RESEARCH

***In vivo* blockade of ovarian sympathetic activity by Neosaxitoxin prevents polycystic ovary in rats**

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Abstract

A high sympathetic tone is observed in the development and maintenance of the polycystic ovary (PCO) phenotype in rats. Neosaxitoxin (NeoSTX) specifically blocks neuronal voltage-dependent Na⁺ channels, and we studied the capacity of NeoSTX administered into the ovary to block sympathetic nerves and PCO phenotype that is induced by estradiol valerate (EV). The toxin was administered with a minipump inserted into the bursal cavity using two protocols: (1) the same day as EV administration and (2) 30 days after EV to block the final step of cyst development and maintenance of the condition. We studied the estrous cycling activity, follicular morphology, steroid plasma levels, and norepinephrine concentration. NeoSTX administered together with EV decreased NA intraovarian levels that were induced by EV, increased the number of corpora lutea, decreased the number of follicular cyst found after EV administration, and decreased the previously increased testosterone plasma levels induced by the PCO phenotype. Estrous cycling activity also recovered. NeoSTX applied after 30 days of EV administration showed near recovery of ovary function, suggesting that there is a specific window in which follicular development could be protected from cystic development. In addition, plasma testosterone levels decreased while those of progesterone increased. Our data strongly suggest that chronic inhibition of sympathetic nerves by a locally applied long-lasting toxin is a new tool to manage the polycystic phenotype in the rat and could be applied to other mammals depending on sympathetic nerve activity.

Key Words

- ▶ polycystic ovary syndrome
- ▶ Neosaxitoxin
- ▶ norepinephrine
- ▶ ovulation

Journal of Endocrinology
(2020) **244**, 523–533

Introduction

High testosterone plasma levels and the presence of fluid-filled follicles (cysts) that do not ovulate characterize polycystic ovary syndrome (PCOS). PCOS is the most prevalent ovarian pathology in women, affecting 5–12% of women of reproductive age, and it is characterized by many endocrine/metabolic associated pathologies.

The most important component of these disorders is the anovulation, which results in irregular menstruation and infertility. In addition, PCOS patients are associated with having masculinizing effects, which result from the hyperandrogenism. It is known that the aberrant polycystic morphology can be restored to normal

follicular development by the following: (1) suppression of sympathetic activity resulting from surgical resection of the superior ovarian nerve (SON; the principal sympathetic nerve controlling follicular development and steroid secretion (Lara *et al.* 1990a, 1993, Flores *et al.* 2011, Del Campo *et al.* 2019); (2) administration of the β -adrenergic antagonist (Luna *et al.* 2012); and (3) blockade of the nerve activity with a locally implanted microstimulator (Pikov *et al.* 2018). For humans, acupuncture (to block the ovarian sympathetic nerves) was found to reduce the PCOS symptoms (Stener-Victorin *et al.* 2008). Current PCOS treatments include estrogen receptor modulators, gonadotropins, laparoscopic ovarian drilling, weight loss, and exercise (Banaszewska *et al.* 2019). However, these treatments are focused on the symptoms rather than the underlying cause and are moderately effective.

The current PCOS therapies do not represent the brain–ovary connection in the human body. While ovulation is controlled by pituitary-secreted gonadotropins (luteinizing hormone and follicle-stimulating hormone), it is also strongly influenced by the sympathetic neural control (Lara *et al.* 2002). The mammalian ovary receives well-organized innervation of postganglionic sympathetic nerves, which originates from the ovarian ganglion and from the celiac and renal plexi (Burden *et al.* 1985, Curry *et al.* 1985). Sympathetic innervation reaches the ovary via the following two nerves: (1) the ovarian plexus nerve (OPN), which is associated with the ovarian branch of the uterine artery (Neilson *et al.* 1970); and (2) the SON, which is at least partially associated with the ligament of the ovary (Neilson *et al.* 1970, Lawrence & Burden 1980). The SON fibers predominantly innervate the secretory components of the ovary, i.e. interstitial glands and follicles, whereas the OPN fibers are mostly perivascular (Lawrence & Burden 1980). Although there is some variation in the way that sympathetic nerves reach the ovary, no differences have been found in the intraovarian distribution of sympathetic fibers, which is similar in all mammalian species, although the density of the network varies considerably among them (Jacobowitz & Wallach 1967). Norepinephrine is the main neurotransmitter that is present in the ovary (Lara *et al.* 1990b, 2002, Greiner *et al.* 2005). The innervation of the gland has been shown to be involved in the regulation of ovarian-specific functions, such as steroidogenesis and early follicular development (Lara *et al.* 2002, Greiner *et al.* 2005) by activating β -adrenergic receptors that are present in cells of the ovarian follicle.

Transection of the SON, which carries the bulk of the sympathetic innervation to the ovarian endocrine

cells, has been observed to restore estrous cyclicity and ovulation (Barria *et al.* 1993). However, a sustained increase in sympathetic activity by estradiol administration (Lara *et al.* 2000), chronic sympathetic stress (Dorfman *et al.* 2003), or pharmacological β -adrenergic receptor activation (Luna *et al.* 2012) causes a polycystic phenotype in the rat ovary, which, in many aspects, resembles PCOS in women.

In this study, we evaluated the effect of suppressing the sympathetic nerve activity in the ovary by local chronic administration of Neosaxitoxin (NeoSTX), a toxin that specifically blocks the voltage-dependent Na⁺ channels with a high affinity and long half-life (Llewellyn 2006, Alonso *et al.* 2016). NeoSTX was administered using a mini-osmotic pump for 30 days as a potential treatment for PCOS. NeoSTX has been previously shown to produce an effective and reversible block of nerve conduction in many sensorial and autonomic nerves and its successful use as a therapeutic agent to treat multiple pathologies in human and veterinary medicine is extensive and well documented (Rodriguez-Navarro *et al.* 2007a,b, Lobo *et al.* 2015, Manriquez *et al.* 2015, Riquelme *et al.* 2018).

Materials and methods

Study design

All animal studies were performed in accordance with the animal protocols that were approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the Universidad de Chile (UCh), Code CBE2017-05. They also complied with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals).

NeoSTX purification

NeoSTX was isolated and purified in the Membrane Laboratory of the Physiology and Biophysics Department, Faculty of Medicine, Universidad de Chile. NeoSTX is produced by dinoflagellates from the genus *Alexandrium*, and it was purified from shellfish that have accumulated this drug and were collected by our laboratory in the southern macrozone of Chile (Manriquez *et al.* 2015). The structure and purity was tested using high-pressure liquid chromatography with a post column derivatization, as previously described (Zamorano *et al.* 2013, Garcia *et al.* 2015). This preparation has been used in many clinical studies as a local anesthetic over the last 10 years.

Animals

We used Sprague–Dawley female rats from our facilities. The rats were maintained under a 12-h:12-h dark:light cycle at $21 \pm 1^\circ\text{C}$ during all the experiments. They were fed Labdiet rat food (Brentwood, MO, USA), provided with enrichment, and co-housed with two other rats of the same age to maintain normal behavior. In control experiments, 3-month-old rats were hemi-ovariectomized (ULO) during the minipump implantation process or sham operation to eliminate a putative effect of the contralateral ovary on steroid secretion that could modify the hypothalamic activity. Some asymmetry was described between the right and left ovary regarding the hypothalamic control of the ovary (Pastelin *et al.* 2017, Rosas *et al.* 2018). Each ovary has a different capacity to release steroids; thus, a hormonal signal from one ovary could affect the contralateral ovary by altering the hormonal signals that arrive at the ovary via the circulation. This was the reason that all control (either sham-injected or sham-denervated) rats underwent ULO to compare the same conditions in all the groups. In the experiments with the PCOS phenotype (Group 3), rats (24 days old) were injected with estradiol valerate (EV) in a single i.m. dose of 10 mg/kg of EV (Sigma Chemical Co.) dissolved in sesame oil. This age group was selected because we previously demonstrated (Cruz *et al.* 2012) a developmental window in which the administration of a single EV solution injection produced a permanent effect on the ovary. Twenty-four days of age was selected as the age limit for reversion, and the PCO phenotype was established when the rats were killed. Control rats were injected with sesame oil. To eliminate the hypertrophy that was derived in the contralateral ovary after ULO (Pastelin *et al.* 2017, Rosas *et al.* 2018), we used both ovaries in this experiment. Thus, we implanted one minipump in each ovary.

The rats were randomly assigned to the following groups, as described below.

Group 1: Effect of NeoSTX on the ovarian noradrenaline (NA) after transmural stimulation *in vitro*

Rats were divided into NeoSTX and Sham groups. Six adults rats in estrous were killed and the two ovaries were incubated in Krebs Ringer Phosphate buffer gassed with 95% O_2 and 5% CO_2 and incubated at 37°C for 10 min in the presence of 10 mM Tetraethylammonium (TEA), which causes profound facilitation of neurotransmitter release and a significant reduction in tissue NA content

when the organ is electrically stimulated (Wakade 1979, 1980, Lara *et al.* 1985). The left ovary was incubated with 10 nM NeoSTX, and the right ovary was incubated without NeoSTX.

The ovaries were placed in a perfusion chamber for transmural stimulation using a SD5 Grass stimulator (Grass Instruments, Quincy, Mass, USA), with trains of 150 pulses (80 V, 1 ms duration), at 5 Hz every 30 s for 90 min, separately, with infusion (2 mL/min) of the respective buffers. At the end of the stimulation period, the ovaries were harvested for NA determination.

In the basal group, four adults rats in estrous were euthanized and the two ovaries were incubated in TEA-Krebs-ringer-phosphate buffer under the same experimental conditions as the experimental groups for 90 min, separately, before being stored.

Group 2: Chronic effect of NeoSTX on the ovary of adult control rats

Two experimental series were performed. In the first test, ten adult rats were randomized and separated into two groups of five rats (sham and NeoSTX groups). During the surgical process, the right ovary was removed and frozen at -80°C for noradrenaline (NA) determination. The left ovary from the rats in the NeoSTX group was implanted with a osmotic mini pump (Alzet osmotic pump, Model 2004, Durect Corporation Cupertino, CA, USA) filled with a solution of NeoSTX dissolved in saline. The pump delivered 0.2 nmol/day of the toxin for 28 days. The mini pump was inserted with a cannula that was placed directly onto the bursal cavity, cemented with surgical glue (Histoacryl blue, B Braun Surgical, Rubi, Spain), and maintained for 28 days. Sham-operated rats were subjected to the same procedure, but a cannula was inserted in the bursal cavity with no drug. Rats were also killed on day 30 after implantation. The left ovary was used for NA determination.

In the second group, 14 adults rats were randomized and separated into two groups of seven rats each (sham and NeoSTX groups). The same surgical procedure was followed as for the first experimental series, except that plasma from all rats was collected and saved for hormone determination when the rats were killed. The left ovary was halved, and one half was fixed in Bouin's fixative for morphological analysis and the other half was used for NA determination. One of the ovaries per group was lost in the sample processing for morphological analysis.

Group 3: The effect of NeoSTX on the PCO-induced phenotype by estradiol valerate

The objective of this experiment was to determine if blocking NA activity can prevent the development of the PCO phenotype or reverse PCOS that was previously induced by the EV administration in rats. Because of a possible reversion of the EV effect over time after EV (90 days), the rats used in this experiment were injected when they were 24 days old. We have shown that rats injected with EV at this age had no reversion of the PCO phenotype (Cruz *et al.* 2012). Control rats were injected with sesame oil and the EV-treated rats received a single dose of 10 mg/kg of EV (Sigma Chemical Co.) dissolved in sesame oil.

For the study of PCO phenotype prevention, 24-day-old rats were injected with EV. On the same day, an osmotic pump containing NeoSTX was implanted in both ovaries and it delivered the toxin for 28 days. Rats were killed 60 days thereafter.

For the study of PCO phenotype reversion, 24-day-old rats were injected with EV, and after 30 days (rats were 54 days old), an osmotic minipump with NeoSTX was implanted in both ovaries.

These experimental groups were compared with rats that were injected with EV at 24 days old and rats that were injected with sesame oil. In addition, in rats injected only with the vehicle, the mini pump with NeoSTX was placed on day 54 in both ovaries (Table 1). Plasma was collected and the left ovary was preserved in Bouin's fixative to evaluate the morphology and the right ovary was used for NA determination.

Our primary outcome measures were evaluated in the animals from the following experimental groups: Estrous cycle phase, evaluated daily by vaginal cytology; Ovarian morphological phenotype; Ovarian NA concentration; and Steroid plasma levels.

Estrous cycling activity

Estrous cycling activity was assessed daily beginning at puberty to the end of the procedure, and this included

a fertility test. Vaginal fluid was obtained daily using a plastic pipette and it was placed on the glass slide and observed under a light microscope. Epithelial cells (large, round, nucleated), cornified cells (irregular, without nucleus), and leukocytes (small, round) were identified, and their proportion was analyzed to define proestrus during the night of ovulation (Dissen *et al.* 1996), estrus (the day after ovulation), and diestrus (two previous days before P), as previously described (Marcondes *et al.* 2002).

Analysis of follicular structures

One of the halves or the entire ovary was fixed in Bouin's fixative, cut into 6- μ m slices, stained in hematoxylin-eosin, and analyzed. The presence of precystic structures or follicular cyst and corpora lutea was quantified. These structures were dominant in the area that was covered in all the ovaries that were analyzed. The following criteria were defined to quantify follicular structures: cystic follicles were devoid of oocytes and showed a large antral cavity; a well-defined thecal cell layer; and a thin (mostly monolayer) granulosa cell compartment containing apparently healthy cells. Type III follicles have oocytes that are not completely surrounded by granulosa cells, which have discontinuous and uneven layers, with a predominant antrum. The corpora lutea showed the absence of oocytes and the presence of luteal cells instead of granulosa cells (Lara *et al.* 2000).

Quantification of intraovarian NE levels

Quantification of NE levels was performed using the competitive norepinephrine ELISA kit-Research® (IMMUSMOL, Pessac, France), according to the manufacturer's instructions. NE was extracted using a cis-diol-specific affinity gel, acylated, and then derivatized enzymatically. The antigen was bound to the solid phase of the microtiter plate. The derivatized standards, controls, and samples, as well as the solid-phase-bound analyte, competed for a fixed number of antiserum-binding sites. The antibody bound to the solid phase was detected using an anti-rabbit IgG-peroxidase conjugate

Table 1 Experimental groups to study the reversion or prevention of the EV-induced PCO phenotype by *in vivo* local delivery of NeoSTX using mini-osmotic pumps.

N	Age	24 days old	54 days old	84 days old
	Group	Drug administration	Drug administration	Euthanasia
5	Sham	Sesame oil injected	–	EU
5	NeoSTX	Sesame oil injected	NeoSTX	EU
5	EV	EV	–	EU
5	EV + NeoSTX (24 d)	EV + NeoSTX	–	EU
5	EV + NeoSTX (54 d)	EV	NeoSTX	EU

and tetramethylbenzidine (TMB) as a substrate. The reaction is monitored at 450 nm. The sensitivity was 2 pg/mL, and the intra and interassay variability were 8.4 and 8.0%, respectively. The cross-reactivity found was 0.14% for adrenaline and 1.8% for dopamine.

Plasma levels of steroid hormones

Plasma levels of steroid hormones testosterone and progesterone were measured. Serum testosterone and progesterone levels were determined using an EIA, according to the manufacturer's instructions. The catalog number of the test kits were 11-TESHU-E01 and 11-PROHU-E01 for T and P4, respectively (Alpco Diagnostic, Windham, NH, USA). Intra- and interassay variations were less than 6% for T, and less than 5% for P4. The minimal detectable values were 0.02 ng/mL, and 0.1 ng/mL, respectively.

Statistical analysis

Differences between the control and treatment groups were analyzed using a paired *t*-test or unpaired *t*-test, according to the comparison group. For within-animal comparisons, the two-tailed *t*-test was used. Significance was set at $P < 0.05$. The minimum number of rats that were used for the experiments was calculated as described by Zar (Zar 1984). The Shapiro–Wilk test was applied to verify the normal distribution of the data. All statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software).

Results

The effect of NeoSTX on ovarian noradrenaline levels

To determine if NeoSTX blocks ovarian sympathetic activity, we designed an *in vitro* assay in which we incubated rat ovary to apply a transmural train of 150 pulses at 5 Hz every 30 s of 1 min for 90 min (80 V; duration, 1 ms). The acute stimulation in the presence of TEA (a blocker of the K-repolarizing current) was used by Wakade (Wakade 1980) to effectively decrease the NA content in the vas deferens. Stimulation for 90 min decreased the NA content in the ovary by 50% (Fig. 1A). When the same experiment was performed in the presence of NeoSTX (100 nM), there was no decrease in NA content and a small increase in the neurotransmitter (Fig. 1A).

Figure 1B shows the results that were obtained after intraovarian chronic administration of NeoSTX *in vivo*

Figure 1

The effect of NeoSTX on ovarian norepinephrine *in vitro* and *in vivo*. (A) Norepinephrine content in ovaries exposed to transmural stimulation (S), with or without NeoSTX in TEA-Krebs buffer, for 90 min. The basal level corresponds to incubation of ovaries without stimulation. (B, C and D) Norepinephrine content in ovaries that received chronic (28 days) administration of NeoSTX (0.1 µg/day) compared to the sham group; (B) compared to the contralateral ovary that was removed on day 0 (Ov 1) with that removed on day 28 (Ov2); and (C) compared to both ovaries of the sham group (D). * $P < 0.05$, *t*-test.

using an osmotic minipump for 28 days. Rats underwent ULO at time 0 and the ovary was used as a 0 time control either for sham or NeoSTX rats. Ovarian noradrenaline decreased by 30% by the presence of NeoSTX (Fig. 1B). In Fig. 1C and D, the individual response of the ovaries after exposure or no exposure to NeoSTX for 28 days.

The effect of NeoSTX on the changes induced by EV on the ovarian corpora lutea, cyst, and plasma levels of steroid hormones

NeoSTX administered to control adults rats also modified the follicular development. There was an increase in the number of corpora lutea and a decrease in the number of follicular cysts that were normally seen in the control ovaries (Fig. 2A and B). Plasma levels of progesterone were also increased in the ovaries of rats treated with NeoSTX (Fig. 2C). Although NeoSTX reduced the number of cysts, there was no decrease in plasma testosterone levels (Fig. 2D).

The effect of NeoSTX on the development and maintenance of the polycystic phenotype

This experiment was designed to analyze if NeoSTX could prevent (P) or reverse (R) the PCO phenotype that was induced by EV. In this case, we used five groups: (1) Sham; (2) NeoSTX; (3) EV; (4) EV+NeoSTX (to prevent);

Figure 2

Effect of chronic NeoSTX administration over the follicular development and steroid plasma levels in adult control rats. (A and B) Quantification of the follicular morphology of luteal bodies (A) and cysts (B). (C and D) Progesterone (C) and testosterone (D) plasma levels. ** $P < 0.01$, t -test, * $P < 0.05$.

and (5) EV+NeoSTX after 28 days (to reverse). EV or vehicle were administered at 24 days old and the experiment was complete after 60 days (Table 1).

A representative image of the ovaries from EV-treated rats is shown in Fig. 3. In the ovaries of the EV group, there were almost no corpora lutea, but the ovary was filled with follicular cysts. Under conditions in which EV and NeoSTX were used, the most prevalent structure was the corpora lutea.

Figure 4A shows the estrous cycling activity of the EV-injected rats. All rats had an open vagina (a signal of puberty in the rat) between 24 and 26 days old. EV rats did not show a regular estrous cycle (transition from diestrus to proestrus, estrus, and metestrus). In the rats that received NeoSTX administration at 24 days old (when EV was administered), the estrous cycling activity was reversed to an almost regular profile for rats that showed 4–5 days for the length of each cycle during all observation periods. NeoSTX administration

28 days after EV administration (i.e. when the polycystic condition is almost developed) did not restore estrus cycling activity up to when the toxin was applied at 28 days, after which there was a reversion of the acyclic condition to a regular profile. Quantification of the number of regular estrous cycles that occurred during the experimental period is shown in Fig. 4B. After NeoSTX administration, either at the beginning or after 28 days of EV administration, there was a recovery of the estrous cycling activity.

Corpora lutea quantification was also consistent with the ovary image (Fig. 4C). The simultaneous administration of NeoSTX to the EV-treated rats resulted in a greater number of corpora lutea compared to the rats treated with EV only. The same effect was found when NeoSTX was applied 30 days after EV. Follicular cysts decreased when the NeoSTX was simultaneously administered with EV (Fig. 4D). The same result but at a lower magnitude was found when the NeoSTX was administered 28 days after the EV-induced cyst formation, which supports that in this case, the ovary function is oriented toward ovulation and not toward PCO development. Rats treated with NeoSTX had a follicular morphology where the corpora lutea predominated, and it was similar or greater in number than the sham control.

Prevention of PCO by NeoSTX (EV+simultaneous NeoSTX) was accompanied by a significant decrease in the ovarian norepinephrine concentration (Fig. 5A). There was no significant decrease in ovarian norepinephrine when NeoSTX was administered after 30 days of EV administration. Similarly, the simultaneous administration of EV and NeoSTX increased the plasma levels of progesterone (Fig. 5B). A similar situation was found with testosterone plasma levels (Fig. 5C). Testosterone plasma levels decreased when NeoSTX was administered on the same day as EV, but it did not recover when the toxin was administered 30 days after EV (Fig. 5C). Figure 6 shows a proposed model to explain the principal findings of this work.

Figure 3

(C) Morphological appearance of the ovaries treated with NeoSTX to prevent (P) or reverse (R) the cystic phenotype generated by EV. CL, corpus luteum; C, cyst; FTIII, follicle type III. A representative image per group is shown. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-545>.

Figure 4

Effect of chronic NeoSTX administration on the prevention (P) and reversal (R) of the cystic phenotype generated by EV. (A) is a representative profile of the estrous cycle. Estrous cycles were analyzed in 25-day-old rats (from the vaginal opening). EV was administered to all groups. The upper representative pair of rats in the upper part of the (A) correspond to NeoSTX administered to group R (R: reversal) on day 28 after EV and the minipump remained patent for 28 more days. The middle pair of estrous cycles corresponds to NeoSTX that is applied to the group P (P: prevention). NeoSTX was administered together with EV and minipump remained patent for 28 days. On day 84, rats were euthanized (EU). The pair of rats at the bottom represent the sham-operated rats that only received the initial EV administration on day 24 (sham without cannula). Two representative panels are shown per group. P, Proestro; E, Estro; M + D, Metaestro and Diestro. (B) Quantification of the number of estrous cycles in 4 or 5 days per group. (C) Quantification of the number of corpora lutea. (D) Quantification of the number of cysts. ** $P < 0.01$; * $P < 0.05$, *t*-test.

Discussion

In this research, we studied the effect of NeoSTX, a toxin that naturally accumulates in shellfish (Manriquez *et al.* 2015). NeoSTX presents a high affinity for neuronal Na channels that are involved in neuronal depolarization of the rat ovary in the control and PCO phenotype that was induced by EV administration. The NeoSTX acts as a specific

inhibitor of the neurotransmitter release. In the first part of the work, we tested the capacity of the toxin to regulate the sympathetic nerve terminal activity *in vitro* and by chronic exposure *in vivo*. Transmural electric stimulation with supramaximal stimulation led to a decrease in the norepinephrine content by increasing neurotransmitter release. We did experiments in the presence of TEA to block the K⁺-repolarizing channel and, thus, maintain the nerve terminals in the depolarized state so they continued to release more neurotransmitter (Wakade 1979, 1980,

Figure 5

Effect of chronic NeoSTX administration in the concentration of intraovarian NA (A) and on progesterone (B) and testosterone (C) plasma levels when NeoSTX was administered to prevent (P) or to reverse (R) the cystic phenotype generated by EV. ** $P < 0.01$; * $P < 0.05$, *t*-test.

Figure 6

Proposed model: NeoSTX blocks the voltage dependent Na⁺ channel in ovarian neurons and sympathetic nerve activity, and a- prevent the development of PCO phenotype when NA increases as a consequence of EV administration. The second process is when the NeoSTX is applied at the time of development of the first cohort of cystic follicles (30 days after EV), it was able to block cyst production and, thus, reverse the PCO phenotype. The data are supported by the *in vivo* decrease of intraovarian NA, the increase in corpora lutea, and decrease in the cystic follicles that were previously increased by EV. It also increases progesterone and decreases plasma testosterone, which strongly suggests the recovery of follicular steroid synthesis. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-545>.

Lara *et al.* 1985). Using this system, we can rapidly test the size of the releasable pool of neurotransmitter and, thus, the activity of the nerves. Ninety minutes of stimulation in this condition decreased the NA content by 50%, which represents a supramaximal stimulation, indicated that it was a good model in which to study the effect of NeoSTX as a blocker of the Na⁺ channel to eliminate the action potential generated by depolarization in the tissue. The finding that NeoSTX blocked the decrease in ovarian NA induced by stimulation means that the toxin was effective at blocking ovarian nerve activity. Because we found a similar decrease in NA after the chronic implantation of a minipump with the toxin for 28 days, this suggests that there is a decrease of the sympathetic tone in the ovary in a chronic fashion that covers the entire development of a ovarian follicle cohort. This also suggests that study of the effect on ovarian steroids secretion is required.

The increase in the number of corpora lutea and the decrease in follicular cyst found after 30 days of chronic NeoSTX exposure suggests that that, under normal conditions, the sympathetic activity in the ovary maintains a basal tone that is necessary to control follicular development and in steroid secretion. Similar results to those obtained after surgical denervation of the hyper-noradrenergic state after EV administration (Lara *et al.* 1993). This effect was similar to the changes in follicular development that we recently described when we increased the cholinergic tone to the ovary of normal rats keeping norepinephrine constant (Urrea *et al.* 2016, Riquelme *et al.* 2019). It is likely that the ovary function is mainly controlled by the hypothalamic–pituitary axis and the role of the autonomic tone results from a balance between cholinergic and noradrenergic activity. For example, a change in this balance to a hyper-noradrenergic state turns the ovary into a polycystic ovary, while a change to a cholinergic predominance is the healthy condition (Riquelme *et al.* 2019). The concept that seems new for a typically endocrine organ is commonly found in all organs receiving autonomic control such as heart or the renal system (Velasquez *et al.* 2019, Grisk 2020). Independent of the impact on ovarian function, this strengthens the concept that endocrine pathologies can be managed by modifying the autonomic tone to the organ (Lansdown & Rees 2012).

Recently, neural regulation was developed as a new therapeutic tool to treat different pathologies (Birmingham *et al.* 2014, Chavan & Tracey 2017, Pavlov & Tracey 2017, Velasquez *et al.* 2019, Grisk 2020). The ovary is one of the organs in which sympathetic activity has been studied (Lara *et al.* 2002, Lansdown & Rees 2012).

This situation has been explored especially because of the close relationship between stress and reproductive function (Toufexis *et al.* 2014). Many studies that were done in rodents have described the organization of sympathetic innervation of the ovary, their activity, and the relationship of the nerves to specific functions of the ovary such as steroid secretion and follicular development. Although there is strong evidence of a hyper-noradrenergic tone as a part of the causative factors in the development of the PCO phenotype in rodents (Barria *et al.* 1993), there are few studies on the role of sympathetic nerves in the PCOS in humans (Stener-Victorin *et al.* 2008, 2009, Zheng *et al.* 2015). These authors published some studies where acupuncture methods were used, which can lower the sympathetic nerve activity and increase the rate of ovulation in women with PCOS. The most recent studies, however, have described that this technique is not as effective as what was previously described (Kuang *et al.* 2018, Chen & Lim 2019, Lim *et al.* 2019). Most promising are recent studies in rodents in which electronic devices were implanted in the SON to regulate the nerve rate of discharge and thereby regulate the neuronal input to the ovary (Pikov *et al.* 2018).

β-Adrenergic blockers such as propranolol have been used to block the PCO phenotype induced by β-adrenergic stimulation induced by isoproterenol in rats (Luna *et al.* 2012). They have the problem of a short half-life (4–5 h, which requires repeated doses as we did in the study), and because of the systemic administration, many tissues are targeted for its action. Interestingly recently appeared a paper in which an acute dose of locally applied propranolol facilitates ovulation (Venegas *et al.* 2019). Although the delivery system could be improved using targeted nanodelivery systems, the short half-life of the drug in solution limits the development of new products. The advantage of using NeoSTX is the very good affinity for the Na⁺ channel and its long-lasting effect. Thus, it could be a good alternative to block neuronal systems for longer times and, therefore, be useful in various pathologies to decrease the sympathetic over-activity of the PCO. In this sense, NeoSTX has been frequently used in veterinary and human clinics as a long lasting local anesthetic that has many advantages over traditional local anesthetic (Rathmell *et al.* 2015). Although Nav1.7 has an IC₅₀ 100 times higher than that for Nav1.6, the administrated dose of NeoSTX is sufficient to inhibit both channels in nerve terminals of the SON nerve fibers, NeoSTX acts specifically on Nav1.6, which is most highly expressed in peripheral neurons, Ranvier nodules, and dendrites (Caldwell *et al.* 2000). This channel has an IC₅₀ of 0.86 nM.

In addition, Nav 1.7 is highly expressed in peripheral neurons with a IC₅₀ of 81.6 nM for NeoSTX (Alonso *et al.* 2016). It is also interesting that the ovary has been described Nav–endocrine sensitive to tetrodotoxin (which has the same molecular target as NeoSTX) in ovarian granulosa cells. This suggests a role in the stimulation of luteolysis and thus tetrodotoxin preserves the luteal phenotype (Bulling *et al.* 2000). Thus, NeoSTX can regulate sympathetic activity and extend the half-life of the corpora lutea.

Our *in vivo* studies with NeoSTX were designed to study the role of the toxin to prevent development of the PCO phenotype or to study the capacity of the toxin to reverse the PCO phenotype at the first steps of cyst formation (Lara *et al.* 2000). To prevent PCO development, we applied the NeoSTX at the same time as EV. Blocking noradrenergic neurons by NeoSTX prevented development of the PCO phenotype, which is shown as the recovery in corpora lutea, decrease in follicular cyst, the increase in plasma levels of progesterone, and decrease in testosterone. All of these factors represent the recovery of estrous cycling activity and probably ovulatory function (Barria *et al.* 1993). The advantage of using the toxin, especially the local intraovarian delivery, was the method's low invasiveness and reversible. Studies with pulses of the toxin to determine the effective time of action when it was locally administered to the ovary will strengthen the effectiveness of the method. We demonstrated that the NeoSTX decreased norepinephrine in the control rats, and it was also able to decrease norepinephrine in the EV-treated ovaries. It is interesting to show that when NeoSTX was administered 30 days after the EV administration, i.e. when the ovaries also present the previous steps of follicular cyst formation (Lara *et al.* 2000), rats quickly recovered their estrous cycling activity, indicating that blocking the neurons could be effective. However, some diffusion of the toxin from the ovary into general circulation may have occurred, and thus, had an unspecific effect on the endocrine system. Our calculation of the toxin dose took into account that the dilution in plasma would result in a concentration that was far from an effective dose. Preventing the development was more effective than the reversion effect. This can be explained because after 30 days of EV administration, follicular cyst formation began before the toxin was applied, and thus, a new cohort of follicles will be blocked. However, the reversion is consistent with the effect on steroid secretion. We cannot discard a putative effect of the toxin on the nerve terminals from the plexus nerve that could participate in the effect on steroid secretion (Uchida & Kagitani 2014).

Altogether, these results strongly suggest that NeoSTX could be an innovative drug to block the PCO phenotype, permitting the rats to ovulate and probably recover from decreased fertilization capacity that occurs after a sympathetic discharge, such as chronic sympathetic stress (Riquelme *et al.* 2019), and thus, providing support for the use of this drug as a therapeutic agent for PCOS.

Participation in the study

MdC participated in the experimental part of the study, biochemical determination data collection, analysis of data and manuscript preparation. NL was in charge of the preparing the toxin and testing the biological activity and purification. HEL conceived the idea and equally contributed to the study design, data collection, analysis, and manuscript preparation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by grant Fondecyt 1170291 (to H E L).

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Received in final form 5 January 2020

Accepted 16 January 2020

Accepted Manuscript published online 20 January 2020