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## Central Evidence That Neuronal Nitric Oxide and Muscarinic Receptor Influence the Salivary Secretion Induced by Pilocarpine

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**Abstract:** In this study we focused on the effect of injection of 7-nitroindazol (7NI) a neuronal nitric oxide synthase inhibitor (nNOSI), L-arginine (LA) a nitric oxide donor agent and atropine a muscarinic receptor antagonist, on the salivary secretion, induced by pilocarpine injection into antero ventral third ventricle (AV3V). Rats were anesthetized with 2,2,2-tribromoethanol (200 mg kg<sup>-1</sup> b. wt.) and a stainless steel cannula was implanted into their AV3V. The amount of saliva secretion was studied over a ten minute period after injection of pilocarpine into AV3V. Injection of pilocarpine (10, 20, 40, 80, 160 µg/0.5 µL) into AV3V produced a dose-dependent increase in salivary secretion. 7-NI (40 µg/0.5 µL), was injected into AV3V prior to the injection of pilocarpine produced an increase in salivary secretion due to the effect of pilocarpine. LA (30 µg/0.5 µL) was injected into AV3V prior to pilocarpine attenuated the increase in salivary secretion induced by pilocarpine. The injection of atropine (20 µg/0.5 µL) prior to pilocarpine blocked the sialogogue effect of it. All these roles of pilocarpine depend on the release of nitric oxide into the AV3V and circumventricular structures of central nervous system that are implicated in the control of hydroelectrolytic balance of the body. We may also conclude that these structures are involved with the cholinergic excitatory mechanism that induces salivary secretion by implication of muscarinic receptors.

**Key words:** Pilocarpine, nitric oxide, atropine, AV3V, salivation

### INTRODUCTION

Involvement of the central nervous system in the salivary secretion induced by pilocarpine in rats has been demonstrating<sup>[1]</sup>.

Pilocarpine, a muscarinic cholinergic agonist, induces copious salivation when administered systemically, simulating an activation of the parasympathetic system<sup>[2]</sup>. Pilocarpine HCL stimulates labial (minor) salivary gland flow in patients with Sjögren's syndrome<sup>[3]</sup>. On the other hand, stimulation induced by pilocarpine prior to irradiation led to a marginal degranulation of the parotid gland and protected only 13% of it<sup>[4]</sup>. Recent studies of our laboratory investigated the effect of cholinomimetic agonist pilocarpine injected into 3rd V on salivary secretion of rats with anteroventral third ventricle (AV3V) electrolytic lesion demonstrating that the central nervous

system, particularly the AV3V region, is important for the effect of pilocarpine on salivary secretion in rats<sup>[1]</sup>. Both the histochemical and functional results have suggested that nitric oxide (NO) plays an excitatory role in the regulation of parasympathetic nerve inducing salivary secretion in the submandibular gland of rats<sup>[5]</sup>. L-NG-nitro-arginine-methyl-ester (L-NAME), a nitric oxide synthase inhibitor, increased the salivation induced by pilocarpine<sup>[6]</sup>. NO plays an important role in the hydromineral regulation<sup>[7]</sup>. However, the role of NO and its interaction with muscarinic receptor of AV3V region on saliva secretion and the effects of pilocarpine have not been studied before. The aim of this study was to evaluate whether NO found in the region of AV3V interferes with the sialogogue effect of pilocarpine interacting with muscarinics receptors.

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## MATERIALS AND METHODS

**Experimental protocol:** The study of salivary flow measurement was started 5 days after the brain surgery. Each animal was submitted to 3 or 4 experimental sessions at 3 day intervals. These parameters were obtained from different experimental sessions and from several groups of animals after the injection of the following drugs into the AV3V.

1. Saline 0.15 M NaCl (control)
2. Pilocarpine (10, 20, 40, 80 and 160  $\mu\text{g}/0.5 \mu\text{L}$ )
3. 7-nitroindazol (40  $\mu\text{g}/0.5 \mu\text{L}$ ), injected prior to pilocarpine
4. L-arginine (30  $\mu\text{g}/0.5 \mu\text{L}$ ), injected prior to pilocarpine
5. Atropine (20  $\mu\text{g}/0.5 \mu\text{L}$ ) injected prior to pilocarpine and 7NI

**Animals:** Male Holtzman rats (250-300 g) were housed in individual metabolic cages, with free access to food pellets and tap water.

**Brain surgery:** The rats were anaesthetized with 2,2,2-(200 mg  $\text{kg}^{-1}$  b.wt., intraperitoneal) and restrained in a stereotaxic apparatus (David Kopf model for rats). A stainless steel cannula (14x0.7 mm o. d.) was introduced into the AV3V. The skull was positioned using bregma and lambda at the same level. The coordinates for approaching the AV3V were obtained from Paxinos and Watson atlas<sup>[9]</sup>.

**Drug injection:** The pilocarpine, 7-nitroindazol, L-arginine, atropine and NaCl 0.15 M (as control) were injected into the AV3V by using a Hamilton micro syringe (5  $\mu\text{L}$ ) connected by a PE 10 polyethylene tubing (25 cm) to a needle (0.3 mm o. d.) which was introduced into the brain through the cannula previously fixed to the animals' head. The volume of injection was always 0.5  $\mu\text{L}$  injected over a period of 30-60 sec.

**Salivary secretion:** Salivary flow was stimulated by pilocarpine (10, 20, 40, 80 and 160  $\mu\text{g} \mu\text{L}^{-1}$ ) injected into AV3V. The animals were anaesthetized with ketamine 1.25 g  $\text{Kg}^{-1}$  b. wt. intraperitoneally (i.p.). Saliva was collected with pre weighed small cotton wool balls inserted into the animals' mouth, a technique slightly different from that used by Schallert<sup>[9]</sup>. Such technique led us to collect the whole saliva. Saliva was collected with four cotton balls weighing approximately 20 mg each, two of which were placed on either side of the oral cavity, with the other two placed under the tongue. The amount of saliva secreted was measured 10 min before the injection

of pilocarpine (baseline saliva secretion) and 5 min after the injection of pilocarpine (stimulated salivary secretion). 7NI, LA and atropine were injected into the AV3V 5 min before pilocarpine.

**Statistical analysis:** The results are reported as mean $\pm$ SEM. The ANOVA and Dunnett's test were used to determine the significance. The values were considered statistically significant when  $p < 0.05$ .

**Histology:** After the experiments, the animals were anaesthetized with ether and perfuse through the heart with saline and 10% formalin. The brain was removed and stored in 10% formalin for at least 1 week. The brain was then frozen and the coronal section (20-30  $\mu\text{m}$ ) was cut and stained with hematoxylin-eosin for examination with a light microscope. Only the results of animals who's AV3V were reached by the injections were used for data analyses.

## RESULTS AND DISCUSSION

**Effect of treatment with 7NI, LA and atropine on salivary secretion, induced by injection of pilocarpine into AV3V:** The basal levels of saliva secretion after injection of NaCl 0.15 M as a control range from  $5 \pm 2$  to  $18 \pm 7$  mg/10 min. The injection of pilocarpine (10, 20, 40, 80, 160  $\mu\text{g} \mu\text{L}^{-1}$ ) induced a dose-dependent increase in the salivary secretion with values of  $28 \pm 13$ ,  $44 \pm 18$ ,  $121 \pm 16$ ,  $219 \pm 14$  and  $365 \pm 10$  mg/10 min, respectively. ANOVA showed significant differences among all doses  $F(4,28) = 44.17$ ,  $p < 0.01$ . 7NI (40  $\mu\text{g}/0.5 \mu\text{L}$ ) injected prior to pilocarpine produced an increase in this sialogogue effect of pilocarpine with values of  $43 \pm 15$ ,  $82 \pm 12$ ,  $172 \pm 17$ ,  $327 \pm 16$  and  $449 \pm 18$  mg/10 min,  $F(4,28) = 35.22$ ,  $p < 0.01$ . LA (30  $\mu\text{g}/0.5 \mu\text{L}$ ) injected prior to pilocarpine attenuated this sialogogue effect of pilocarpine with values of  $19 \pm 11$ ,  $31 \pm 10$ ,  $52 \pm 12$ ,  $98 \pm 14$  and  $139 \pm 12$  mg/10 min. ANOVA showed significant differences among those various doses  $F(4,28) = 39.39$ ,  $P < 0.01$ . Atropine (20  $\mu\text{g}/0.5 \mu\text{L}$ ) injected prior to pilocarpine blocked the sialogogue effect of pilocarpine with values of  $19 \pm 11$ ,  $21 \pm 12$ ,  $22 \pm 14$ ,  $28 \pm 15$  and  $29 \pm 12$  mg/10 min. ANOVA showed significant differences among those various doses  $F(4,28) = 39.39$ ,  $p < 0.01$ . Atropine plus 7NI injected prior to pilocarpine blocked the effect of 7NI on pilocarpine sialogogue action with values of  $32 \pm 14$ ,  $39 \pm 16$ ,  $99 \pm 13$ ,  $198 \pm 17$  and  $331 \pm 12$ . ANOVA showed significant differences among those various doses  $F(4,28) = 421.23$ ,  $p < 0.01$  (Fig. 1).

The present results show that the injection of pilocarpine into the AV3V affects the salivary flow in a dose-dependent manner. The dose of 40  $\mu\text{g} \mu\text{L}^{-1}$

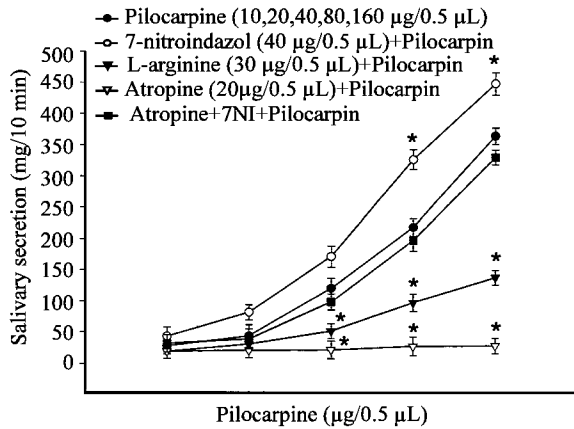


Fig. 1: Effects of 7-nitroindazol, L-arginine and atropine on salivary flow during injection of pilocarpine into AV3V. Results are reported as mean  $\pm$  SEM. \* $p < 0.001$  different from Pilocarpine

produced a medium salivary flow. It has been demonstrated that pilocarpine, when injected intracerebroventricularly produced salivary secretion at a level significantly different from that of control<sup>[11]</sup>. It has been also noticed that the electrolytic lesion of the AV3V produced a decrease in salivary flow induced by pilocarpine injected intracerebroventricular (i.c.v)<sup>[11]</sup>. The present results showed the participation of the areas of the central nervous system in attenuating the decrease in salivary secretion, demonstrating that a controlled-release form of pilocarpine may overcome the therapeutic weaknesses of current pilocarpine preparation by prolonging salivary secretion and reducing undesirable side effects. These results are important, since several medicines used by patients with cardiovascular diseases may alter salivary secretion and interfere with the effects of pilocarpine when it is used as a medicine. The recognition of the role of nitric oxide in cell-to-cell communication has changed the concept of traditional neurotransmission. N-methyl-D-aspartate receptors mediate dipsogenic response *c-Fos* expression induced by i.c.v. infusion of angiotensin II<sup>[9]</sup>. The presence of nitric oxide in many structures of the central nervous system has been described<sup>[10-13]</sup>. Nitric oxide centrally or systemically plays an important role in the regulation of MAP, heart rate and water and electrolyte excretion. During aphagia after lateral hypothalamic lesion it has been demonstrating saliva hypersecretion<sup>[14]</sup>. Nitric oxide, superoxide and hydrogen peroxide production in brain mitochondria after haloperidol treatment has been demonstrated<sup>[15]</sup>. 7NI inhibits the cerebellum nitric oxide synthase (NOS) in rats<sup>[13]</sup>. I.c.v. injection of 7NI demonstrated that NO of the

paraventricular neurons of the hypothalamus, has important role in the central regulatory mechanisms such as the temperature regulation<sup>[9]</sup>. Pharmacodynamic and pharmacokinetics studies of the 7NI also proved the pharmacological NO participation in the CNS<sup>[16]</sup>. L-NAME, increased the salivary flow induced by pilocarpine<sup>[6]</sup>. The present results showed that 7NI when injected into the AV3V previously to pilocarpine injection increased the salivary flow induced by pilocarpine. NO contributed to the control of vascular tone in salivary glands of rats<sup>[7]</sup>. NO also played an important role in the central and peripheral effects of pilocarpine. This data was confirmed by the results using LA that produce a decreased in the saliva flow induced by pilocarpine. The muscarinic receptors are implicated in this mechanism. This affirmation is supported by the findings utilizing the atropine, which blocked totally the siologogue effect of pilocarpine. AV3V region play an important role in the central mechanism that regulated salivary secretion, inhibiting or releasing NO. Our results are supported by the research of Vaucher *et al.*<sup>[18]</sup> and Saad *et al.*<sup>[19]</sup>. The main finding of this study is that 7NI has more effective effect on pilocarpine induced increase in salivary flow than L-NAME that is demonstrated by Saad *et al.*<sup>[20]</sup>. These results allowed us to infer that in the central area of the brain the neuronal NO plays a more important role than endothelial NO in the regulation of salivary secretion induced by pilocarpine. Other important finding is the interference of muscarinic receptors on the NO effect.

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