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Grassi, Daniela; Lagunas, Natalia; Pinos, Helena; Panzica, Giancarlo; Garcia-Segura, Luis Miguel; Collado, Paloma. NADPH-Diaphorase colocalizes with GPER and is modulated by the GPER agonist G1 in the supraoptic and paraventricular nuclei of ovariectomized female rats. NEUROENDOCRINOLOGY. 104 (1) pp: 94-104.
DOI: 10.1159/000445190

Neuro endocrinology

**Neuroendocrinology (DOI:10.1159/000445190)**
(Accepted, unedited article not yet assigned to an issue)

Advanced Release: March 9, 2016



Received: June 24, 2015
Accepted after revision: March 2, 2016

**Original Paper**

# NADPH-diaphorase colocalizes with GPER and is modulated by the GPER agonist G1 in the SON and PVN of ovariectomized female rats

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**Short title**: GPER activity modulates NADPH-diaphorase and pERK 1/2 in the SON and the PVN

**Keywords:** GPER, ERK, NOS, Estrogen, Hypothalamus, PVN, SON, NADPH-diaphorase

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**Abstract**

Nitric oxide is produced in the brain by the neuronal Nitric Oxide Synthase (nNOS) and carries out a wide range of functions by acting as neurotransmitter-like molecule. Gonadal hormones are involved in the regulation of the brain nitrergic system. We previously demonstrated that estradiol, via classical estrogen receptors (ERs), regulates NOS activity in the supraoptic (SON) and paraventricular nucleus (PVN) of the hypothalamus, acting through both ERα and ERβ. Magnocellular and parvocellular neurons in the SON and in the PVN express also the G protein-coupled ER (GPER). In this study we have assessed whether GPER is also involved in the regulation of NADPH-diaphorase in the SON and the PVN. Adult female ovariectomized rats were treated with G1, a selective GPER agonist, or with G1 in combination with G15, a selective GPER antagonist. G1 treatment decreased NADPH-diaphorase expression in the SON and in all PVN subnuclei. The treatment with G1+G15 effectively rescued the G1-dependent decrease of NADPH-d expression in both brain regions. In addition, the activation of ERK 1/2, one of the kinases involved in the GPER–dependent intracellular signaling pathway and in NOS phosphorylation, was assessed in the same brain nuclei. Treatment with G1 significantly decreases the number of pERK 1/2 positive cells in the SON and PVN, while the treatment with G1+G15 significantly recovered its number to control values. These findings suggest that the activation of GPER in the SON and the PVN inhibits the phosphorylation of ERK 1/2, which induces a decrease in NADPH-diaphorase expression.

## 1. Introduction

Nitric oxide (NO), an inorganic and unstable free radical gas, plays a wide range of functions in the central nervous system (CNS) [1] generally by acting as a neurotransmitter-like molecule. In the nervous system, NO is produced by the oxidation of L-arginine by the neuronal Nitric Oxide Synthase (nNOS or NOS type I), which is expressed in the brain and peripheral nerves [2-3] Generation of NO from L-arginine requires several cofactors, including nicotinamide adenine dinucleotide phosphate (NADPH). NADPH-diaphorase histochemical method detects cells with NOS activity (nNOS, eNOS and iNOS) [4] and is considered a reliable indicator of NOS presence and activity in the hypothalamus [5-12]. Since nNOS is the main NOS isoenzyme expressed in the hypothalamus, nNOS has the same distribution than the enzyme NADPH-diaphorase [4, 12].

Gonadal hormones play an important role in the regulation of the brain nitrergic system [13-15]. In particular, estradiol regulates NOS activity in the supraoptic nucleus (SON), decreasing the number of NADPH-d positive cells through both estrogen receptor (ER) $\alpha$ and ER$\beta$. In contrast, the activation of ER$\alpha$ increases NADPH-d expression in all the paraventricular (PVN) subnuclei, while the activation of ER$\beta$ has the opposite effect [16]. Rodent magnocellular and parvocellular neurons in the SON and in the PVN express not only the classical ERs [17] but also the more recently discovered G protein-coupled estrogen receptor 1 (GPER 1), formerly referred as G protein-coupled receptor 30 (GPR30) [18-21]. GPER is expressed in the brain and peripheral tissues [22-23] and it is known to be involved in physiological and pathological events regulated by estradiol in the central nervous, immune, reproductive and cardiovascular systems (for reviews see [24]). GPER-dependent intracellular signaling has been described in tumor cells as well as in the brain [23, 25-29]. Moreover, it has been shown that GPER is involved in the regulation of blood pressure, since its chronic activation by G1 reduces blood pressure in hypertensive rats [30] and a hypofunctional genetic variant of GPER is associated with increased blood pressure in women [31]. Since SON and PVN are known to be involved in the regulation of blood pressure, we were interested to elucidate whether GPER is functionally active in these

nuclei. Extracellular signal-Regulated Kinase (ERK)-1 and ERK-2 has been identified as one of the mediators of GPER signaling [23]. In particular, estrogen, through the GPER-dependent stimulation of adenylyl cyclase and cAMP-dependent signaling, inactivates Raf-1 and, in turn, induces an attenuation of ERK 1/2 activity in breast cancer cells. On the other hand, pERK 1/2 is involved in the phosphorylation of NOS on Ser1416 in the NTS for the regulation of blood pressure in male spontaneously hypertensive rats [32].

The main aims of the present study were to investigate: i) whether GPER is involved in the regulation of NADPH-diaphorase expression in the SON and the PVN subnuclei, and ii) if there is a relationship between the phosphorylation of ERK 1/2 and the expression of NADPH-diaphorase in the SON and the PVN subnuclei in response to the activation of GPER.

## 2. Experimental procedures

### 2.1 Animals and experimental treatments

Wistar albino female rats from our in-house colony were kept on a 12:12-h light–dark cycle and received food and water ad libitum. Animals were handled in accordance with the guidelines published in the “NIH Guide for the care and use of laboratory animals”, the principles presented in the “Guidelines for the Use of Animals in Neuroscience Research” by the Society for Neuroscience, and following the European Union (2010/63/UE) and the Spanish legislation (L6/2013; RD53/2013). Experimental procedures were approved by our Institutional Animal Use and Care Committee (UNED, Madrid). Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

Female rats were bilaterally ovariectomized at the age of 3 months under isoflurane anesthesia. They were then housed in plastic cages and randomly assigned to the different treatments. Seven days after surgery 4 rats per each group received one i.p. injection of vehicle (corn oil) or the selective GPER agonist G1((±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-

benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone (BiogenCientifica, Madrid, Spain; 2,4 nmol/animal), or/and the selective GPER competitive antagonist G15 ((3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (BiogenCientifica, Madrid, Spain; 7,4 nmol/animal). Doses of GPER ligands were based on a previous *in vivo* study [33].

Twenty-four hours after the injection of the estrogenic compounds all the animals were deeply anesthetized with pentobarbital (Normon Veterinary Division, Madrid, Spain, 50 mg/kg) and perfused through the left cardiac ventricle with 50 ml of saline solution (0.9% NaCl) followed by 250 ml of fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were quickly removed and immersed for 4–6 hours at 4ºC in the same fixative solution and then rinsed with phosphate buffer. Brains were placed for 72 hours in a 30% sucrose solution in PBS, frozen in liquid isopentane at −35ºC, and stored in a deep freezer at −80ºC until sectioning. Brains were serially cut in the coronal plane at 25 µm thickness with a cryostat, obtaining 4 series of adjacent serial sections. In each series, each section was 100 µm distant from the following one. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the rat brain atlas of [34]. Sections were collected in multiwell plates with a cryoprotectant solution [35] and kept at -20°C. Immunohistochemical assay for GPER and p-ERK 1/2 and histochemistry for NADPH- diaphorase were performed on different series.

## 2.2 Immunohistochemistry

The presence of GPER and p-ERK 1/2 were detected by immunohistochemistry performed on free-floating sections according to the following steps. Before the reaction, the sections collected in the cryoprotectant solution were washed overnight at 4ºC in PBS 0.1M, pH 7.3–7.4. The following day, free floating sections were first washed for 30 min at room temperature in PBS 0.1M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA. Sections were then treated for 10 min with a solution of PBS 0.1M, pH 7.3–7.4, containing methanol/hydrogen peroxide (PBS/methanol 1:1 with 0.3% hydrogen peroxide) to quench

endogenous peroxidase activity. Sections were washed for 30 min at room temperature in PBS 0.1M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA then incubated for 48 hours at 4°C with a rabbit polyclonal pERK 1/2 antibody (Cell Signaling, Millipore, Madrid, Spain, reference 9101S) diluted 1:2,000 or rabbit polyclonal GPER (ABCAM, Cambridge, UK, reference ab39742) diluted 1:250 in 0.1M PBS, pH 7.3–7.4, containing 0.2% Triton X-100, 0.2% BSA and 3% normal serum goat. A biotinilated goat anti-rabbit secondary antibody (Thermo scientific, Pierce, Rockford, IL, USA) was then used at a dilution of 1:300 for 120 min at room temperature. The antigen–antibody reaction was revealed by incubation with avidin-peroxidase complex (Thermo scientific, Pierce, Rockford, IL, USA) for 90 min. The peroxidase activity was visualized with a solution containing 0.187 mg/mL 3,3- diamino-benzidine (Sigma, Madrid, Spain) in PBS 0.1M, pH 7.3–7.4. The sections were washed in the same buffer and collected on chromallum coated slides, air dried, cleared in xylene, and cover slipped with Depex (VWR International Eurolab, Barcelona, Spain) for quantitative analysis.

Specificity of p-ERK 1/2 and GPER antibodies were reported in previous studies (Cell Signaling data sheet, [36]). Even though, additional assays were performed in order to test their specificity in our material: (a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); and (b) the secondary antibody was omitted. In these conditions cells and fibers were totally unstained. Moreover, in order to test the specificity of the GPER antibody an additional experiment using a specific blocking peptide (ABCAM, Cambridge, UK, reference ab39742) was performed. Before the primary antibody hybridization, the same antibody was preabsorbed with the corresponding blocking peptide diluted 1:10 during 18 hours at 4ºC followed by the same protocol of immunohistochemistry described above. Cells and fibers were then unstained (Fig 1B,D).

2.3 NADPH-diaphorase histochemistry

The presence of NADPH-diaphorase, a specific marker for nitric oxide-producing neurons [4, 36], was detected by histochemistry performed on free-floating sections. Before the reaction, the sections collected in the cryoprotectant solution were washed for 30 min at room temperature in PBS 0.01 M, pH 7.3–7.4 and then overnight at 4ºC in phosphate buffer 0.1 M, pH 7.4. The following day, free floating sections were washed at first in TRIS buffer 0.1 M, pH 8.0 for 15 min at room temperature, then in TRIS buffer, pH 8.0 containing 0.5% Triton X-100 for 10 min. The sections were incubated for 30 min at 37°C in a solution of 0.8 mM nitrobluetetrazolium (Sigma, Madrid, Spain) and 1 mM reduced NADPH (Sigma, Madrid, Spain) in TRIS buffer, 0.1 M, pH 8.0, containing 0.5% Triton X-100. The sections were washed in the same buffer and collected on chromallum coated slides, air dried, cleared in xylene, and cover slipped with Depex (VWR International Eurolab, Barcelona, Spain) for quantitative analysis. Controls were performed by omitting reduced b-NADPH in the incubation procedure.

2.3.1- Double staining of NADPH-diaphorase and GPER

To investigate the possible co-existence of NADPH-diaphorase and GPER, one series for each animal was stained with the NADPH-diaphorase protocol, washed 3 times with PBS 0.1M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA, and finally incubated with a rabbit polyclonal anti-GPER antibody (ABCAM, Cambridge, UK) diluted 1:250 in 0.1M PBS, pH 7.3–7.4, containing 0.2% Triton X-100, 0.2% BSA and 3% normal serum goat. The following steps followed the immunohistochemical protocol described for the detection of GPER.

2.3 Morphometric analysis

The quantitative analysis of NADPH-diaphorase staining, GPER or pERK immunostaining was performed on coded sections without knowledge of the experimental group. Two types of NAPDH-diaphorase positive neurons were detected: (i), neurons intensely stained in their perikarya and in their cell processes and (ii), neurons with moderate staining in the perikarya with no staining in the cell processes. Both types of neurons were included in the quantification. Selected fields were acquired by a digital camera (Olympus DP25) connected to a Nikon eclipse E600 microscope using a x20 objective. The number of NADPH-diaphorase, GPER or pERK 1/2 positive cells was assessed in the SON and the PVN, using four coded sections per animal. Data presented are the total number of immunolabeled cells for each rat for the series of four sections. For NADPH-diaphorase and GPER colocalization, data presented are the total number of neurons positive for the two markers. Sections selected for analysis corresponded to ca. -1.3 mm from bregma for the SON and to ca. -1.8 mm from bregma for the PVN (Paxinos and Watson). Since in previous studies we did not detect a lateralization of the distribution of NADPH-diaphorase positive cells, counts were restricted to the right hemisphere of the hypothalamus. Since the anatomical heterogeneity of the PVN, a grid of 24 square boxes of 8 $\mu m^2$ each were used in each slice and the grid was precisely located ensuring the left side of the grid on the wall of the third ventricle. The grid overlaps the total area of the PVN. Cell counting was performed for each square box within the boundaries of the nucleus. The PVN subdivisions were identified following the detailed anatomical description provided for the rat by [37] based on Nissl-stained material. The boxes corresponding to the same PVN subdivision, along the four coded sections, were grouped for the statistical analysis (see Fig. 1 [17]). In the SON, instead, all NADPH-diaphorase or pERK 1/2 positive cells within the anatomical boundaries of the nucleus were counted together.

2.4 Statistical Analysis

Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, using the SPSS-17.0 software (SPSS Inc, Chicago, USA). A value of $P < 0.05$ was considered statistically significant. Data are presented as the mean±SEM.

## 3. Results

*3.1 Co-localization of GPER and NADPH-diaphorase positive cells in the SON and the PVN*

In the control animals (ovariectomized females), GPER immunoreactive neurons were observed within the SON and in all the subdivisions of the PVN, with a higher staining in the PaLM and the PaMM (Fig 1A-C).

In double stained sections we observed 3 types of arrangements: cells showing only NADPH-diaphorase positivity, cells showing only immunoreactivity for GPER and cells showing both markers (Fig 2A, 2B). The number of NADPH-diaphorase positive and/or GPER positive cells varied from animal to animal and depending on the brain region (Table 1). In the SON, 80-100% of the NADPH-diaphorase positive were also GPER immunoreactive and 40-50% of GPER immunoreactive cells were also NADPH-diaphorase positive. In the PVN, 70-100% of NADPH-diaphorase positive cells were also GPER immunoreactive in the PaDC, 99-100% in the PaLM and 85-100% in the PaMM and PaV. The cells expressing GPER were also NADPH-diaphorase positive in a percentage of 30-50% in the PaDC, 20-30% in the PaLM, and 50-70% in the PaMM and PaV.

*3.2 NADPH-diaphorase and pERK 1/2 positive cells in the SON*

Both NADPH-diaphorase and pERK 1/2 positive elements were present in the SON. pERK 1/2 immunoreactive cells were more abundant than NADPH-diaphorase positive cells. Qualitative observation of NADPH-diaphorase and pERK 1/2 positive cells in the SON revealed differences in the pattern of staining among the different experimental groups. Representative examples of NADPH-diaphorase and pERK 1/2 positive cells in the SON are shown in Fig. 3.

Regarding NADPH-diaphorase positive cells, one-way ANOVA showed a significant effect of the hormonal treatment [$F_{(2,9)}$= 61.40; P<0.0001]. The post hoc Bonferroni's test showed that the number of NADPH-diaphorase positive cells was significantly decreased by treatment with GPER agonist G1 (P<0.001) and significantly increased by the administration of G1 in combination with the GPER antagonist G15 (P<0.01), compared to control animals (Fig 3 A-C, Fig. 4 A).

Regarding pERK 1/2 positive cells, one-way ANOVA showed a significant effect of the hormonal treatment [$F_{(2,9)}$= 39.40; P<0.0001]. The post hoc Bonferroni's test showed that the number of pERK 1/2 positive cells was significantly decreased by G1 (P<0.01) and significantly increased by the co-treatment with G1 and G15 (P<0.01), compared to control animals (Fig 3 D-F, Fig. 4 B).

*3.3 NADPH-diaphorase positive cells in the PVN*

NADPH-diaphorase positive cells were observed in the four subdivisions of the PVN: PaDC, PaLM, PaMM and PaV. Qualitative observation of NADPH-diaphorase positive cells in the different PVN subnuclei revealed differences in the pattern of staining among the different experimental groups. Representative examples of NADPH-diaphorase positive cells in the different PVN subdivisions are shown in Fig. 5 A, B, C.

One-way ANOVA was performed for each subnucleus of the PVN. Significant effects of the hormonal treatment were detected in all the subdivisions [PaDC, $F_{(2,9)}$= 79.10; P<0.0001; PaV, $F_{(2,9)}$= 49.51; P<0.0001; PaLM, $F_{(2,9)}$= 86.62; P<0.0001; PaMM, $F_{(2,9)}$= 11.73; P=0.0031].

The Bonferroni's post-hoc test showed that the number of NADPH-diaphorase positive cells was significantly decreased by G1 in the PaDC (P<0.001), the PaV (P<0.001), the PaLM (P<0.01) and the PaMM (P<0.01). This effect of G1 was blocked by G15 in all PVN

subdivisions. In addition, the co-treatment with G1 and G15 resulted in a significant increase in the number of cells in the PaDC ($P<0.05$), the PaLM ($p<0.001$) and the PaV ($p<0.01$) versus control values (Fig. 5 A-C, Fig. 6).

*3.4 Number of pERK 1/2 positive cells in the PVN*

pERK 1/2 immunoreactive cells were observed in the four subdivisions of the PVN. Representative examples in the different PVN subdivisions are shown in Fig. 5 D, E, F.

A significant effect of the hormonal treatment on the number of pERK immunoreactive cells was detected in all the PVN subdivisions [PaDC, $F_{(2,9)}= 31.85$; $P<0.0001$; PaV, $F_{(2,9)}= 53.47$; $P<0.0001$; PaLM, $F_{(2,9)}= 92.26$; $P<0.0001$; PaMM, $F_{(2,9)}= 15.59$; $P=0.0012$]. The Bonferroni's post-hoc test showed that the number of pERK immunoreactive cells was significantly decreased by G1 in the PaDC ($P<0.01$), the PaV ($P<0.001$), the PaLM ($P<0.01$) and the PaMM ($P<0.01$). This effect of G1 was blocked by G15 in all PVN subdivisions. In addition, the co-treatment with G1 and G15 resulted in a significant increase in the number of cells in the PaDC ($P<0.05$), PaV ($P<0.05$) and PaLM ($P<0.001$) versus control values (Fig. 5 D-F, Fig. 7).

## DISCUSSION

The presence of GPER [19-21] and NADPH-diaphorase [2, 38-40] has been demonstrated in rodent SON and PVN. However, to our knowledge this is the first time in which co-expression of both molecules has been reported in the brain. In previous studies we demonstrated an estradiol-dependent regulation of NADPH-diaphorase in the SON and the PVN of ovariectomized females [17, 40]. The co-expression of GPER and NADPH-diaphorase observed in the present study suggests a possible involvement of GPER in the modulation exerted by estradiol on NADPH-diaphorase expression in the SON and PVN. Previous reports have established that pERK 1/2 is involved in GPER-dependent beneficial effects in cerebrovascular insufficiency [41]. On the other hand, pERK 1/2 is known to

modulate NADPH-diaphorase expression in the nucleus of the tractus solitarius (NTS), located in the dorsal medulla and involved in the central control of blood pressure [32]. Our data show that pERK 1/2 and NADPH-diaphorase respond in a similar manner to the activation/inhibition of GPER in the SON and the PVN. The stimulation of the GPER activity by the selective agonist G1 significantly decreased the expression of both pERK 1/2 and NADPH-diaphorase in the SON as well as in all PVN subnuclei. On the contrary, when the activity of the GPER is blocked by the selective inhibitor G15, the G1-dependent decrease of pERK 1/2 and NADPH-diaphorase expression is recovered even over control values in all the structures studied, except for the PaMM where the expressions was similar to the control levels. The increased pERK 1/2 and NADPH-diaphorase levels over control values after G15 treatment may reflect the action of endogenous estradiol acting on classical ERs in the absence of GPER signaling. Previous studies have shown that aromatase, the enzyme involved in estradiol synthesis, is expressed in the SON and PVN [42-43]. In addition, estradiol, acting on ERα and ERβ regulates NADPH-diaphorase in the SON and PVN [17]. However, the effect of ERα is different to the effect of ERβ. Our present findings suggest that the final regulation of NADPH-diaphorase will depend on the balance of the action of exogenous and endogenous estradiol on ERα, ERβ and GPER.

In contrast to our previous data showing a different role of the classical estrogen receptors on the modulation of NADPH-diaphorase expression in the SON and the PVN [9], our present findings indicate that G1 had the same inhibitory effect on the number of NAPDH-diaphorase positive cells in both nuclei. Furthermore, G1 also induced a similar decrease in the number of pERK 1/2 immunoreactive cells in the SON and PVN. These findings suggest that pERK 1/2 may be involved in the GPER-dependent physiological functions in these hypothalamic nuclei. This possibility is in agreement with the fact that pERK 1/2 is involved in the phosphorylation of NOS on Ser1416 in the NTS [32] and that GPER modulates pERK 1/2 decreasing its phosphorylation in human breast cancer cells [25]. Therefore, we could suggest that in the SON and the PVN the activation of GPER inhibits the phosphorylation of ERK 1/2, which induces a decrease in the NADPH-diaphorase activity.

By comparing the present data with those previously obtained through the action of agonists and antagonists of classical estrogen receptors (ERα and ERβ) [17] we may have a picture of the complexity of the estrogenic action on the NADPH-diaphorase system in the PVN and SON of ovariectomized female rats. In the SON, NADPH-diaphorase is modulated in a similar way by the agonists and antagonists of GPER, ERα and ERβ. Activation of these three receptors in the SON consistently downregulates the number of cells expressing NADPH-diaphorase. The situation is different in the PVN, where the activation of ERβ and GPER downregulates and the activation of ERα upregulates NADPH-diaphorase expression. This regional specificity could be due to the neuroanatomical and functional heterogeneity of the PVN. It is important to note that in the rat, GPER as well as ERβ are expressed in both SON and PVN, while ERα is not expressed in these nuclei [44]. However, neurons expressing ERα are projecting to both the PVN [45-46] and the SON [47]. Therefore, the final response of the nitrergic system through ERs would probably be dependent on the balance of a local direct action (GPER and ERβ) and an indirect action through the afferent neurons regulated by ERα, similarly to what has been hypothesized for the vasopressin system [48].

Previous studies demonstrated that nNOS regulation of blood pressure is mediated by pERK 1/2 [32] and, moreover, in a previous work, we showed the estradiol regulates NADPH-diaphorase expression in the SON and the PVN nuclei under acute osmotic stress. Taking all these data together, it may be suggested that GPER, together with the classical ERs, is involved in the regulation of NADPH-diaphorase in the SON and PVN, probably through the modulation of ERK 1/2 activity.

## AUTHOR CONTRIBUTIONS

D.G., G.C.P., L.M.G.S. and P.C. designed and supervised the experiments; D.G., N.L. and H.P. performed the experiments; D.G. prepared the figures for publication; D.G. and L.M.G.S. wrote the first draft of the manuscript; all authors read and approved the manuscript.

## Acknowledgements

We thank Ana Belen Camara Ruiz and Luis Troca de Castro for their generous help during the immunohistochemistry and the NADPH-diaphorase histochemical protocol.

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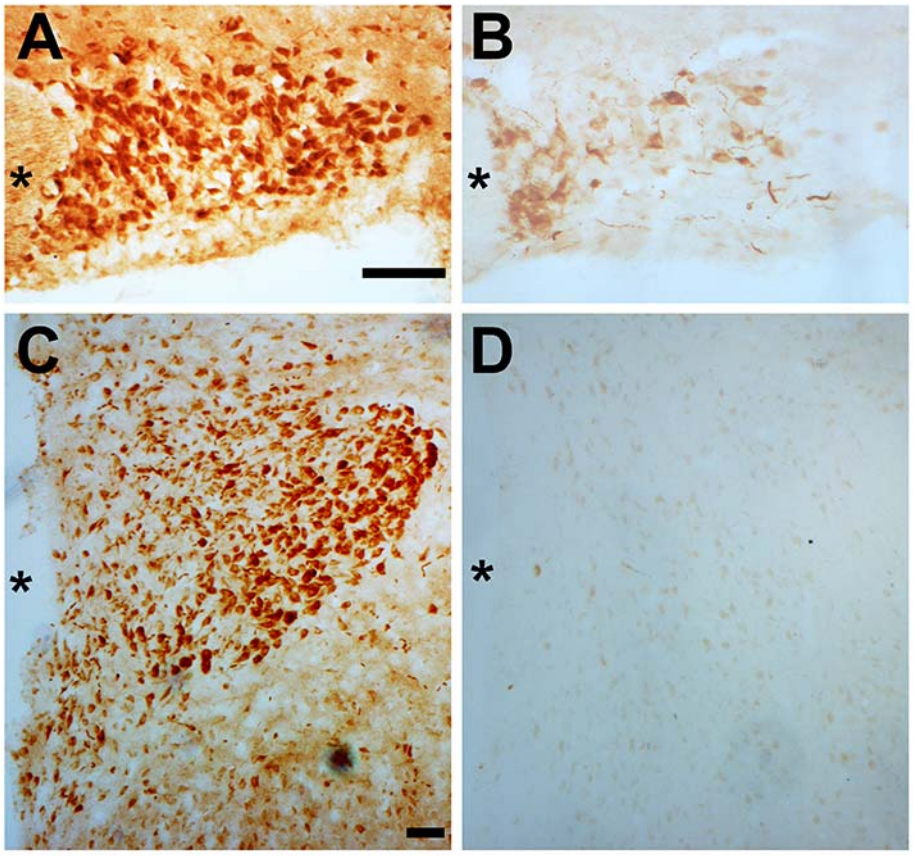



Figure 1. Representative microphotography of GPER immunostaining **A-B** in the supraoptic (SON) and **C-D** in paraventicular nucleus (PVN) of control ovariectomized rats. A-C panels show GPER immunostaining and B-D GPER immunostaining using a preabsorbed antibody with the specific blocking peptide.

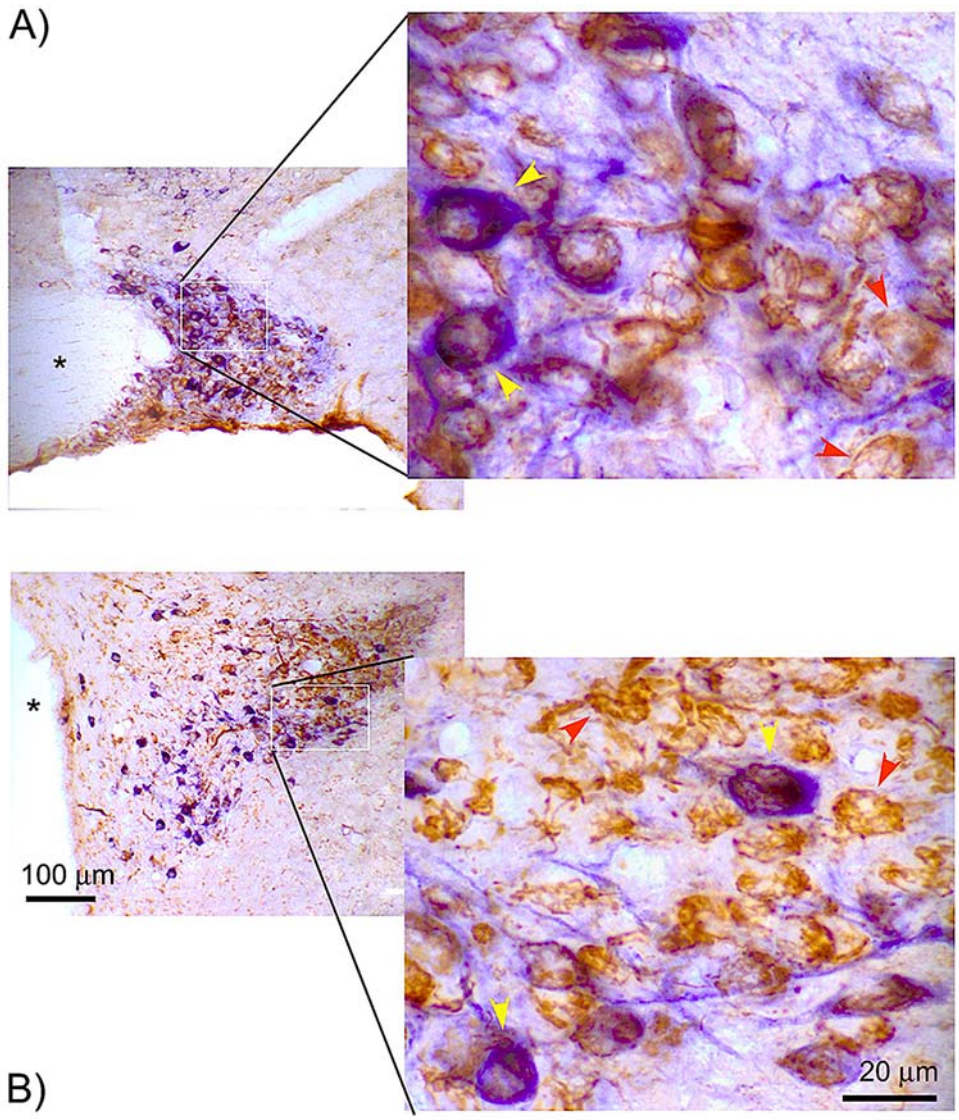



Figure 2. Representative microphotographs of co-localization of NADPH-diaphorase expressing cells and GPER immunoreactive cells within the SON (A) and the PVN (B) of control ovariectomized rats. Yellow arrows indicate double positive staining for NADPH-d and GPER, red arrows indicate GPER staining. (A)* Optic Chiasma (B) * 3rd Ventricle.

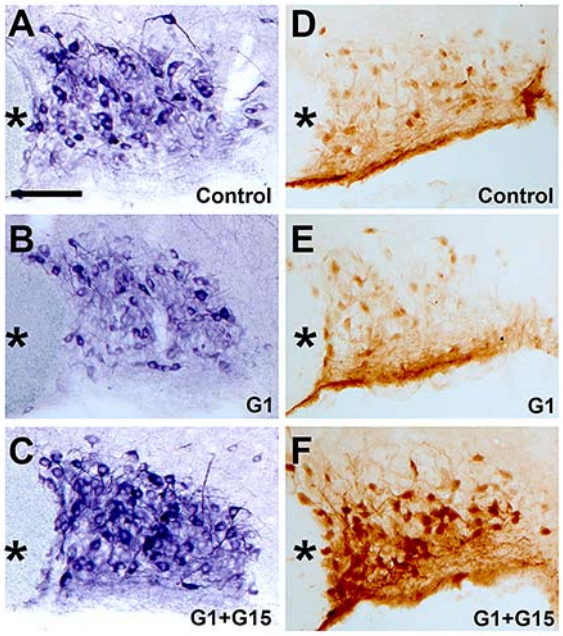



Figure 3. Representative microphotography of NADPH-diaphorase **A-C** and pERK 1/2 immunostaining **D-F** in the supraoptic nucleus. * Optic Chiasma Scale Bar 100µm

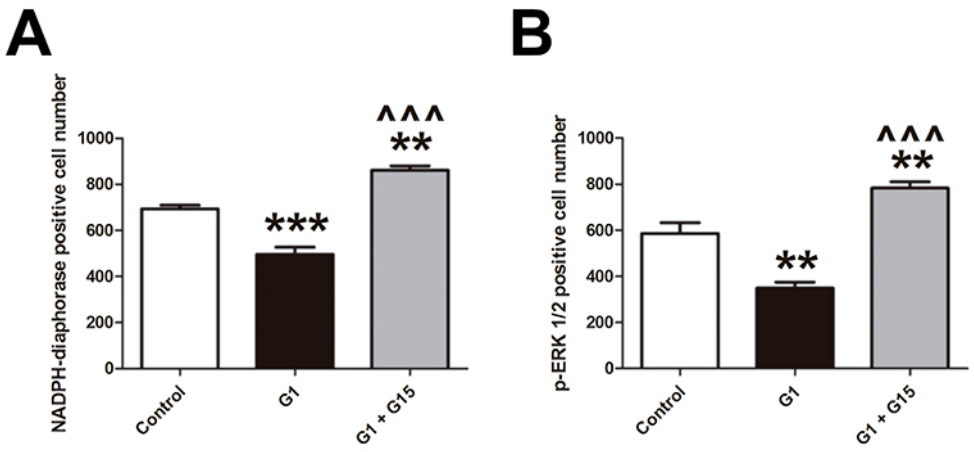



Figure 4. (A) Number of NADPH-diaphorase expressing cells (B) number of pERK 1/2 expressing cells in the supraoptic nucleus in animals treated with vehicle (control), GPER selective agonist G1((±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]- ethanone) or GPER selective agonist G1 in combination with the GPER selective antagonist G15 ((3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline) Significant difference ** ($P < 0.01$) *** ($P<0.001$) versus control values, ^^^ ($P<0.001$) versus G1 values. Data represents the mean ± SEM of the total number of cells per animal in the four sections evaluated.

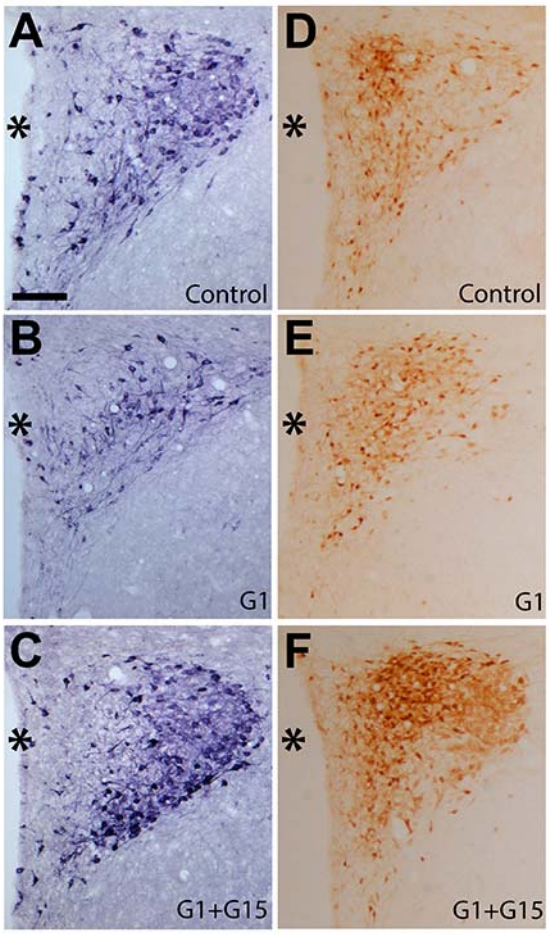



Figure 5. Representative examples of NADPH-diaphorase **A-C** and pERK 1/2 immunostaining **D-F** in the paraventricular nucleus. * 3rd Ventricle Scale Bar 100μm

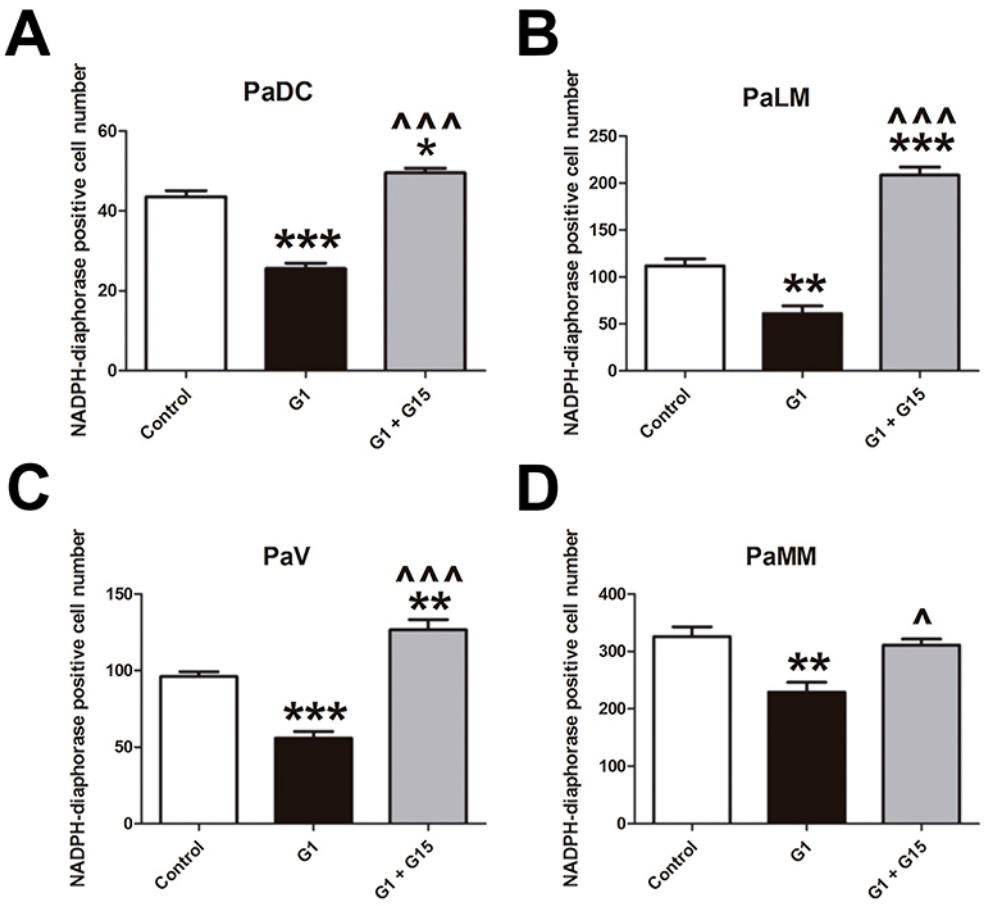



Figure 6. Number of NADPH-diaphorase expressing cells in the paraventicular subnuclei in animals treated with vehicle (control), GPER selective agonist G1((±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone) or GPER selective agonist G1 in combination with the GPER selective antagonist G15 ((3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline). (A) Paraventricular dorsal cap (PaDC). (B) paraventricular lateral magnocellular subdivision (PaLM). (C) paraventricular parvocellular subdivision (PaV) and (D) paraventricular medial magnocellular subdivision (PaMM). Significant difference * (P<0.05) ** (P < 0.01) *** (P<0.001) versus control values, ^ (P<0.05) ^^^ (P<0.001) versus G1 values. Data represents the mean ± SEM of the total number of cells per animal in the four sections evaluated.

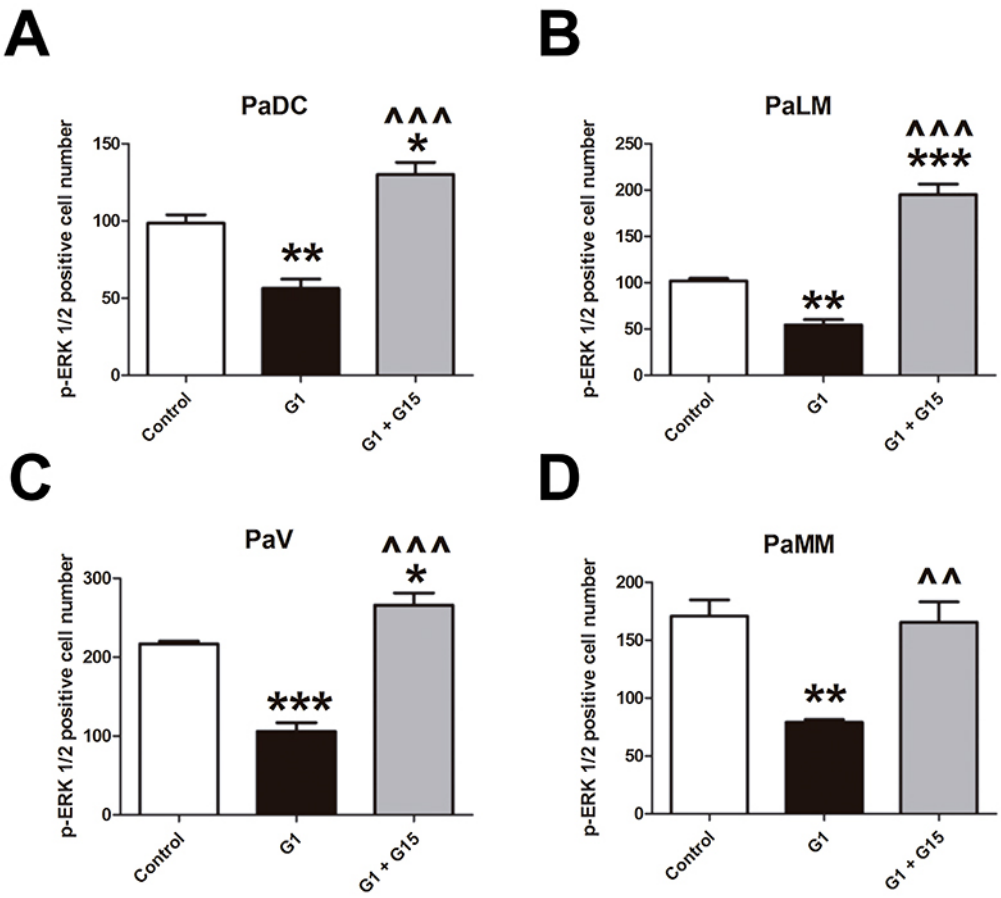
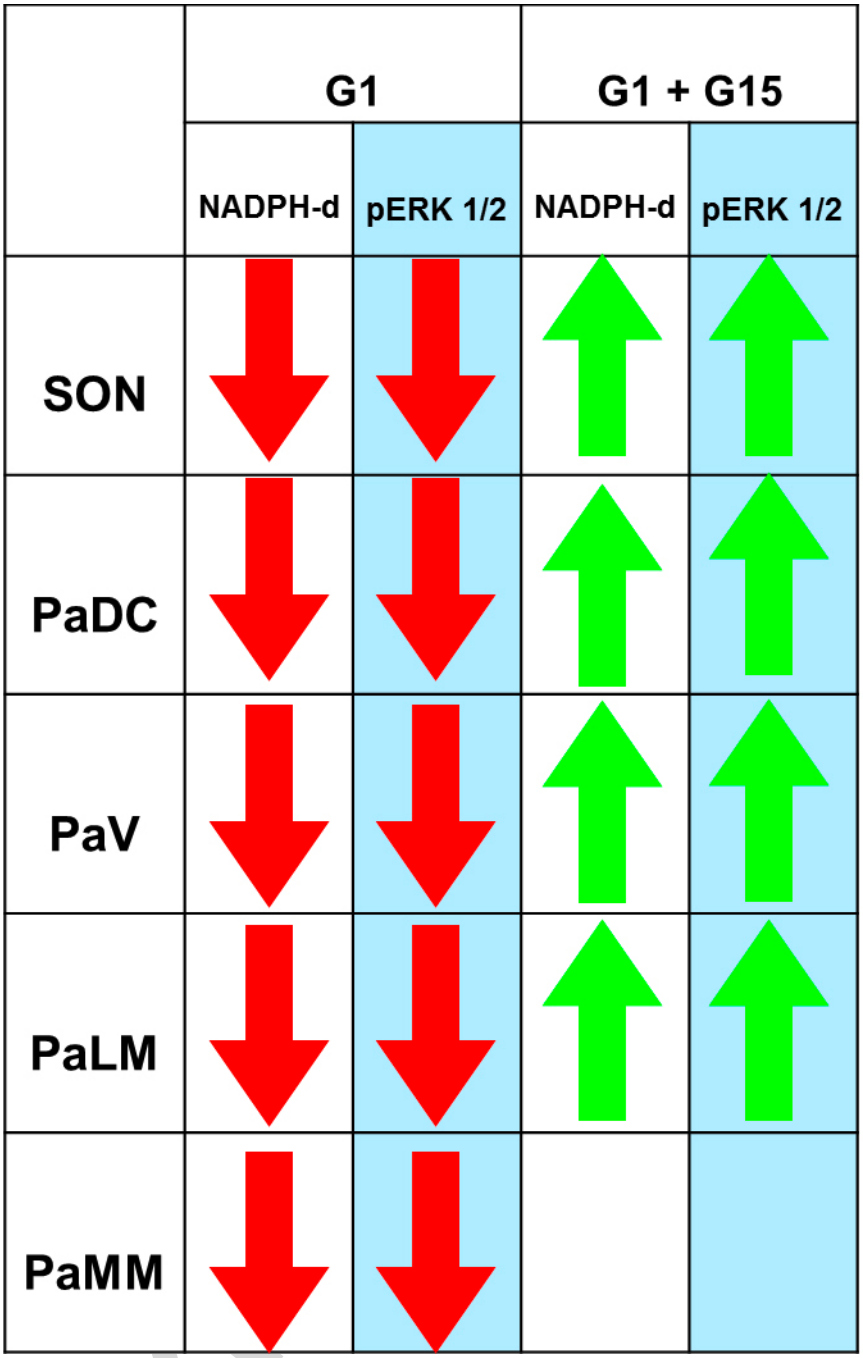



Figure 7. Number of pERK 1/2 expressing cells in the paraventicular subnuclei in animals treated with vehicle (control), GPER selective agonist G1((±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]- ethanone) or GPER selective agonist G1 in combination with the GPER selective antagonist G15 ((3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline). (A) Paraventricular dorsal cap (PaDC). (B) paraventricular lateral magnocellular subdivision (PaLM). (C) paraventricular parvocellular subdivision (PaV) and (D) paraventricular medial magnocellular subdivision (PaMM). Significant difference ** ($P < 0.01$) *** ($P<0.001$) versus control values, ^^ ($P<0.01$) ^^^ ($P<0.001$) versus G1 values. Data represents the mean ± SEM of the total number of cells per animal in the four sections evaluated.

Table 1. Total cell number of NADPH-diaphorase positive and/or GPER immunoreactive cells within the SON and the different PVN subnuclei.

| | NADPH-d positive cell number | GPER positive cell number | GPER and NADPH-d positive cell number |
|---|---|---|---|
| PaDC | 40±0.33 | 81±2.96 | 40±0.33 |
| PaLM | 134±0.57 | 131±4.70 | 134±0.41 |
| PaMM | 367±3.48 | 302±21.20 | 356±1.73 |
| PaV | 90±2.08 | 151±5.81 | 83±2.18 |
| SON | 600±25.85 | 823±35.35 | 573±28.30 |

Table 2. The up-arrows indicate a significant increase and down-arrows indicate a significant decrease in the number of NADPH-diaphorase and pERK 1/2 positive cells.




Neuroendocrinology (*International Journal for Basic and Clinical Studies on Neuroendocrine Relationships*)
Journal Editor: Millar R.P. (Edinburgh)
ISSN: 0028-3835 (Print), eISSN: 1423-0194 (Online)
www.karger.com/NEN