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Catecholamine uptake, storage and regulated release by ovarian granulosa cells

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Abstract

Catecholamines present in the mammalian ovary are involved in many normal aspects of ovarian functions, including initial follicle growth, steroidogenesis and pathological states such as polycystic ovary syndrome (PCOS). Sympathetic nerve fibers are the largest source of norepinephrine (NE), but not the only one. Surgical denervation of the rat ovary, reduces, but does not eliminate, the ovarian content of NE. The aim of this work is to explore which intraovarian cells may participate in the ovarian NE homeostasis and the mechanisms involved. It was found that denervated rat ovaries can take up NE and cocaine considerably, decreased its uptake, suggesting involvement of catecholamine transporters. Granulosa cells of rat ovarian follicles present dopamine transporter (DAT) and NE transporter (NET). Their functionality was confirmed in isolated rat granulosa cells while cocaine blocked the uptake of NE. Furthermore, the presence of the vesicular monoamine transporter 2 (VMAT2), together with the exocytotic protein (SNAP25) in granulosa cells, implies catecholamine storage and regulated release. Regulated calcium-dependent release of NE was shown after depolarization by potassium, implying all neuron-like cellular machinery in granulosa cells. These results in rats may be of relevance for the human ovary, as DAT, NET, VMAT2 and SNAP25 protein and mRNA are found in human ovarian follicles and/or isolated granulosa cells. Thus, ovarian non-neuronal granulosa cells, after taking up catecholamines, can serve as an intraovarian catecholamine-storing compartment, releasing them in a regulated way. This suggests a more complex involvement of catecholamines in ovarian functions as is currently being recognized.

Less than 250 Words

Introduction

The sympathetic innervation of the mammalian ovary has been involved in many aspects of the regulation of ovary functions, including steroidogenesis and early follicular development (1, 2). In rats, these actions are performed mainly by norepinephrine (NE) and vasoactive intestinal peptide (VIP), which are contained in and released from nerve fibers. NE acts on ß2-adrenergic receptors present in theca and granulosa cells from rat ovary and stimulates the production of progesterone and androgens, but not the secretion of estradiol (1-4). In contrast to NE and acting on its own receptors, VIP stimulates estradiol and progesterone release from cultured granulosa cells and whole ovaries in vitro (5). Recent evidence has confirmed a role for VIP in the control of ovarian cyclic steroid production in adult cycling rats (6). These neurotransmitters may also facilitate the follicular development, as seen, for example, in the inhibition of follicular growth following ovarian denervation (7-9).

In the rat, a substantial fraction of the sympathetic innervation, targeting the ovary, is provided by neurons of the celiac ganglion and travels through the superior ovarian nerve (SON). Sympathetic innervation represents almost 90% of the NE present in the ovary, and yet, after surgical denervation, NE levels still remain over 10-15% (10), suggesting the existence of intraovarian cells participating in ovarian NE homeostasis. Furthermore, oocytes could be in part responsible for this, as at least in monkeys they showed to take up dopamine, through membrane dopamine transporter (DAT) and synthesize NE (via dopamine-beta hydroxylase) (11). These mechanisms could have special relevance to pathological states, such as polycystic ovary syndrome (PCOS), the most frequent ovarian pathology in women during their fertile years. Using a rat model of PCO, an increased ovarian sympathetic tone has been described, associated with the development and maintenance of PCO (12). In addition, these findings have been recently extended to the women (13), supporting a role for NE and catecholamines in maintaining the pathological condition. It is important to consider

that sympathetic denervation or electrolytic lesion in the sympathetic pathway of the ovarian nerves in the rat reverse the formation of cyst (14, 15). However, the responsible for an increased sympathetic tone in PCOS could also lie within the ovary itself and may include an increased activity of intraovarian neurons (found in some species and Wistar rats) or other unknown intraovarian compartment which could regulate NE homeostasis. The present study was designed to address these issues and to search for others ovarian sources for NE in two strains of rats and extend the study to human beings.

Materials and Methods

Animals

Ovaries from adult and prepubertal Wistar and Sprague-Dawley rats, derived from stocks maintained at the University of Chile, were used. The ovaries of Wistar rats are reported to contain neuron-like cells, while Sprague-Dawley rats lack of these cells (16). The ovaries derived from adult Wistar and Sprague-Dawley rats were used either to quantify NE, incorporate and release NE, RT-PCR procedures and/or fixed for immunohistochemistry (n=30 for each rat strain), whereas the ovaries from prepubertal Wistar and Sprague-Dawley rats were used to isolate granulosa cells (n=12 for each rat strain). Some of the Wistar and Sprague Dawley rats underwent ovarian denervation (SONX) 11-12 days before the experiments. This procedure was intended to eliminate the extrinsic contribution of NE via the sympathetic innervation of the ovary. For the procedure, rats were anesthetized with Xilaxine (10mg/kg) and Ketamine (90mg/kg) and the ovaries were denervated under dorsal approximation as described (14, 17). All the animals were euthanized by decapitation. The ovaries were rapidly removed, cleaned of adherent tissue and immediately used for release and incorporation experiments. Other ovaries of the same age were frozen at -80°C for total RNA isolation and NE quantification or embedded in Zamboni's fixative for

immunohistochemistry. In addition, Bouin's fixed and paraffin embedded ovaries from adult Sprague-Dawley rats we used for the preparation of sections and immunohistochemistry. These samples had been used in previous studies (18). All animal procedures were previously approved by the Institutional Ethic Committee of the Faculty of Chemistry and Pharmaceutical Sciences of the Universidad de Chile and the experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

Rat granulosa cells

Rat granulosa cells were purified as previously described (14, 19). To eliminate the contribution of luteal cells present in the ovary of adult rats, the ovaries of prepubertal (25 days old) Sprague-Dawley rats were used as recommended (19). The ovaries were punched with a needle and squeezed out gently to obtain the granulosa cells. Cells were suspended in 1 ml of Krebs-Ringer Buffer (KRB) and centrifuged at 3000 xg for 5 min and the pellet of cell was suspended in one ml of KRB. Cells were counted and aliquots of 50,000 cells were used for the NE release and incorporation experiments. RNA was isolated from several batches of 100,000 cells each.

Human granulosa cells

Human granulosa cells were obtained from follicular aspirates of women undergoing IVF, as described (20, 21). They were separated by centrifugation at 560 xg for 3 min and subsequently washed in serum-free DMEM/Ham's F-12 medium (1:1, Sigma, Deisenhofen, Germany). Washed cells were suspended in culture medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10 % fetal calf serum as previously described (22). The Ethics Committee of the Ludwig-Maximilians University of Munich had approved the use of the cells for scientific experiments, and

written consent of the patients was obtained. Cells from 2-4 patients were pooled for all experiments.

Uptake and release of NE

The procedure employed has been previously described (23-25). In brief, the ovaries were preincubated for 20 min in KRB, pH 7,4, gassed with 95%O_2-5%CO_2, and then incubated for 30 min at 37°C with 2 µCi ^{3}HNE (Dupont/NEN, Boston, MA). After washing the tissue in KRB (six washes of 10 min each) to remove non-incorporated radioactivity, the ovaries were transferred to a thermorregulated superfusion chamber and perifused at a flow rate of 1.5 ml/min for 10 min with KRB plus 10 mM tetraethylammonium, a potassium channel blocker which enhances the release of NE from nerve terminals in response to electrical stimulation (26). One-minute fractions were collected. After 3 min, the gland was subjected to a train of monophasic electrical pulses (80 V, 10 HZ, 10 msec/pulse for 1 min), delivered through a parallel set of platinum electrodes and generated by a Grass S-4 stimulator (Grass Instruments, Quincy, MA). One minute fractions were collected for 5 min more. In each experiment, ovaries from SONX and control rats were simultaneously stimulated in parallel superfusion chambers. At the end of the experiment, the ovaries were homogenized in 0.4 N perchloric acid, and ^{3}Hcatecholamine remaining in the tissue was determined by scintillation counting (Packard Liquid Scintillation Analyzer 1600TR; 72,5 % efficiency for ^{3}H) to calculate the radioactivity remaining in the tissue after the experiment. To calculate the amount of NE released, the radioactivity present in each of the 1-min samples was determined in the same way. The release, which represents ^{3}HNE overflows from ovaries structures, was then expressed as fractional release, *i.e.* as a percentage of the total radioactivity present in the tissue (24, 25).

Effect of cocaine on ovarian incorporation and release of ^{3}HNE

In order to elucidate the effect of cocaine on the NE incorporation by the ovary, rat ovaries were halved. One half was incubated with KRB and the other with KRB plus 10 µM cocaine (Sigma Chem. Co., St Louis, MO) for 10 min at 37°C. Later, the ovaries were incubated with 2 µCi/ml ^{3}HNE (levo-[7,8-^{3}H]-norepinephrine, 40 Ci/mmol, Amersham Biosciences, UK) for 30 min at 37°C. Each ovary was washed 6 times with KRB for 10 min to eliminate ^{3}HNE excess. Ovarian halves treated initially with cocaine were also washed with 10 µm cocaine the first two times. Finally the halved ovaries were homogenized in 0.4 N perchloric acid and the remaining ^{3}Hcatecholamine in the tissue was determined by scintillation counting (Packard Liquid Scintillation Analyzer 1600TR; 72.5 % efficiency for ^{3}H) to calculate the radioactivity incorporated by the tissue.

Incorporation of NE in isolated granulosa cells

In order to analyze the releasing capacity of NE in granulosa cells, they were isolated them from the Sprague-Dawley rats' ovaries as previously explained. Granulosa suspension was incubated in 1 ml of KRB, containing 0.25 µCi/ml of ^{3}HNE (levo-[7,8-^{3}H]-norepinephrine, Amersham Biosciences, UK) for 30 min at 37°C. Granulosa cell suspension was centrifuged and the cellular pellet was washed 5 times by suspension with 1 ml of KRB during 10 min followed by 5 min centrifugation (3,000 xg) each time to eliminate non-incorporated ^{3}HNE. To generate a depolarizing stimulus, granulosa cell suspension was incubated with KRB supplemented with 80 mM potassium for 10 min, and then it was centrifuged for 5 min at 3,000 xg and supernatant saved for radioactivity counting. A post stimulus fraction was obtained by suspending the cells in KRB during 10 min and then centrifuging them at 3,000 xg for 5 min. The final pellet was homogenized in 0.4 N perchloric acid and the remaining

^{3}Hcatecholamine in the tissue and that of the lavages was determined by scintillation counting (Packard Liquid Scintillation Analyzer 1600TR; 72,5 % efficiency for ^{3}H).

The effect of cocaine and calcium dependence in the ^{3}HNE up-take and release in rat granulosa cells

To evaluate the effect of cocaine in the ^{3}HNE granulosa incorporation, the protocol used was the same as the one explained before, except that 10 µM of cocaine were present during incorporation of ^{3}HNE and during the first two lavages. To study the dependence of the release of NE on extracellular calcium, granulosa cells were incubated in KRB with ^{3}HNE, washed and stimulated with corresponding KRB solutions lacking of calcium and containing 0.1 mM EGTA.

NE determination by HPLC

To quantify the concentration of NE in the ovaries, they were homogenized in 1:4 Dulbecco's phosphate buffered saline (DPBS, pH 7.35) and centrifuged for 15 min (13000 xg) at 4°C. Dulbecco's homogenizated supernatant was acidified with 0.25 N perchloric acid in a relation of 1:4, then centrifuged at 13000 xg for 3 min and stored at –80°C. At the time of quantification, the supernatant was filtered (sterile filter 0.22 µm) and 20 µl of the solution was injected into a Waters HPLC system (Waters P600, Milford, USA) with a C18 reverse phase column (BAS MF 6213 ODS, USA) coupled to an electrochemical detector (Waters 464, Milford, USA). The mobile phase contained 100 mM NaH_2PO_4, 1.29 mM octyl-sulphate, 0.02 % EDTA and 0.5 % acetonitrile (pH 2.5) with a flow rate of 1 ml/min. The potential of the amperometric detector was set at 0.65 V and the sensitivity was of 1 nÅ. Under these experimental conditions, retention time was 4 min for NE. A standard curve between 25-400 pg was measured parallel to NE's sample quantification.

RNA preparation, reverse transcription and polymerase chain reaction (RT-PCR) for rat tissues

RT-PCR was used to determine whether mRNAs encoding *Net* (SLC6A2), *Dat* (SLC6A3) and *Vmat2* (SLC18A2) are present in the rat ovary, granulosa cells and/or celiac ganglion. The celiac ganglion was dissected as described earlier (27). Total RNA was extracted as recommended (28). A total of five µg RNA were subjected to reverse transcription at 42°C for 60 min, using 1.6 mM dNTPs, 10 mM DTT, 176 nM random hexamers (Invitrogen, Carlsbad, CA), 25 U RNaseOUT (Invitrogen, Carlsbad, CA), 125 U reverse transcriptase SuperScriptII (Invitrogen, Carlsbad, CA) and first strand buffer, in a 30 µl volume. The reaction was finished by heating the samples at 75°C for 10 min.

Dilutions of the RT reaction were subjected to PCR amplification using 1 U of DNA Taq polymerase (Promega, Madison, WI), 1 mM dNTPs, and 0.5 µM of each gene-specific primer in a total volume of 30 µl. The PCR reaction consisted of 32 cycles (denaturation at 94°C for 60 sec, annealing at 60°C for 60 sec, and extension at 72°C for 60 sec) using a DNA thermal cycler (MJ Research, Watertown, MA).

The primers for *Net* and *18s* (the latter used as constitutively expressed gene for normalization purposes) were those previously reported by other authors: *Net* (29) and those for *18s* were obtained from Ambion (Ambion, Wiesbaden, Germany). Amplification of *18s* RNA was performed in a different tube to avoid interference with the amplification of the mRNAs of interest. The other primer sequences are detailed in table 1. Reaction tubes lacking of RT product input were used as PCR controls. The RT-PCR products were separated on 2.0 % agarose gels, stained with ethidium bromide, and photographed digitally. The identities of all PCR products were verified by direct sequencing, using one of the specific primers.

RT-PCR for human granulosa cells

Total RNA from several batches of cultured granulosa cells (3 and 4 days after isolation) was prepared using RNEasy kit (Qiagen, Hilden, Germany). As previously described (20), total RNA (200–500 ng) was subjected to reverse transcription, using random primers (pdN6) and Superscript-RT II (Life Technologies, Karlsruhe, Germany). Commercial human brain and ovary cDNAs (BD CLONTECH, Inc., Heidelberg, Germany) were used as well.

PCR amplifications (see table 2) consisted of 34 cycles of denaturing (at 94°C for 60 sec), annealing (at 60°C for 60 sec), and extension (at 72°C for 60 sec). Reaction tubes lacking of RT product input were used as PCR controls. In some cases, control reactions, in which cDNA was replaced by RNA, were also performed. The PCR reaction products were separated on 2.0 % agarose gels and visualized with ethidium bromide. The identities of all PCR products were verified by direct sequencing, using one of the specific primers

Immunocytochemistry of human granulosa cells

DAT protein in cultured human granulosa cells was examined by immunocytochemistry using a commercially available polyclonal antiserum (rabbit anti-DAT, Alpha Diagnostic Intl. Inc, distributed by BIOTREND, Cologne, Germany, 1.200 - 1:500), as described (30). This antiserum recognizes rat and mouse DAT, but cross-reacts with human DAT, as their sequences posses a 72% of homology. Cells were cultured for this purpose on glass coverslips, fixed (4 % paraformaldehyde in 10 mM PBS) and used after rinsing in 10 mM PBS (pH 7.4). Incubations with the antiserum were performed overnight in a humidified chamber at 4°C. After washing steps, a fluorescein isothiocyanate-labeled second goat anti-rabbit antiserum was used. For control purposes, the first antiserum was omitted, and incubations with normal rabbit serum were carried out instead. In a similar way, rabbit anti-NET antiserum /Biozol, Eching, Germany, ab41559; 1.500-1:1000) was employed. Sections were examined

with a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany), equipped with a fluorescein filter set.

Immunohistochemistry for DAT and NET in rat and human ovaries

Rat ovaries sections (Wistar and Sprague-Dawley) and human ovarian sections (from the tissue collection of the Anatomical Institute, Munich; (21, 30-32) were subjected to immunohistochemistry. The same specific DAT and NET antisera used for human granulosa cells were used for these tissues, according to the procedure previously reported (21, 30, 31). In addition, VMAT2 rabbit antiserum was used (Phoenix Pharmaceuticals, Mountain View, CA; 1:1.000 - 1:2:000). In brief, sections of 6 µm were deparaffinized and endogenous peroxidase activity was blocked. After that, those sections were submitted to antigen retrieval by heating in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6.0) at 90°C for 30 min, plus 20 min cooling in the same solution. Sections were incubated overnight at 4°C with the antisera followed by a second antibody (anti-rabbit biotinylated gamma globulin; 1:250) (Vector Laboratories, Inc. Burlingame, CA), and avidine-biotin complex peroxidase (ABC, Vector Laboratories, Inc, Burlingame, CA). The binding was visualized with DAB (Vector Laboratories, Inc, Burlingame, CA). As a control for DAT specificity, some sections were incubated with anti-DAT, which had been preabsorbed with DAT peptide, provided by Alpha Diagnostic. Other controls consisted of replacing the primary antiserum by buffer or incubation with non-immune serum. Sections were observed with a Zeiss Axiovert microscope.

Statistical Analysis

The results were expressed as mean ± standard error. Comparisons between several groups were made using the Student *t* test, considering $p<0.05$ as significant.

Results

Ovarian NE uptake studies and effects of cocaine

Surgical denervation of the ovary by SONX 11- 12 days prior to the NE uptake- and release-experiments was performed in order to eliminate the most important source of extraovarian NE (sympathetic innervation via SON). As shown in figure 1, surgical denervation of the ovary from Wistar or Sprague-Dawley rats decreased the amount of ^{3}HNE released upon electrical depolarization. The effect of denervation on endogenous ovarian NE levels is shown in table 3. In the ovary of Wistar rats, it decreased by an 80% approximately (108±33 ng/mg tissue to 14±5.5 ng/mg tissue ($p < 0.01$)) as consequence of denervation. A similar reduction was found in Sprague-Dawley rats (data not shown, (14)). However, the amount of ^{3}HNE released upon electrical depolarization decreased only by 48% (15.3±1.6 % fractional release to 8.0±1.0 5 fractional release ($p<0.01$)) for Wistar and by 59% (10.3±1.6 vs 4.3±1.5) in Sprague-Dawley rats.

The capacity for incorporating NE after blocking the NET and DAT catecholamine transporters with cocaine, a well-known inhibitor of NE and DA neuronal uptake (33, 34) was then compared. A decrease of an 85% was found in the NE incorporation capacity, an effect which had the same magnitude for control and SONX ovaries (table 3). In the ovary of Sprague-Dawley rats, cocaine produced a similar effect but in a lower magnitude (56% for control and 58% for denervated ovaries). Thus, we conclude that a cocaine-sensitive up-take mechanism exists and, although in the Wistar and Sprague-Dawley rat ovaries the sympathetic innervation is very important as a source of norepinephrine, yet there must be other intraovarian cellular structures which can take up and incorporate NE.

Identification of DAT, NET and VMAT2 in rat granulosa cells

The effects of cocaine, a blocker for NET and DAT, suggested the presence and involvement of these membrane transporters in the ovary. Thus, immunohistochemical studies attempted to localize NET and DAT in the ovary of Wistar and Sprague-Dawley rats (Figure 2Aa and 2Ba). Immunoreactive NET, DAT and VMAT2 proteins were mainly located in granulosa cells and the oocyte in Wistar rats. Signals were found in luteal cells as well, albeit with a lower intensity, Although it was found DAT and VMAT2 immunoreactivity in the ovary of Sprague-Dawley rats, immunoreactivity for NET was not found. Signals were absent in negative controls indicating the specificity of anti-NET, anti-DAT and anti-VMAT2. However, Figure 2Ab and 2Bb shows that *Dat* and *Net* mRNAs are present in the ovary of both strains of rats, in the isolated granulosa cells and, as expected, in the celiac ganglion. Furthermore, mRNA for the vesicular transporter (*Vmat2*) was also detected in both, the ovary and isolated granulosa cells.

Uptake and release studies in rat granulosa: Uptake is blocked by cocaine and the release of NE is calcium-dependent

Because of the presence of NET and DAT (catecholamine membrane transporters), the vesicular transporter VMAT2 and the known presence of the exocytotic protein synaptosome-associated protein of 25 kDa (SNAP25) (18, 35) in granulosa cells, in the present experiment it was tested whether they are able to incorporate and to release ^{3}HNE. In Figure 3, A and B show the percentage of ^{3}HNE fractional release from granulosa cells, which were isolated from Wistar and Sprague-Dawley prepubertal rats. High potassium stimulation (80mM) evoked the release of a substantial quantity of ^{3}HNE, followed by a post stimulation release, higher in magnitude than the basal release, indicating that granulosa cells can take up NE and

can release it in a controlled way.

Figure 4 depicts the incorporation rate of ^{3}HNE in granulosa cells under the presence of cocaine (10 μM). In whole tissue of the Wistar rats cocaine decreased the ^{3}HNE incorporation in granulosa cells (figure 4A upper left), indicating that NE enters to the cells through a cocaine-dependent transporter. Also, cocaine strongly decrease the subsequent fractional release of ^{3}HNE in granulosa cells (Figure 4A, middle), suggesting that an important fraction of ^{3}HNE was incorporated to granulosa cells by DAT/NET transporter and that this way of uptake is a prerequisite for subsequent NE release. Next, it was examined whether calcium was also involved in the release of NE, by removing extracellular calcium during high potassium induced NE release. It was discovered that the induced release from granulosa cells was clearly dependent on extracellular calcium (Figure 4, upper right), as it could be expected for a neuronal release mechanism. The same effect was found in the granulosa cells of the ovary of Sprague Dawley rats (Figure 4B, lower panel).

DAT, NET, VMAT2, SNAP25 in human granulosa cells

Finally, the entire ovary and brain (commercial cDNAs) and isolated luteinized human granulosa cells where screening by RT-PCR, to look for the presence of NET and DAT mRNAs in order to examine if the results obtained in rat granulosa cells could also be of relevance for the human ovary (Figure 5A). *DAT* and *NET* were already found in brain, and using identical PCR condition, *NET* mRNA was detected in entire ovary, but it was present only in traces in human granulosa cells. On the other hand, *DAT* mRNA was strongly present in both ovary and granulosa cells. To further examination of DAT, immunohistochemisty and immunofluorescence methods were employed and revealed that DAT is present in human granulosa cells in vivo (large antral follicle) and in isolated human granulosa cells as well (Figure 5B). RT-PCR

studies revealed that human granulosa cells besides expressing *SNAP25* (18) also express *VMAT2* (Figure 5C). Thus, these results correspond to the ones found in rats and support the existence of a similar uptake, storage and secretory machinery in both rat and human granulosa cells.

Discussion

A large body of evidence supports a role for the sympathetic nervous system in the regulation of steroid production and follicular maturation. Changes in the sympathetic outflow of the ovary could participate in the control of follicular development and, thus, changes in the sympathetic input (by stress or steroidal hormones) could modify ovary function (14, 15). Recent studies in ovaries have shown expression of genes by granulosa cells which are normally associated with nerve cells (36, 37). In addition, differentiation of granulosa cells into tumors, expressing neuronal markers, has likewise been observed (38). These results imply that there could be similarities in the behavior of ovarian cells and neuronal cells.

On the other hand, granulosa cells (through membrane receptors to DA and NE) are under the control of catecholamines, which are mainly derived from extrinsic sympathetic nerves or released from intrinsic sources, present in at least some species (16, 39) and could travel through the blood stream to the ovary from the adrenal. However, neither sympathetic innervation nor neuron-like cells or blood vessels are found in direct contact with granulosa cells and do not penetrate the basal lamina, which separates the granulosa and theca cell compartments. This implies that, first, catecholamines must diffuse through this structural barrier in order to reach granulosa cells. While in small follicles this may be an strong possibility, the diffusion distance increases in multilayered growing follicles.

Here, a novel evidence for granulosa cells of ovarian follicles, including large follicles is shown. These follicles will become a cellular compartment, which is able to regulate intrafollicular catecholamine homeostasis in an unexpected way. We show that granulosa cells take up NE, store it and, upon depolarization, release it. The presence of these mechanisms in the ovary of Wistar and Sprague-Dawley rats and the prerequisites for this mechanism in human ovary strongly suggest that these cells serve as unexplored catecholamine-storing cells within the ovarian follicle, ensuring that NE and/or DA are being present in the granulosa cell compartment, which lacks direct contact to catecholamine sources, such as innervation. This perspective would be in accordance with a role of NE and DA as important factors of the follicular microenvironment.

Many studies have demonstrated that after ovarian denervation (surgical cutting of the sympathetic nerves arriving to the ovary) there is a residual intraovarian NE independent of the rat's strain used in the study (3, 40). This implies the presence of another intraovarian catecholamine storage compartment besides nerve fibers, which degenerate after the denervation procedure. Intraovarian neuron-like cell bodies have been described only in the ovary of Wistar but not in Sprague-Dawley rats (41) and could be used for such storage of NE. As we previously demonstrated in the Sprague-Dawley rat (14) and in the present study for the Wistar rat, we found a similar decrease in endogenous NE after surgical denervation suggesting that the remaining NE is still present in another cellular compartment present in both ovaries.

While granulosa cells are not able to perform *de novo* synthesis of catecholamines, as they do not express tyrosine-hydroxylase (TH), they were found to possess DAT and NET. Interestingly, granulosa cells from both strains of examined rats express DAT and NET. However, the amount of mRNA and protein for NET seem to be low (especially in Sprague-Dawley, where it was not possible to detect NET by

immunohistochemistry). Nevertheless, the use of cocaine, while clearly indicating functionality of DAT and/or NET, does not allow to judge the degree of participation of DAT versus NET, as both transporters can be affected by cocaine (42). In addition to intraovarian neuronal cells (16) only present in Wistar rats, granulosa cells of the two strains may also differ in relation to NET. Yet, they do not differ in relation to DAT as it is a prerequisite for catecholamine uptake, and functional catecholamine up-take, possibly suggesting that this mechanism may be of greater importance.

There are some subtle differences between the capacities for releasing NE between both strains. Sprague-Dawley rats, which do not posses intraovarian neurons, have a higher capacity to release NE under electrical depolarization and sympathetic denervation is more effective to decrease the NE. It remains to be established whether these differences reflect a special contribution of intraovarian neurons or granulosa cells. Nevertheless, it is suggestive that the capacity for releasing NE from granulosa cells is higher in the cells of Sprague-Dawley rats than those of Wistar rats. It is proposed that in the ovary of Sprague-Dawley rats NE comes preferentially from extrinsic sympathetic nerves and granulosa cells while in Wistar they are derived from three different compartments which are: sympathetic nerves, intrinsic neurons and granulosa cells. Granulosa and extrinsic nerves are minor components compared to the innervation of Sprague-Dawley rats. Regarding intraovarian nerves, it is suggested that the differences in intraovarian neurons between Wistar and Sprague-Dawley ovaries may be a result of the actions of different intraovarian regulators, which may include estrogen, on target cells. Supporting this assumption, it was found that TH-immunoreactive cells, (presumably intraovarian neurons) also become detectable in denervated (Guanethidine-treated) Sprague-Dawley rat ovaries after the treatment with estradiol (2). This may be related to the fact that the gene for nerve-growth factor (NGFb), present in the rat ovary, the gene for low affinity NGF receptor (NGFR), which provides trophic support to the sympathetic ovarian nerves (9), and presumably

intraovarian neuron-like cells, contain estrogen-responsive elements (43).

The transmembrane transporters DAT and NET mediate Na^+-dependent re-accumulation of released catecholamines into the presynaptic terminals of neurons (44) in the nervous system. Functionality of ovarian DAT and NET was proved by the ability of cocaine to block NE uptake in isolated rat granulosa cells. NE is the largest catecholamine detected in the rat ovary (45) and that was the reason it was chosen to perform the experimental uptake and release studies using radioactive NE. Even though NET has a preference for NE, and DAT for DA (42), both catecholamines can be taken up by the transporters. As both transporters are inhibited by cocaine, a specific involvement of NET or DAT in the uptake of NE or DA in rat granulosa cells can not be concluded for the reasons mentioned above. However, it is possible to speculate that due to the strong expression of DAT, in contrast to NET, in human granulosa cells, the human ovary besides containing NE also contains a high concentration of DA (46). Thus, in a DAT-mediated uptake, granulosa cells are likely to uptake not only DA, but also NE and could make these cells a storage compartment for these catecholamines.

What is the possible role of a granulosa cells storage compartment for catecholamines? An immediate consequence is a reduced amount of catecholamines in the surrounding extracellular space or the follicular fluid of larger follicles. This may have consequences such as lower concentrations of catecholamines that may reduce the ability of signaling molecules to activate their receptors. Recently, four of the five dopaminergic receptors described in the central nervous system (47) have been described in human granulosa cells and two of them in rat granulosa cells. Granulosa cells also support typical receptors for NE, specifically beta- and alpha-adrenergic receptors (48). Therefore, it is possible that uptake mechanisms compete with the actions performed by catecholamines, after binding to their receptors. This situation is

comparable to the one in the synaptic cleft. In monkeys functional DAT has also been found in another cell type contained in the follicle, specifically the oocyte (11). Furthermore, oocytes possess a catecholamine metabolizing enzyme (dopamine beta hydroxylase; DBH). Thus, it can synthesize NE from its precursor DA. Uptake of catecholamines may be just part of a more complex process supporting intrafollicular catecholamine homeostasis, as experimental evidence for storage and regulated release was found, as well as presence of typical molecules involved in these tasks in neurons, specifically VMAT2 and SNAP25. Presence of the vesicular monoamine transporter VMAT2 in both, rat and human granulosa cells was reported. In neurons, VMATs, which are present in two isoforms (VMAT1/VMAT2) packages monoamines into synaptic and secretory vesicles by exchange of protons (42). A similar role and evidence of a vesicular storage compartment must now be assumed in case of ovarian granulosa cells, as well.

The concept that granulosa cells are targets for neurotransmitters is not a new one. However, the idea that these cells, which are present in a compartment of the ovary and lacks direct innervation, can take up, store and release neurotransmitter, including catecholamines is only an emerging insight. From a pathological perspective, it has been previously postulated that development and maintenance of polycystic ovary is associated with an activation of sympathetic nerves (12). Adult rats exposed to chronic stress develop a polycystic condition similar in morphological aspect to the human PCOS, suggesting a higher sympathetic input and role in the derangement of follicular development characteristically seen in human being with the pathology (15). From this point of view, it has recently been demonstrated that women with PCOS have a higher sympathetic tone (13). Bearing this in mind, granulosa cells as a cellular compartment in general with the ability of storing and releasing NE, could serve as a reserve to maintain a higher availability of catecholamines in the follicles. Experiments are currently in progress to examine this hypothesis.

In summary, this study shows that ovarian non-neuronal, endocrine granulosa cells can take up NE, presumably derived from several potential sources, and can then serve as an intrafollicular catecholamine-storing compartment. Exocytotic NE release from granulosa cells can occur in a regulated manner, as described for neuronal cells. These results provide novel insights to the neuronal nature of granulosa cells and suggest a more complex involvement of catecholamines in normal ovarian functions as they are currently recognized. It remains to be shown whether and how these mechanisms are involved in ovarian disorders,

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Figure Legends

Figure 1: Induced release of ^{3}HNE from the ovary of Wistar and Sprague Dawley rats. Ovaries from control and denervated (SONX) animals were incubated with ^{3}HNE and were stimulated by transmural electrical pulses (80V, 2 mseg, 10Hz) as described in Materials and Methods. The rectangle represents the time of the stimulus and each bar corresponds to the dpm of ^{3}HNE released by ovary per minute. Results are means ± SEM from 4-6 individual experiments for each condition in each rat strain.

Figure 2: Expression of NET and DAT in the ovary of Wistar (A) and Sprague-Dawley (B) rats.

In Panel **A**, on the left: DAT and NET and VMAT2 immunoreactive cells in the ovary of Wistar rats are depicted There is an intense mark in granulosa cells and the oocyte (DAT) which disappears when the specific DAT antiserum is preabsorbed with excess of specific peptide (Co, middle panel). The additional control (right panel) is a consecutive section of the one stained for VMAT2, in which the antiserum was replaced by the buffer. Other controls, such as incubation with non-immune serum are not shown. On the right: Ethidium bromide-stained agarose gels depict results of RT-PCR experiments for *Net, Dat* and *Vmat2.* Cloning and sequencing of RT-PCR products confirmed their identity. OV: ovary; GC: granulosa cells; CGgl: celiac ganglion; Co: PCR negative control without RT product.

In Panel **B**, on the left:: DAT and VMAT2 immunoreactive granulosa cells in the ovary of Sprague-Dawley rats (antral follicle). Control (Co) is a consecutive section of the one stained for VMAT2 (specific antiserum was omitted). On the right: Ethidium bromide-stained agarose gels depict results of RT-PCR experiments for *NET, DAT* and *VMAT2*. Cloning and sequencing of RT-PCR products confirmed their identity. OV: ovary; GC: granulosa cells; CGgl: celiac ganglion; Co: PCR negative control without RT product.

Figure 3: Induced release of ^{3}HNE from Wistar (A) and Sprague-Dawley (B) rat granulosa cells. Granulosa cells were incubated with ^{3}HNE and stimulated with KCl-80mM as described in Materials and Methods. The asterisks represent the significative increase of ^{3}HNE release in response to the stimulus and results are expressed in % fractional release. Results are means ± SEM from 4 individual experiments in each rat strain. *<p 0.05, ***<p 0.001

Figure 4: Effect of 10µM cocaine on incorporation (left) and release (middle) of ^{3}HNE from rat granulosa cells and the effect of the absence of extracellular calcium on KCl-80mM induced release (right). Panel A correspond to the results from Wistar and B to Sprague-Dawley rats. Results are expressed either as % of incorporation in relation to total ^{3}HNE in the incubation medium or as % fractional release calculated as net release (minus basal release). Results are means ± SEM from 4 individual experiments in each rat strain.

Figure 5: Expression of DAT, NET, SNAP 25 and VMAT2 in human ovary and cultured human granulosa cells.

Panel A and C: Ethidium bromide-stained agarose gels depicts results of RT-PCR experiments for *NET* and *DAT* and *VMAT2* in human brain, ovary and granulosa cells (hGC); co(-) control reaction shown without cDNA input.

Panel B shows DAT immunoreactivity in cultured granulosa cells (top) and in granulosa cells (arrow) of a large antral follicle of the human ovary (bottom). There is a distinct mark in granulosa cells that disappears when the first antibody is preabsorbed with excess of specific peptide (Co for human ovary) or when the specific antiserum was replaced by the buffer (Co in granulosa cells).

Table 1:

Primer information for rat *Dat (SLC6A3)* and *Vmat2 (SLC18A2)*

		Genebank accession number
Dat Sense Antisense	 5'-ACC TTC TTC CCG GAG CAC TGG-3' 5'-GCA TTT AAC ACC CTG GTA GCA CAT-3'	**NM_012694**
Vmat2 Sense Antisense	 5'-ACT AAA GCC GAC AAG GGG TGA AA-3' 5'-TGA AGC AGG TGG GAG ATG TGA TAG-3'	**NM_013031**

Table 2:

Primer information for human *SNAP25, DAT (SLC6A3), NET (SLC6A2)* and *VMAT2 (SLC18A2)*

		Genebank accession number
SNAP25		**L19760**
Sense	5'-ATG GCC GAA GAC GCA GAC A-3'	
Anti	5'-ACC ACT TCC CAG CAT CTT TGT-3'	
Nested sense	5'-CTG GAA AGC ACC CGT CGT ATG-3'	
Nested anti	5'-GCA CGT TGG TTG GCT TCA TCA-3'	
DAT		**NM_001044**
Sense	5'-AGC CGG CAC GTC CAT CCT CTT TG-3'	
Anti	5'-GGC GCA CCT CCC CTC TGT CCA C-3'	
Nested anti	5'-ATG CTG ACC ACG ACC ACG A-3'	
NET		**BC060831**
Sense	5'-GCT TCT ACT ACA ACG TCA TCA TC-3'	
Anti	5'-CGA TGA CGA CGA CCA TCA G-3'	
Nested sense	5'-CTG GTC ACT CTA CTA CCT-3'	
Nested anti	5'-GCT GCT CTC GTG AAG GTG-3'	
VMAT2		**BC108927**
Sense	5'-CTT TGG AGT TGG TTT TGC-3'	
Anti	5'-GCA GTT GTG ATC CAT GAG-3'	
Nested sense	5'-TGC AAT TGG AAT GGT GGA T-3'	
Nested anti	5'-GAG AGG GGC AAA AAG AAT A-3'	

Table 3:
Changes in the norepinephrine concentration, ^{3}HNE release, ^{3}HNE incorporation and the effect of cocaine in ovaries of sham and denervated Wistar rats (SONX).

	Sham	SONX	% decrease after SONX
NE (ng/mg tissue)	108 ± 33	14 ± 5.5 **	87
H^3-NE fractional release (%).	15.3 ± 1.6	8.0 ± 1.0 **	48
H^3-NE incorporation (dpm/mg tissue)	3936 ± 1080	1700 ± 490**	56
H^3-NE incorporation + cocaine (dpm/mg tissue)	627 ± 186	271 ± 37**	54
% decrease by cocaine	85	85	

Results are expressed as mean values ± SEM of 4-6 individual experiments in each condition.**$p < 0.01$ vs. sham.

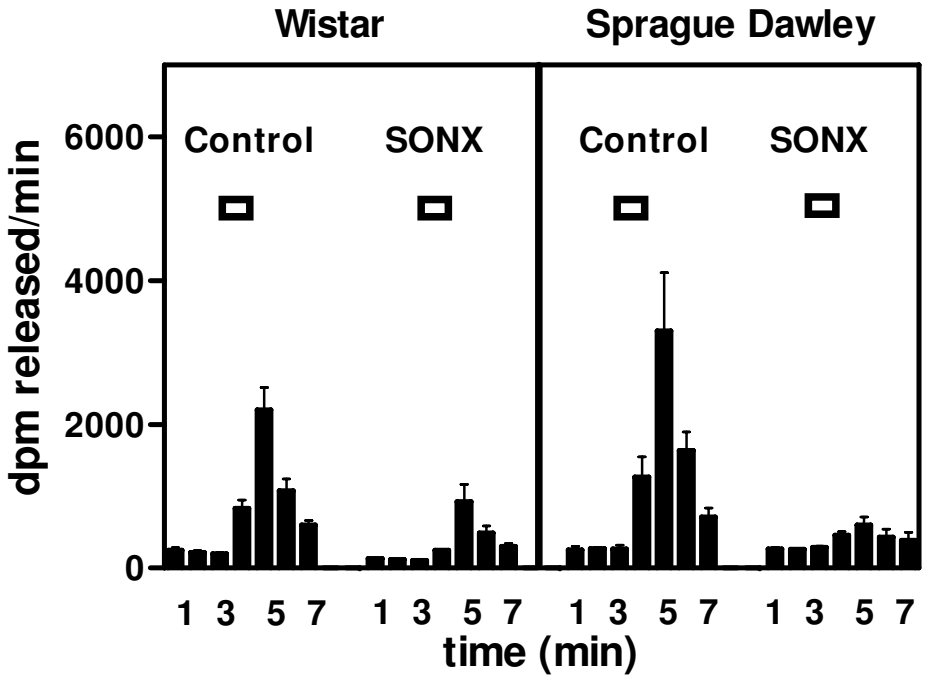

Fig 1 Greiner et al

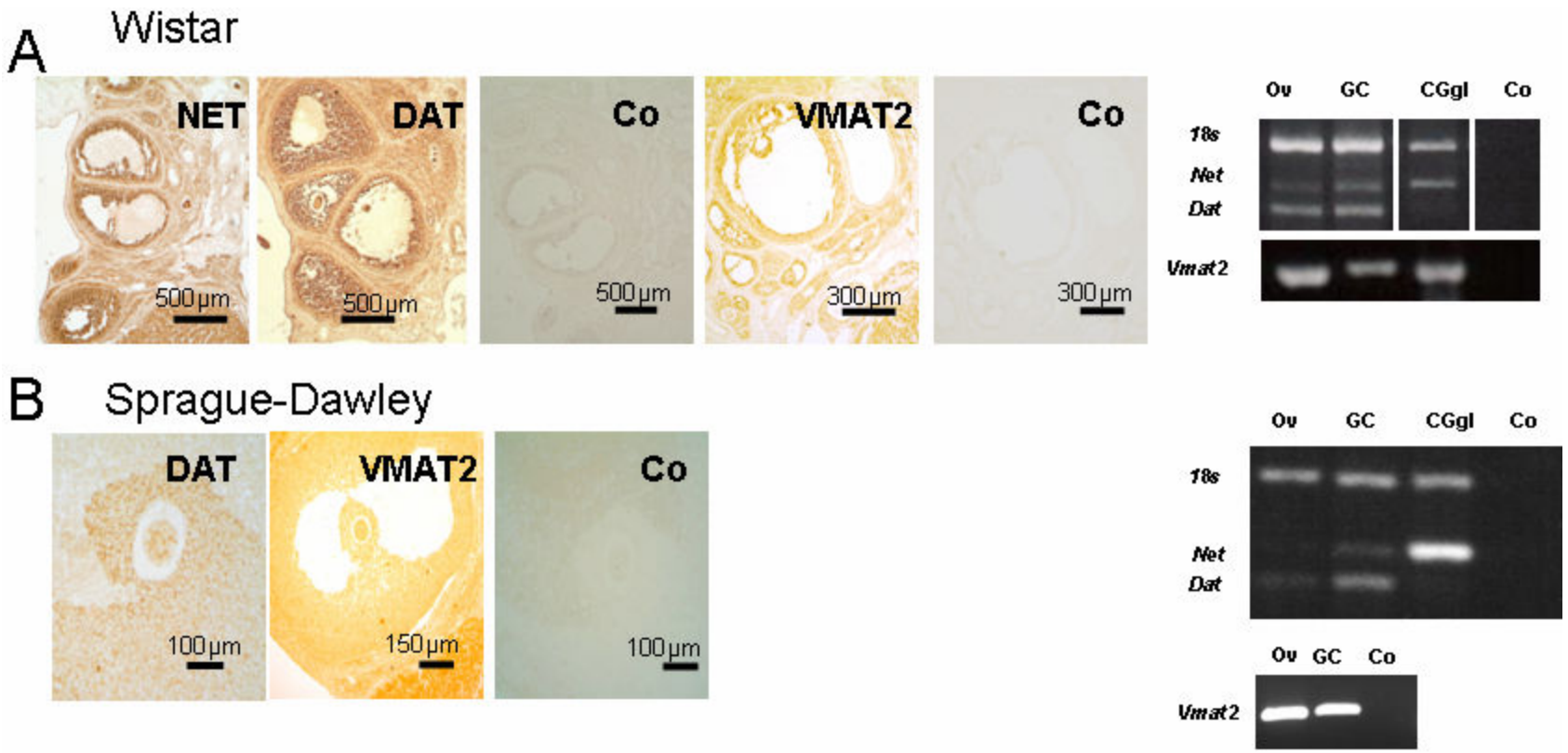

Figure 2: Greiner et al

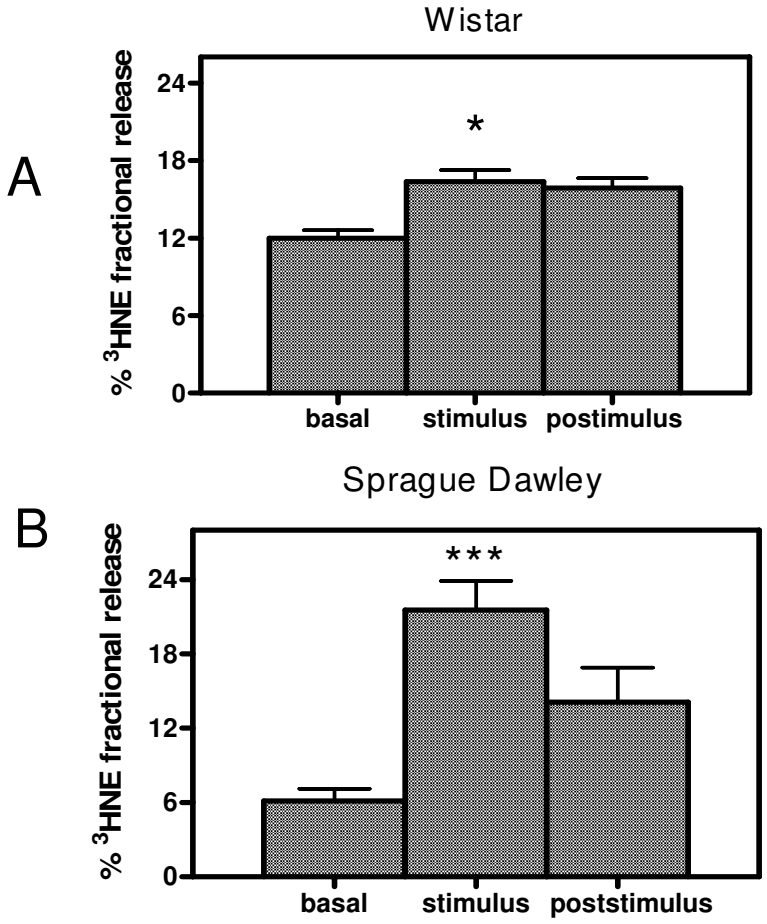

Figure 3, Greiner et al

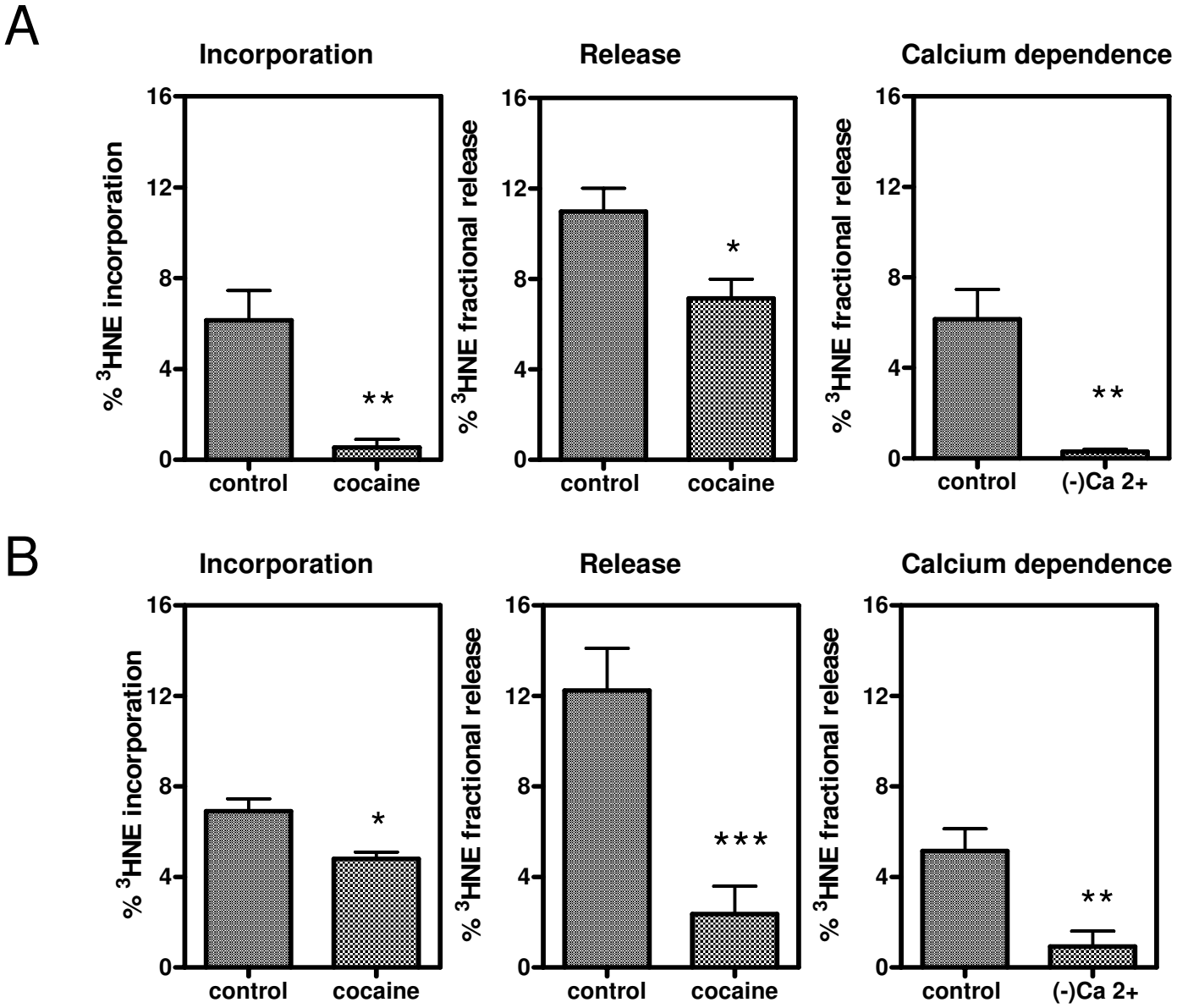

Figure 4, Greiner et al

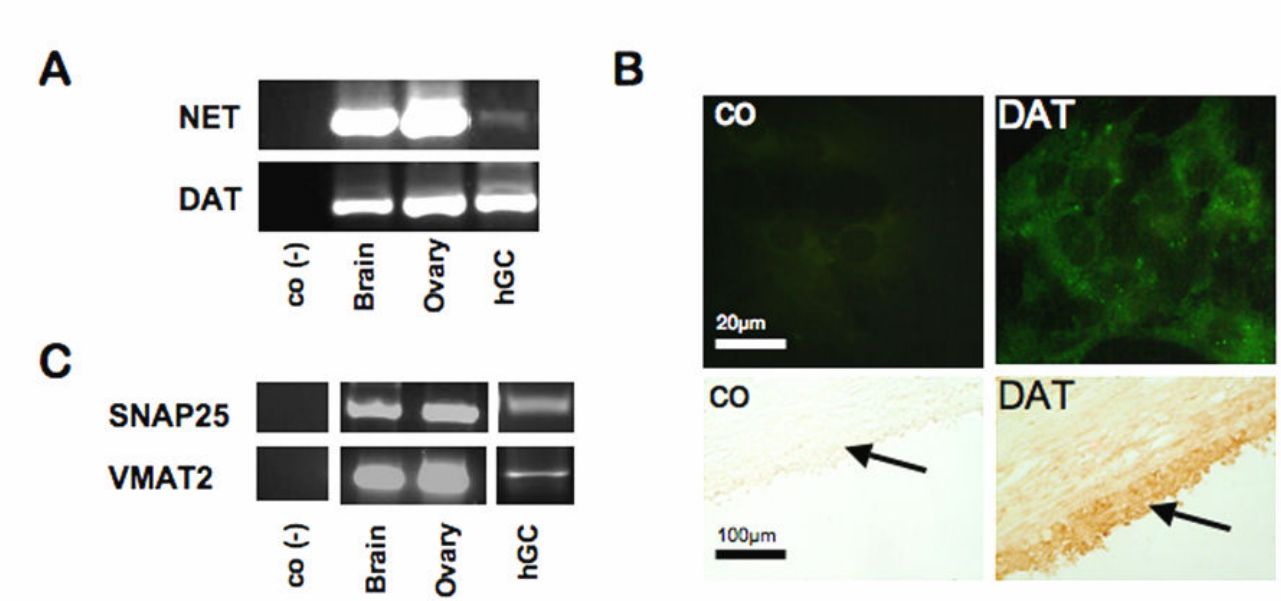

figure 5 Greiner et al