental groups (NMDA + duct-ligation vs. NMDA + subm./subl.) the analyses again found significantly more submandibular-sublingual saliva secretion than parotid secretion ($p<0.0001$). Finally, no significant differences were detected upon comparing the two control groups given buffer (Control + duct-ligation vs. Control + subm./subl., $p = 0.99$).

These data clearly indicate that the cell bodies activated during the stereotaxic surgery control mainly the submandibular-sublingual salivary glands secretory activity. Therefore, these results support the idea that the region activated corresponds functionally to the SSN. Nonetheless, in the present study, the activation of the lateral reticular formation does cause a small significant effect on the parotid glands. In relation to this last finding, although the inferior salivatory nucleus, which houses the parasympathetic preganglionic cell bodies that innervate the parotid salivary glands, has been located anatomically in the reticular formation surrounding the nucleus ambiguus and caudally to the site activated in the present study, some of its rostralmost somata reach the central portion of the SSN [10, 13, 51]. So, the overlap observed between superior and inferior salivatory cell bodies, or the possible presence of interconnections between the two salivatory nuclei, might explain why activation of the lateral reticular formation in the present study affects the secretory activity of both types of salivary glands, although submandibular-sulingual secretory activity was significantly greater than parotid salivary flow.

2.2.3. Long-term changes in drinking behavior

To analyse the amount of water consumed during the 2-h period in which the animals were presented with dry food on experimental days 16 and 17, we performed a 2-way mixed ANOVA (4 group x 2 day). The analysis found a significant effect in the group factor ($F_{3, 26} = 12.10$, $p<0.0001$, $\eta^2_p = 0.58$), but not in the day factor ($F_{1, 26} = 0.15$, $p = 0.69$) or in the group x

day interaction ($F_{3, 26} = 0.02$, $p = 0.99$). These data are presented in Table 1. To further examine these data we used Tukey tests to analyse the group factor. The analysis revealed that during the two days of exposure to dry food, NMDA + duct-ligation rats drank significantly more water than the Control + duct-ligation group ($p<0.003$). However, when comparing NMDA + subm./subl. vs. Control + subm./subl. groups, no significant differences whatsoever were found ($p = 0.99$). Importantly, the NMDA + duct-ligation group consumed significantly more water than the NMDA + subm./subl. rats ($p<0.0002$). Lastly, the two control groups consumed similar amounts of water ($p = 0.61$). In contrast with the foregoing, as shown in Table 1, on experimental day 18 when the animals were exposed to wet food during the 2-h daily feeding period, the differences in drinking behavior disappeared. In this case, a one-way ANOVA found no significant differences between groups ($F_{3, 26} = 1.22$, $p = 0.31$, range from 3 to 4.8 ml). Finally, the 4 groups consumed similar amounts of food on experimental days 16, 17 and 18 ($F<1$ on all three days).

During the 22-h food deprivation period on experimental days 16 and 17, the amount of water consumed by all the animals was also measured (Table 1). A 2-way mixed ANOVA found significant differences only in the group factor ($F_{3, 26} = 29.37$, $p<0.0001$, $\eta^2_p = 0.81$), not in the day factor ($F_{1, 26} = 0.16$, $p = 0.68$) or in the group x day interaction ($F_{3, 26} = 2.39$, $p = 0.09$). The analysis of the group factor revealed that the NMDA + duct-ligation group drank significantly less water than any other group (NMDA + duct-ligation vs. NMDA + subm./subl., $p<0.0001$; NMDA + duct-ligation vs. Control + duct-ligation, $p<0.0001$; NMDA + duct-ligation vs. Control + subm./subl., $p<0.0001$). However, neither the comparison of NMDA + subm./subl. vs. Control + subm./subl. groups ($p = 0.59$) nor of the comparison Control + duct-ligation vs. Control +subm./subl. groups ($p = 0.99$) showed significant differences.

The results show an increase in the consumption of water only in animals that had both a lesion to the SSN and duct-ligated parotid glands (NMDA + duct-ligation group). In this case the desalivation experienced by the animals must be almost total, given that the brain lesion affected the SSN, which controls the submandibular-sublingual glands, and the duct-ligation

suppressed all parotidic secretion. Previous studies in our lab and others have shown that partial desalivation does not cause a significant increase in the intake of water associated with the intake of dry food. So, for secondary hyperdipsia to develop following a deficit in saliva secretion, it is necessary to affect, in general, all three pairs of salivary glands [38, 44, 46, 47, 52, 53]. According to the foregoing, in the NMDA + duct-ligation group, the cerebral lesions must have profoundly reduced the secretion of the submandibular-sublingual glands, otherwise the animals would not have developed polydipsia. Taken together, only in the NMDA + duct-ligation group is there a combination of central and peripheral interventions that result in total desalivation. In the rest of the groups the desalivation caused is only partial, as one pair of salivary glands remains completely functional (either submandibular-sublingual or parotid glands). In the latter cases the saliva present, although lesser in quantity, is still enough for the animals to be able to swallow the food satisfactorily [44, 45] and the onset of hyperdipsia is therefore unnecessary. Thus, the present data can be taken as behavioral proof that the cell bodies lesioned in the lateral reticular formation constitute the SSN.

3. <u>Experiment 2</u>: *Parasympathetically-mediated submandibular-sublingual salivation after activation of salivatory somata: effect of cholinergic and adrenergic-receptors blockade.*

The aim of the present exp. was to investigate the parasympathetic versus sympathetic nature of submandibular-sublingual salivary secretion induced following lateral reticular formation activation. The salivary glands are doubly innervated, as they are controlled by both parasympathetic and sympathetic fibers. The SSN controls submandibular-sublingual glands through efferent preganglionic parasympathetic fibers that are incorporated to the chorda tympani. In rats peripheral stimulation of these salivatory nerves produces a potent hypersecretory effect, which is blocked by muscarinic antagonists such as atropine [54-57]. Sympathetic secretion, on the other hand, is mediated in rats by both α- and β-adrenergic receptors [56, 58]. In this case the salivary secretion observed following stimulation of the superior cervical ganglion is completely abolished by the simultaneous blockade of α- and β-adrenergic glandular receptors [56, 59, 60]. Based on the foregoing, we hypothesized that the

blockade of the cholinergic receptors of the submandibular-sublingual salivary glands would abolish the saliva hypersecretion observed after the microinjection of NMDA into the SSN. The blockade of adrenergic receptors, however, should not affect the secretory effect at all.

3.1. Methods

3.1.1. Subjects

Twenty-one male Wistar rats from Charles River Laboratories (France) were used in this experiment. Three rats died following the neurotoxic lesions and were therefore excluded. In order to dissociate between the three classically autonomic neurotransmitter receptors described in the submandibular-sublingual salivary glands (α-adrenergics, β-adrenergics and muscarinic-cholinergics), the animals were divided randomly into three groups. In the first group, prior to the NMDA microinjection into the SSN the animals were administered i.p. buffer (NMDA + buffer, n = 5). In the second group, prior to the NMDA microinjection the animals were given i.p. α- and β-adrenergic receptor antagonists (NMDA + dihydroergotamine/propranolol, n = 7). Last of all, in the third group, an antagonist of the cholinergic receptors was administered i.p. prior to the NMDA microinjection in the brainstem (NMDA + atropine, n = 6). The housing conditions were identical to those described in exp. 1.

3.1.2. Surgery

The conditions of analgesia and anesthesia were identical to those described in exp. 1. Once all the animals were anesthetised the parotid ducts were ligated bilaterally to ensure that in all the rats the saliva collected in the mouth following the NMDA microinjection came exclusively from the submandibular-sublingual glands. About 15 minutes later, once the animal had been placed on the stereotaxic apparatus, the stereotaxic surgery was performed. The surgical procedure followed in both interventions, parotid duct ligation and stereotaxic surgery, was identical to that described in exp. 1.

3.1.3. Procedure

During the stereotaxic surgery, specifically 15 min before NMDA microinjection into the SSN, the rats in each group received the following drugs intraperitoneally:

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 the 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 submandibular-sublingual saliva secretion [27, 56, 57, 60].

The procedure used to collect the S-S saliva from the oral cavity, during the hour following the activation of the SSN (1, 20, 40 and 60 min), was the same as followed in exp. 1. Baseline saliva secretion was also measured in each rat immediately before lowering the cannula into the brainstem.

3.1.4. Histology

The procedures were the same as those followed in exp. 1, but on this occasion micrographs were obtained using a Nikon bellows PB-6 for microphotography and a digital Nikon D700 camera. The images were developed digitally using Adobe Photoshop, version 13.0 x 64, 2012.

3.1.5. Data analyses

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

3.2. Results

3.2.1. Histological findings

The localization and extension of the lesions was practically identical to exp. 1 and no important differences were observed between groups in the size and distribution of the lesions. Figure 3 shows a detailed representation of the extension of the NMDA neurotoxic lesions.

3.2.2. Short-term saliva secretion

During the baseline period the three groups secreted similar amounts of saliva ($F_{2, 15} = 0.33$, $p = 0.72$, range 3.2 to 4.9 mg). Figure 4 shows the magnitude of submandibular-sublingual 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 ($F_{2, 15} = 35.63$, $p<0.0001$, $\eta^2_p = 0.82$), time factor ($F_{3, 45} = 25.25$, $p<0.0001$, $\eta^2_p = 0.62$) and group x time interaction ($F_{6, 45} = 6.47$, $p<0.0001$, $\eta^2_p = 0.46$). Analysis of the interaction using Tukey tests revealed that only atropine administration blocked hypersecretion. Specifically, upon comparing the NMDA + atropine group vs. NMDA + buffer group, the latter secreted significantly more saliva 1 min ($p<0.0001$) and 20 min ($p<0.0001$) after NMDA microinjection, but not 40 min ($p = 0.18$) or 60 min ($p = 0.15$) after it. Similarly, the NMDA + atropine group secreted significantly less saliva than the NMDA + dihydro/propranolol group 1 ($p<0.0001$) and 20 min ($p<0.001$) after, but not 40 ($p = 0.20$) or 60 min ($p = 0.48$) after NMDA microinjection. Finally, no significant differences were detected between NMDA + buffer vs. NMDA + dihydro/propranolol groups in any of the time periods measured (p from 0.74 to 1.0).

These results support the parasympathetic nature of the submandibular-sublingual salivary secretion observed following lateral reticular formation activation with NMDA neurotoxin. These results, together with those of exp. 1, serve to confirm functionally that the cell bodies activated following NMDA application correspond to and are part of the SSN.

4. Discussion

In the present study we used NMDA-neurotoxin to activate/lesion the cell bodies of the parvocellularis lateral reticular formation in rats, the proposed SSN, in order to demonstrate functionally that this region is secretory in nature. Results indicate a high secretion of submandibular-sublingual saliva during the hour after the microinjection of NMDA and a change in food-associated drinking behavior 16 days later. In relation to the latter, the animals exhibit hyperdipsia associated with the intake of dry food, but the hyperdipsia disappears in the presence of wet food. In the second experiment, the administration of atropine, but not the administration of dihydroergotamine plus propranolol, blocks the secretion of saliva induced by the activation of the n. parvocellularis, thus confirming the cholinergic/parasympathetic nature of hypersalivation.

The findings provide physiological and behavioral evidence that the somata of the parvocellular lateral reticular formation constitute the SSN. Indeed, the area of the brainstem affected in our experiments corresponds closely to the region that has repeatedly been identified anatomically as the SSN using modern tracing methods in the rat and other rodents [10, 12, 13, 18-21, 61, 62], cat [14, 16, 17], dog [9], rabbit [11] and monkey [15]. In addition, it is well known that the SSN controls only submandibular-sublingual salivation, not parotid salivation [55]. In relation to the foregoing, the results of exp. 1 show submandibular-sublingual salivary hypersecretion but minimal, yet significant, salivation from the parotid glands. The fact that activation of the SSN in our study evokes a certain amount of parotid secretion might be explained by an overlap of the somas of the superior and inferior salivatory nuclei. In support of this possibility, anatomical evidence has shown that the rostral portion of the inferior salivatory nucleus reaches the central/caudal region of the SSN, although at this level the density of inferior somata observed is lower than the density of superior salivatory cells [51]. Additionally, one study demonstrated that inferior salivatory neurons express NMDA receptors with the same characteristics as those observed in superior salivatory cells [63]. Therefore, the differential secretory activity registered in submandibular-sublingual vs. parotid glands in our exp. 1 agrees

with existing anatomical and neurochemical data and, furthermore, it functionally supports the proposal that the brainstem lateral reticular formation corresponds closely to the SSN.

The procedure we have used to produce hypersecretion of saliva in exp. 1 contrasts with the procedure used in classic physiological studies, which have not been able to functionally identify the precise location of the salivatory centers [11, 27-31]. In these classic studies electrical stimulation was applied in numerous regions of the medulla and saliva secretion was observed, mainly, along a band of neural tissue beginning dorsomedially to the genu of the facial nerve and extending laterally to the spinal nucleus of the trigeminal nerve [27, 28, 31]. Other authors have also found salivary secretion following electrical stimulation of points within the lateral reticular formation [11, 27]. However, the latter are not at all conclusive since salivary secretion may be provoked by direct stimulation of the efferent fibers from the salivatory nuclei and/or the afferent fibers connected to the salivatory centers [32, 33]. With respect to the first possibility, it is important to bear in mind that the efferent axons from the salivatory cells of the SSN course dorsally within the reticular formation until they form a genu located laterally to the somatomotor genu [12, 19]. They then travel ventrally along the medial and ventral borders of the spinal nucleus of the trigeminal nerve to exit the brainstem just ventral to the incoming afferents of the chorda tympani [19]. Therefore, the fact that in some of the aforementioned classic studies the points at which saliva hypersecretion was observed coincide with the trajectory of the efferent fibers from the SSN makes it difficult to functionally determine the precise location of the SSN. It is also important to consider, in studies using electrical stimulation, that due to the high diameter of the electrode in comparison with the size of the salivatory cell bodies (between 7-40 μm diameter, according to Hiura and Mitchell & Templeton [61, 12, respectively], it is possible that efferent fibers, dendrites or afferent axons of the cell bodies are being stimulated [27].

In relation to the possibility of evoking salivary secretion after electrical stimulation of afferent fibers to the SSN, some studies have shown saliva hypersecretion after stimulation of afferent gustatory fibers or afferent somatosensory oral fibers [14, 36, 64]. In addition, other

studies have shown using electrophysiological methods, that both gustatory and oral somatosensorial information converges in SSN cells [25, 26]. This is why 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 [55]. In the present series of experiments, however, we have eliminated the possibility of activating afferent fibers to the SSN, by applying an agonist of NMDA-receptors into the parvocellular reticular formation which affects only the cell bodies [35]. In effect, a high number of salivatory neurons express NMDA-receptors in their soma [40, 41]. The activation of these receptors evokes an excitatory response in SSN neurons [42, 43]. For this reason, as far as we know, the present study is the first to activate exclusively the cell bodies of the superior salivatory neurons, thus allowing the precise functional location of the SSN to be determined. An additional benefit of the method used in our study is that hours after injecting NMDA-neurotoxin into the SSN, salivatory cells are permanently lesioned, which allows us to examine long-term changes in drinking behavior due to a deficit in saliva secretion.

In the long term, the results indicate that of the four groups used in exp. 1, only the animals belonging to the NMDA + duct-ligation group developed polydipsia during the 2-h period in which they were exposed to dry food. The explanation for this could be that in these animals the degree of desalivation is greater than in the rest. In effect, these animals present a complete deficit in parotid secretion, caused by the parotid duct-ligation performed prior to the brain surgery, and in addition they present a deficit in submandibular-sublingual secretion due to the neurotoxic lesions of the n. parvocellularis. In the three other groups, the animals had only a partial saliva deficit that affects just the submandibular-sublingual glands (NMDA + subm./subl.; Control + subm./subl.) or the parotid glands (Control + duct-ligation). The fact that these last three groups do not exhibit polydipsia is in agreement with previous studies that have shown that partial desalivation (either of the submandibular-sublingual or of the parotid glands) does not induce a prandial style of drinking and so the typical associated polydipsia does not appear [37, 46, 47]. Therefore, in the animals of the NMDA + duct-ligation group the high

water intake arises from the use of water to facilitate the swallowing of dry food in the absence of saliva [44, 45, 65-67]. Proof of this is that this style of drinking and its associated polydipsia disappear completely in the presence of wet food (experimental day 18), suggesting that the polydipsia developed by the experimental rats of the NMDA + duct-ligation group is of non-homeostatic nature. In support of the non-homeostatic nature of the polydipsia it should also be noted that the high intake of water during the 2-h period of dry food was completely reversed during the following 22-h period of food deprivation on experimental days 16 and 17, when the experimental rats of the NMDA + duct-ligation group show hypodipsia in comparison with the rest of the groups. Taken together, this behavioral data lead us to the conclusion that the neurotoxic lesions of the nucleus parvocellularis really are damaging a secretory center directly involved in the control of the submandibular-sublingual salivary glands, that is, the SSN.

With respect to exp. 2, the results obtained indicate that the parvocellularis nucleus controls the submandibular-sublingual glands via parasympathetic nerve pathways whose main neurotransmitter is acetylcholine [56]. In effect, the cholinergic blockade of the submandibular-sublingual glands with atropine eliminates all saliva secretion evoked by overactivation of the nucleus parvocellularis. As for the joint blockade of the receptors α- and β-adrenergics of the glands, it does not reduce the secretion evoked after NMDA-microinjection in the slightest, despite other authors having shown that this same treatment is capable of completely blocking sympathetic salivary secretion after superior cervical ganglion stimulation [59, 60]. These data are in agreement and replicate previous results obtained in our lab using electrolytic lesions targeting the parvocellular nucleus [68, 69]. The present study, however, takes it one step further, by demonstrating that the salivation evoked by activating the parvocellular region is due exclusively to the activation of cell bodies and not to efferent or afferent fibers connected to the SSN.

In summary, in the present study we have, for the first time, specifically activated/lesioned the cell bodies of the brainstem region proposed as the SSN based on anatomical investigations. Our data, viewed from a functional perspective, suggest that the somata of the parvocellular

reticular formation control the secretory activity of the submandibular-sublingual salivary glands via a cholinergic pathway and they therefore constitute the SSN.

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Data availability:

Data will be made available on request.

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Table 1
Drinking behavior

1A) Water intake (mean ± SEM) during the 2-h period of dry food on days 16 and 17

Group	Day 16	Day 17
NMDA + duct-ligation	25.16 (±2.62)**	24.38 (±4.64)**
NMDA + subm./subl.	11.00 (±0.49)	10.78 (±0.43)
Control + duct-ligation	13.91 (±2.04)	13.50 (±1.62)
Control + subm./subl.	10.21 (±0.52)	10.04 (±0.36)

1B) Water intake (mean ± SEM) during the 2-h period of wet food on day 18

Group	Day 18
NMDA + duct-ligation	3.90 (±0.63)
NMDA + subm./subl.	4.85 (±0.58)
Control + duct-ligation	3.94 (±0.86)
Control + subm./subl.	2.97 (±0.69)

1C) Water intake (mean ± SEM) during the 22-h of privation of food, days 16 and 17

Group	Day 16	Day 17
NMDA + duct-ligation	4.38 (±0.56)**	4.15 (±0.55)**
NMDA + subm./subl.	12.88 (±0.51)	12.97 (±0.61)
Control + duct-ligation	11.25 (±1.42)	12.52 (±0.83)
Control + subm./subl.	12.64 (±0.84)	10.88 (±0.49)

Groups:
- NMDA + duct-ligation = NMDA microinjection + duct-ligated parotid glands.
- NMDA + subm./subl. = NMDA microinjection + submandibular-sublingual glands extirpated.
- Control + duct-ligation = buffer microinjection + duct-ligated parotid glands.
- Control + subm./subl. = buffer microinjection + submandibular-sublingual glands extirpated.

**$p<0.0001$

FIGURE 1

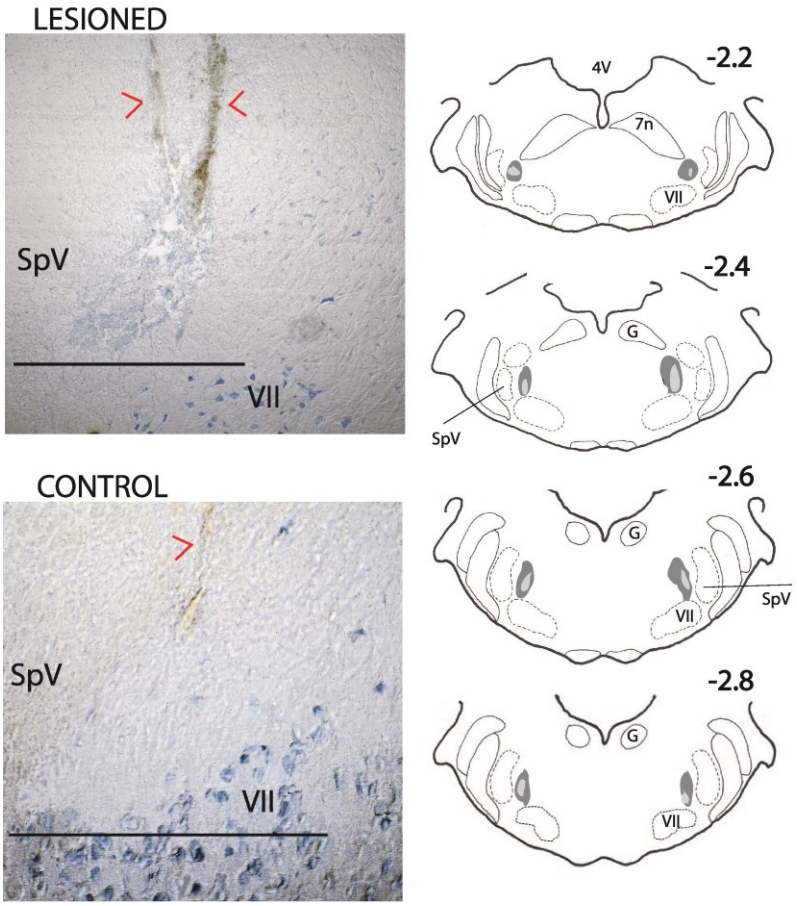

Figure 1. Histology exp. 1. *Left*: Photomicrographs of coronal sections from a representative lesioned and control rat. In the lesioned rat the lesion can be seen just dorsal to the nucleus of the facial nerve. The horizontal bar equals 1 mm. The arrows (red) mark the trajectory of the cannula in its descent towards the nucleus to perform each of the lesions in that hemisphere. *Right*: Coronal sections showing the largest (grey) and smallest (clearer central area) superior salivatory nucleus lesions. Abbreviations: G, genu of the facial nerve; 4V, ventricle; SpV, spinal nucleus of the trigeminal nerve; VII, nucleus of the facial nerve; 7n, descending root of the facial nerve. AP coordinate with reference to the auditory meatus according to the atlas of Pellegrino et al. [48].

FIGURE 2

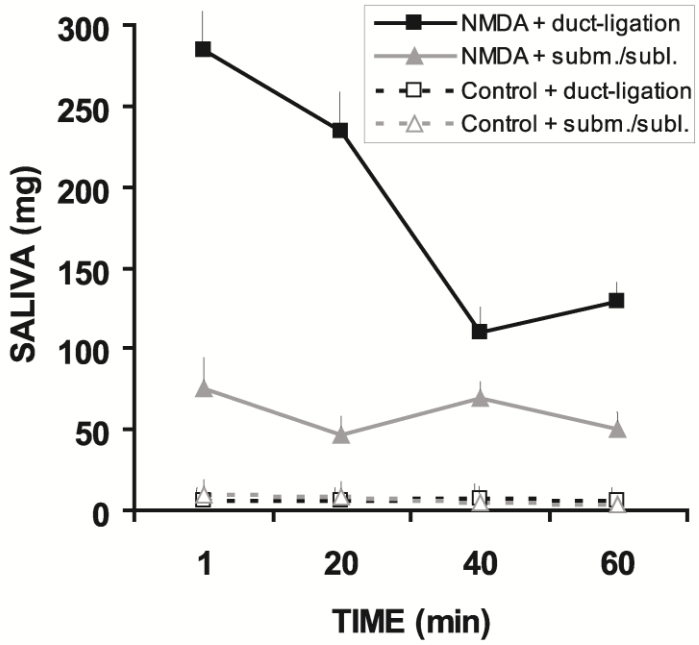

Figure 2. Saliva hypersecretion: Mean amount (±SEM) of submandibular-sublingual and parotid saliva secreted in experimental and control groups during the hour following NMDA or buffer microinjection, respectively, into the nucleus parvocellularis of the lateral reticular formation. NMDA + duct-ligation (NMDA microinjection + duct-ligated parotid glands), n = 8; NMDA + subm./subl. (NMDA microinjection + submandibular-sublingual glands extirpated), n = 8; Control + duct-ligation (buffer microinjection + duct-ligated parotid glands), n = 7; Control + subm./subl. (buffer microinjection + submandibular-sublingual glands extirpated), n = 7.

FIGURE 3

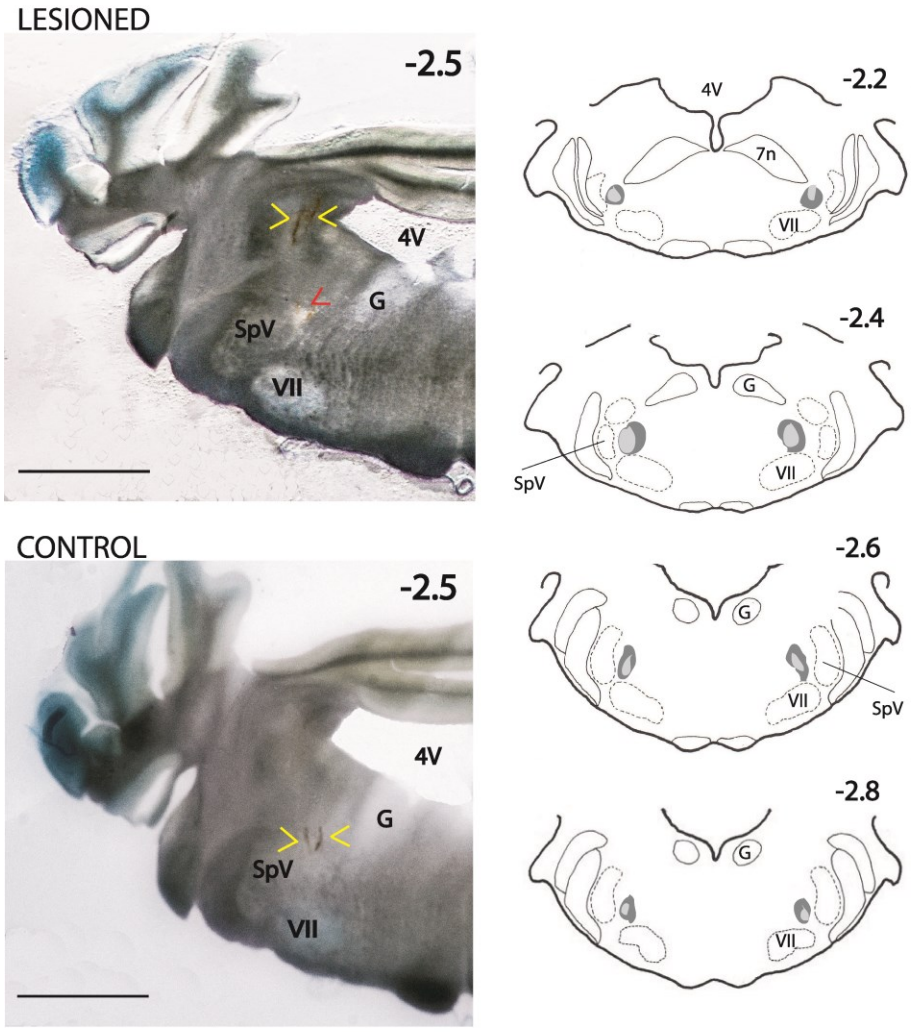

Figure 3. Histology exp. 2. *Left*: Photomicrographs of coronal sections from a representative lesioned and control rat. The horizontal bar equals 1 mm. The two arrows (yellow) mark the trajectory of the cannula in its descent towards the nucleus to perform each one of the lesions in that hemisphere. *Right*: Coronal sections showing the largest (grey) and smallest (clearer central area) superior salivatory nucleus lesions. Abbreviations: G, genu of the facial nerve; 4V, ventricle; SpV, spinal nucleus of the trigeminal nerve; VII, nucleus of the facial nerve; 7n, descending root of the facial nerve. AP coordinate with reference to the auditory meatus according to the atlas of Pellegrino et al. [48].

FIGURE 4

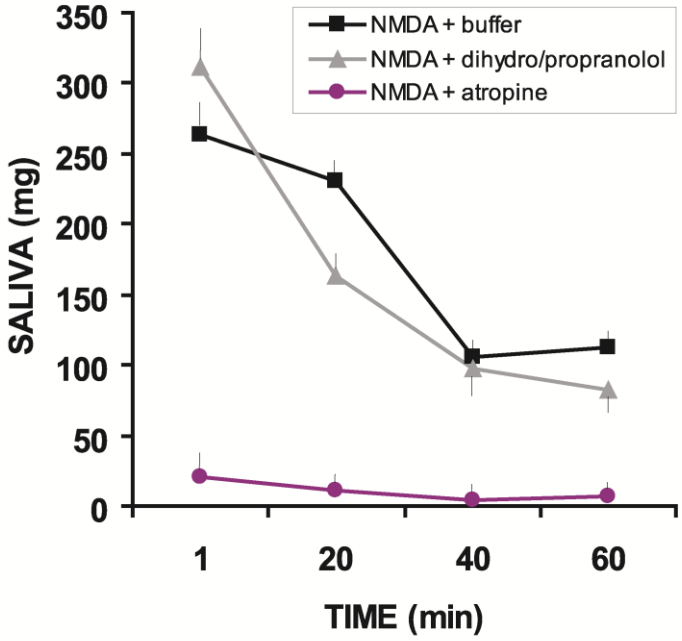

Figure 4. Saliva hypersecretion: In all the animals the ducts of the parotid glands were ligated prior to the stereotaxic surgery. The data indicate the mean amount (±SEM) of submandibular-sublingual saliva secreted in the three groups used in exp. 2 during the hour following NMDA microinjection to the nucleus parvocellularis of the lateral reticular formation. NMDA + buffer (NMDA microinjection + buffer i.p.), n = 5; NMDA + dihydro/propranolol (NMDA microinjection + dihydroergotamine plus propranolol i.p.), n = 7; NMDA + atropine (NMDA microinjection + atropine i.p.), n = 6.