FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2010/2011

João Manuel Palmeira da Rocha Neves

EFFECTS OF CHRONIC ETHANOL TREATMENT AND WITHDRAWAL ON THE EXPRESSION OF NEUROPEPTIDE Y IN THE RAT NUCLEUS ACCUMBENS: AN UNBIASED STEREOLOGICAL STUDY

Abril, 2011

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Mestrado Integrado em Medicina

Área: Neurociências

Trabalho efectuado sob a Orientação de:

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Revista Neuroscience

Abril, 2011

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

Faculdade de Medicina da Universidade do Porto

2010/2011

Unidade Curricular "Dissertação/Monografia/Relatório de Estágio Profissionalizante"

Projecto de Opção do 6º ano – DECLARAÇÃO DE REPRODUÇÃO

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Número do Bilhete de Identidade: 13278664

Título da Dissertação/Monografia/Relatório de Estágio Profissionalizante (cortar o que não interessa):

Effects of chronic ethanol treatment and Withdrawal on the expression of neuropeptide y in the rat nucleus Accumbens: an unbiased stereological study

Orientador:

Professora Doutora Maria Dulce Cordeiro Madeira

Ano de conclusão: 2011

Designação da área do projecto:

Neurociências

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PROJECTO DE OPÇÃO
DISSERTAÇÃO/MONOGRAFIA/RELATÓRIO DE ESTÁGIO

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EFFECTS OF CHRONIC ETHANOL TREATMENT AND WITHDRAWAL ON THE EXPRESSION OF NEUROPEPTIDE Y IN THE RAT NUCLEUS ACCUMBENS: AN UNBIASED STEREOLOGICAL STUDY

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Abbreviations:

ACh, acetylcholine

ANOVA, analysis of variance

C.A.S.T., computer assisted stereological toolbox

CE, coefficient of error

CET, chronic ethanol treatment

CV, coefficient of variation

DA, dopamine

GABA, *gamma*-aminobutyric acid

i.c.v., intracerebroventricular

NAc, nucleus accumbens

NGF, nerve growth factor

NPY, neuropeptide Y

NPY-ir, neuropeptide Y immunoreactive

PB, phosphate buffer

PBS, phosphate-buffered saline

SD, standard deviation

TrkA, tyrosine kinase receptor A

VTA, ventral tegmental area

W, withdrawal

ABSTRACT

The nucleus accumbens (NAc) is known for its role in mediating the reinforcing effects of drugs of abuse. Neuropeptide Y (NPY) displays a pivotal role in anxiety, stress, rewarding processes and drug abuse. It may influence ethanol intake by regulating basal levels of anxiety and by modulating the sedative effects of ethanol and/or its rewarding properties. Nerve growth factor (NGF), whose levels are decreased during chronic ethanol treatment (CET) and withdrawal (W), plays a key role in NPY expression. We examined the effects of CET, W and NGF on the total number of NPY-immunoreactive (NPY-ir) neurons in the NAc. Rats were assigned to adult control, CET and W groups and to an old CET group. Stereological methods and NPY immunostained material were used to estimate the total number and the somatic volume of NPY-ir neurons. We found an increase in NPY expression in adult CET rats. Furthermore, we found that W reduces the expression of NPY to control levels. On the contrary, in old CET rats the expression of NPY was reduced to about 48% of the levels in adult CET animals. It was also found that NGF treatment of W rats significantly increased the expression of NPY in the NAc relative to non-treated W and control rats. These results demonstrate that age interferes with the effects of CET in the expression of NPY in the NAc and that NGF regulates the phenotype of NPY-ir neurons. This study might help to explain the cryptic role of NPY in alcohol intake, dependence and W.

KEY WORDS

Nucleus accumbens, neuropeptide Y, nerve growth factor, alcohol, withdrawal, stereology.

The nucleus accumbens (NAc) is a central component of the limbic system (Goto and Grace, 2008) and a major terminal area of the dopaminergic mesocorticolimbic system, known to play a critical role in the motivational and addictive properties of drugs of abuse (Ochoa et al., 1990; Kelley et al., 1997). It is immunohistochemically divided into three subterritories: in the caudal three fourths it is composed by a central "core" and a surrounding "shell" and in the rostral one-fourth, where the core and shell cannot be identified, by the "rostral pole" (Zahm and Heimer, 1993). It is one of the brain regions with highest concentrations of neuropeptide Y (NPY; Salin et al., 1990), a 36 amino acid peptide that has remained extremely well conserved throughout vertebrate evolution (Barreiro-Iglesias et al., 2010) and is abundantly expressed in the central nervous system, most notably in the limbic structures (Heilig, 2004). NPY displays a pivotal role in anxiety, stress, rewarding processes, neurobiological responses to ethanol and drug abuse (Heilig et al., 1992; Pandey et al., 2003; Cippitelli et al., 2010).

Nerve growth factor (NGF) is a member of the neurotrophin family. Neurotrophins are a family of growth factors that critically regulate many aspects of neuronal development and function, exerting their effects via two types of receptors, the tyrosine kinase receptor (TrK) and the p75 neurotrophin receptor (Huang and Reichardt, 2003, Hempstead, 2006, Zampieri and Chao, 2006). There is growing evidence that neurotrophins regulate the gene expression and the protein levels of neuropeptides under several conditions (Lindsay and Harmar, 1989, Croll et al., 1994, Nawa et al., 1994, Carnahan and Nawa, 1995). With respect

to NGF, it has been recently shown that it can restore the activity of NPYergic neurons in the NAc (unpublished results) and somatosensory cortex (Cardoso et al., 2006) of aged rats.

In rodents, chronic ethanol treatment (CET) and withdrawal (W) alter NPY levels in several brain areas, such as the arcuate nucleus and the cingulate gyrus (Clark et al., 1998; Ehlers et al., 1998; Roy and Pandey, 2002). Previous studies have also shown that, in adult rodents, CET reduces NGF and TrkA levels in several regions of the brain (Aloe and Tirassa, 1992; Aloe et al., 1993; Heaton et al., 2003). On the grounds of these data, we have hypothesized that CET and W might alter NPY expression in the NAc through a mechanism mediated, in part at least, by changes in NGF levels. To test this possibility, we have examined the effects of CET and W on the total number of NPY–immunoreactive (NPY-ir) neurons in the NAc, and investigated if the administration of NGF interferes with the expression of NPY by its neurons. Because the levels of NGF and its mRNA have been reported to be reduced in several regions of the aged brain (Terry et al., 2011), we have additionally investigated whether there are age-dependent effects of CET on the NPY expression in the NAc.

EXPERIMENTAL PROCEDURES

Animals and treatments

A total of 25 Wistar male rats were used. Food and water were available *ad libitum* until rats were 2-months old. Then, rats were assigned to control (n = 5), adult CET (n = 15) and old CET (n = 5) groups. Adult CET rats received a 20% (V/V) aqueous ethanol solution as their only available liquid source for 6 months, starting at 2 months of age. At 8 months of age, 10 rats were smoothly shifted from ethanol treatment to water intake during further 2 months.

Five of these rats (W group) received no further treatment whereas the remaining (n = 5; W+NGF group) were submitted to surgery for implantation of osmotic minipumps that allowed intracerebroventricular (i.c.v.) delivery of NGF. Because there is evidence that this surgical procedure does not interfere with the central NPYergic system (Cardoso et al., 2006), vehicle-treated rats were not included in this study. The old group of CET rats was treated as the adult one, but from 18 to 24 months of age.

The experiments were performed in accordance with European Communities Council Directives of 24 November 1996 (86/609/EEC) and Portuguese Act no. 129/92. All efforts were made to minimize the number of animals used and their discomfort and suffering.

Surgical procedures and drug treatment

For i.c.v. administration of NGF, rats were anesthetized by sequentially injecting, at intervals of 10 min, solutions in physiological saline of 0.25% promethazine (0.4 ml/kg body weight, subcutaneous; Fenergan, Laboratórios Vitória, Amadora, Portugal), followed by 2% xylazine (0.132 ml/kg body weight, intramuscular; Sigma – Aldrich, Madrid, Spain) and, finally, 10% ketamine (0.5ml/kg body weight, intramuscular; Imalgene 1000, Merial Portuguesa, Rio de Mouro, Portugal). Then, they were placed on a stereotaxic apparatus with bregma and lambda in the same horizontal plane. After a midline incision on the scalp, the calvaria was exposed. For i.c.v. delivery of NGF, permanent stainless steel cannulae (Alzet brain infusion kit) were stereotaxically placed in the right lateral ventricle at the following coordinates (Paxinos et al., 1998): 1.1 mm posterior to the bregma, 1.7 mm lateral to the midline, and 4.0 mm below the surface of the skull. The cannulae were connected to Alzet osmotic minipumps (model 2002; Alza Corporation, Palo Alto, CA, USA) and filled with methylene blue (0.01%, Sigma – Aldrich, Madrid, Spain) via sterile coiled polyethylene tubing (PE-60; Intramedic, Becton Dickinson, Sparks, MD, USA). This tubing was filled with air–oil spacer at the pump end

(Vahlsing et al., 1989), and with NGF at the cannula end (150 μg diluted in 150 μl of vehicle). Osmotic minipumps were pre-tested to confirm their delivery rate, and implanted subcutaneously in the neck. In all operated rats, incisions on the skin of the scalp were closed with surgical stitches and treated with local antiseptic. After surgery, rats were individually housed and maintained in a warm place until waking up. Postoperative care consisted of subcutaneous injections of 0.9% saline (2 ml) to prevent dehydration and weight loss. Twelve days after the beginning of the i.c.v. infusions, the animals were killed and the total infusion volume calculated. The total infusion volume was 135.31 ± 27.31 μl of NGF per animal.

Tissue preparation

At the end of the experimental periods, animals were deeply anesthetized by intraperitoneal injection of a solution (3 ml/kg body weight) containing 1% sodium pentobarbital and 4% chloral hydrate in physiological saline. Then, they were perfused transcardially with 150 ml of 0.1 M phosphate buffer (PB; pH 7.6) for vascular rinse, followed by 250 ml of a fixative solution containing 4% paraformaldehyde in PB, at pH 7.6. The brains were removed from the skulls, coded, immersed for 1 h in the same fixative, and maintained overnight in a solution of 10% sucrose in PB, at 4 ºC. The brains were placed on a vibratome and serially sectioned in the coronal plane at 40 μm through the NAc. The sections were collected in phosphate-buffered saline (PBS).

From the entire set of sections obtained from each brain, four series were formed by using a systematic, random sampling procedure. Specifically, the first section of each set was randomly selected, and the remaining were sampled, along the entire rostrocaudal extent of the NAc, at regular intervals of 160 μm, that is, by sampling 1 out of 4 sections. The first set thus formed was used for Nissl staining, whereas the other, composed of sections adjacent to those used for Nissl staining, were used for NPY immunostaining.

Nissl staining

Sections were mounted serially on gelatin-coated slides. After air-drying overnight at room temperature, they were stained with Cresyl Violet, dehydrated, and coverslipped with Histomount (National Diagnostics, Atlanta, GA, USA). The observations of these sections under light microscopy allowed to accurately establish the boundaries of the NAc in the immunostained sections (Fig. 1A).

Immunostaining for NPY

Sections were washed twice in PBS, treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase, and incubated overnight at 4 ºC with the primary antiserum against NPY (Chemicon, Temecula, CA, USA; 1: 10,000 dilution in PBS). Biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA, 1 : 400 dilution in PBS) was used as the secondary antibody. Sections were then treated with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA, 1 : 800 dilution in PBS). In the last two steps, the incubation was carried out for at least 1 h at room temperature. Following treatment with peroxidase complex, sections were incubated for 10 min in 0.05% diaminobenzidine (Sigma – Aldrich, Madrid, Spain) to which 0.01% H_2O_2 was added. Sections were rinsed with PBS for at least 15 min between each step. Tissue penetration was increased by the inclusion of 0.5% Triton X-100 in all immunoreactions and washes. All procedures were performed on a rocking table.

Immunostained sections were mounted on gelatin-coated slides and air-dried. They were then dehydrated in a series of ethanol solutions (70%, 90%, 95% and 100%), cleared in xylol and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA). To prevent variability in staining, sections from all groups analyzed were processed in parallel at

the same time. The same procedure was followed for control sections, which were incubated without antiserum; no immunostaining was observed in these sections.

Stereological analysis

The optical fractionator method (West et al., 1991; Madeira et al., 1997) was used to estimate the total number of NPY-ir neurons on blind-coded slides. The boundaries of NAc were consistently defined on the basis of cell morphology (Fig. 1) and cytoarchitectonic criteria. The method for carrying out this delimitation was the systematic identification of a high cell density area around the anterior commissure, and located beneath the rostral caudate-putamen and above the olfactory tubercle. The cellular pattern of this area resembles that of the caudate-putamen and olfactory tubercle to such an extent that it is difficult to define the border between them. Likewise, the caudal parts of the nucleus merge without clear boundary into the bed nucleus of the stria terminalis. Conversely, the medial part of the NAc is separated from the septum-diagonal band complex by a distinct, arciform boundary, whereas rostrally and laterally it extends until the external capsule (Zahm and Brog, 1992).

Cell counting was carried out using an Olympus C.A.S.T.-Grid system (Olympus DK A/S, Denmark) and a Hiedenhain MT-12 microcator (Heidenhain, Germany). All the estimations were performed at a final magnification of 2000x. All the sections previously immunostained for NPY and containing the NAc were used, which provided an average of 13 sections per animal. In each section, fields of view were systematically sampled using a step size of 250 μm along the x and y axes; the disector used had a counting frame area of 8436 μm^2 at the tissue level and a fixed depth of 10 μm. Immunostaining of the perikaryal cytoplasm with a relatively unstained nucleus was the criterion used for the identification of NPY-containing neurons (Fig. 2). By applying this sampling scheme, an average of 211

NPY-ir cells was counted per nucleus. The mean coefficient of error (CE) of the estimates was 0.08.

The mean somatic volume of NPY-ir neurons was estimated by applying the optical rotator method (Tandrup et al., 1997). Neurons used for measurements were selected by means of the optical disector (West and Gundersen, 1990; Madeira et al., 1995; Leal et al., 1998; Paula-Barbosa et al., 2001), as described above, but by using a step size of 350 μm along the x and y axes. These procedures were implemented with the C.A.S.T.-Grid system software, which allows the estimation of the mean somatic volume using a spatial line grid. Measurements of intersections between the cell membrane and the spatial line grid were performed using a two-grid line and two focal planes per each cell, at magnification of 2000x. The mean CE of the estimates was 0.07.

Statistical analysis

The precision of individual estimates of neuron numbers was evaluated as the CE, as described by Gundersen et al. (1999). The precision of individual estimates of mean somatic volumes was calculated by applying the equation: $CE^2 = CV^2/n$ (Gundersen and Jensen, 1987), in which CV denotes the intraindividual coefficient of variation (CV = Standard deviation/mean) and n is the number of observations in one animal. The mean CE was calculated from estimates for an individual, as described by West et al. (1991). Differences across groups were assessed by one-way analysis of variance (ANOVA). Whenever significant effects were detected by ANOVA, pair-wise comparisons were done by using the Tukey's HSD test. Comparisons between adult and old CET rats were done using the Student's t-test for independent variables. Differences were considered significant if $P < 0.05$.

RESULTS

Qualitative observations

No differences in the location and packing density of NPY-ir neurons were noticed among the groups studied.

Total number of NPY-immunoreactive neurons

The estimates of the total number of NAc NPY-ir neurons are shown in Figure 3. ANOVA revealed that the variations in the NPY expression were dependent on the effect of treatment ($F_{(3,16)} = 5{,}301$; $P = 0.011$). The statistical analysis revealed that CET and W+NGF produced a significant increase in the total number of NPY-ir neurons relatively to control (Tukey´s post hoc test: $P = 0.017$ and $P = 0.038$, respectively) and W (Tukey´s post hoc test: $P = 0.018$ and $P = 0.042$, respectively) rats. However, no significant differences were detected in the total number of NAc NPY-ir neurons between control and W rats. Additionally, in old CET rats the total number of NPY-ir neurons was reduced by 48% relative to adult CET rats (Student`s t-test, $P < 0{,}0001$).

Somatic size of NPY-immunoreactive neurons

Statistical analysis (ANOVA) revealed a significant effect of treatment on the mean somatic volume of neurons immunostained for NPY ($F_{(3,16)} = 2.015$; $P = 0{,}0452$) and the Tukey´s post hoc test revealed no significative differences between groups. Likewise, Student`s t-test also revealed no differences ($P = 0{,}056$) in the somatic volume of NPY-ir neurons between adult and old CET animals (Fig. 4).

DISCUSSION

The present study reveals an age-dependent effect of ethanol on the total number of NPY-ir neurons in the NAc. Indeed, the results of the present study show that in adult CET rats the number of NPY-ir neurons is higher than in controls and old CET rats. This study also indicates that W restores the number of NPY-ir neurons to control numbers and that NGF administration to W rats leads to an increase in the expression of NPY in the NAc.

Additionally, it is also shown that there are no differences in the somatic size of NPY-ir neurons among all groups analyzed. The effect of CET and W on somatic volumes seems to be region-specific. In fact, no CET- or W-induced changes have been noticed in the hypothalamic parvicellular neurons (Silva et al., 2002; Paula-Barbosa et al., 2003), as opposed to the hypothalamic magnocellular neurons where CET increases and W restores neuronal volumes (Madeira et al., 1993) and to the basal forebrain cholinergic neurons, where CET induces a reduction in the somatic volume that is further aggravated by W (Cadete-Leite et al., 2003). The absence of variation in neuronal size of W rats treated with NGF is interesting in light of data showing that administration of NGF to W rats leads to neuronal hypertrophy in the suprachiasmatic nucleus and basal forebrain (Paula-Barbosa et al., 2001; Cadete-Leite et al., 2003). Similar to this work, it was previously reported that NGF treatment of old rats does not induce somatic size changes in NPY-ir neurons of the NAc (unpublished results).

Previous investigations have shown that CET, as well as W, alter central NPY expression. CET and W have been found to reduce NPY signaling in the cortex, amygdala and hypothalamus (Roy and Pandey, 2002). On the contrary, it has also been demonstrated that CET (Clark et al., 1998) and W (Bison and Crews, 2003) induce a significant increase in NPY in the hypothalamus and hippocampal formation, respectively. However, these

investigations were restricted to biochemical data and qualitative observations of immunostained material. In our study, an increase was found in the total number of NPY-ir neurons in adult CET rats. It has been suggested that increased NPY activity in response to ethanol may serve as a protective mechanism to further limit ethanol intake (Thiele et al., 2000). This occurrence might be explained by the long-term increase in cholinergic activity parallel to a similar raise in the dopaminergic neurotransmission in the NAc due to CET (Nestby et al., 1997; Nestby et al., 1999). Accumulated evidence suggests a crucial role for central dopaminergic and dopamine (DA)-sensitive neurons in mediating natural as well as drug-rewarded behavior (Nestby et al., 1997). Expression of NPY in the rat NAc is under the influence of the dopaminergic mesencephalic pathway, through D1 postsynaptic receptors (Lindefors et al., 1990; Salin et al., 1990; Midgley et al., 1994). The mechanism underlying ethanol activation of this dopaminergic system is not clearly understood. However, it has been suggested that ethanol elevates DA levels by either disinhibiting GABAergic (*gamma*-aminobutyric acid) interneurons due to direct activation of $GABA_A$ receptors, thus removing tonic inhibitory influences, or by influencing afferents, that will modulate the firing properties of the ventral tegmental area (VTA) pathways (Grobin et al., 1998; Floresco et al., 2003). So, the cholinergic excitatory input to the dopaminergic neurons in the VTA might be an important part of this neuronal circuits (Larsson and Engel, 2004). Mesencephalic dopaminergic neurons receive cholinergic inputs from the pedunculopontine and the laterodorsal tegmental nuclei (Woolf, 1991), and the mesencephalic pathway is the main known dopaminergic afferent system of the NAc (Chen et al., 2009). It has been shown that cholinergic agonists administered into the VTA increase extracellular DA levels in the NAc (Westerink et al., 1996). Subsequently, it was demonstrated that ethanol increases extracellular acetylcholine (ACh) levels in the VTA, and that this increase leads to a raise in DA release in the NAc, through activation of nicotinic ACh receptors located in the VTA

(Larsson et al., 2005; Löf et al., 2007). However, other explanations might exist since the regulation of NPY levels in the NAc is believed to present a high level of complexity.

As mentioned before, this study revealed that W restored the total number of NPY-ir neurons to control values. This was an interesting finding in view of the existence of studies reporting an increase in the NPY-ir content of the hippocampal formation of W rats, being hypothesized that it would exert protective effects against seizure activity (Bison and Crews, 2003).

Concerning the increased NPY expression in W+NGF rats, there is evidence from *in vivo* studies that many developing neurons express NPY transiently early during development (Wahle et al., 2000; Wirth et al., 2005). Thus, it is possible that in adult rats these neurons might be capable of expressing NPY when exposed to higher than normal steady-state levels of neurotrophins (Wirth et al., 2005). Indeed, in the visual cortex and geniculate nucleus, it was demonstrated that increased levels of NGF are associated with an up-regulation of cells immunoreactive for NPY (Aloe et al., 2001). A possible explanation for this finding is the activating effect of this neurotrophin on the cholinergic interneurons of the NAc, which in turn modulate NPY expression in this nucleus. Cholinergic interneurons are the only known direct source of ACh in the rat NAc (Ligorio et al., 2009). There is a growing body of evidence showing that NGF increases the choline acetyltransferase content and the cell body size of cholinergic interneurons of the neostriatum and NAc (Fischer et al., 1988; Hagg et al., 1989), and regulates the phenotype of NPY-ir neurons. (Barnea et al., 1996; Cardoso et al., 2006). In the few studies that have analysed the distribution of NGF receptors in the NAc, it was demonstrated that almost all TrkA-immunoreactive neurons co-express choline acetyltransferase (Sobreviela et al., 1994). Furthermore, it has been shown that NAc contains no immunoreactive cells for p75 neurotrophin receptor (Barrett et al., 2005). Since cholinergic and NPYergic cells are morphologically different, large and medium size

respectively (Kawaguchi et al., 1995, Pickel et al., 1998), it is not plausible that there would be co-expression of ACh and NPY in the same neuron. Thus, our results might derive either from a direct role of ACh in the control of NPYergic neurons (Wettstein et al., 1995, Cardoso et al., 2006) or from the cholinergic control of DA release in the NAc (Del Arco et al., 2008).

In this study, we found a significant decrease in the number of NPY-ir neurons in old CET rats when compared to adult CET rats. A previous study has shown that aging leads to an approximately 18% decrease in the levels of NPY in the NAc (unpublished results). Thus, the finding in the present study of 48% reduction in the number NPY-ir neurons shows that there is an age-dependent effect of CET on the total number of NPY-ir neurons in the NAc. One possible explanation is that CET leads directly to death of this neuronal population in aged rats. However, this is not likely because in an earlier study (unpublished results) it was shown that NGF is fully capable of restoring the age-associated changes in the chemical phenotype of these NAc neurons. However, we cannot exclude this possibility because the total number of NAc neurons was not estimated in the present work. In addition, a previous study demonstrated a statistically significant neuronal loss in the locus ceruleus neurons of old CET rats, as opposed to what was observed in adult CET rats (Jaatinen et al., 2003). Another possibility is that the ethanol-induced DA release in the NAc is reduced in aged rats compared with younger animals, though the basal extracellular DA levels is increased in older groups (Yoshimoto et al., 1998).

CONCLUSIONS

This study might help to explain the cryptic role of NPY in alcohol intake, alcohol dependence and W. A direct link between NPY signaling and regulation of alcohol

consumption was first shown in a study where transgenic mice with over-expression of NPY consumed less alcohol (Thiele et al., 1998). It was proposed that the up-regulation of NPY expression may contribute to the successful behavioral adaptation to stress (Thorsell, 2008). The NPY system may well be one of the most interesting target systems for the development of treatments for alcohol dependence as well as mood disorders, such as depression and anxiety syndromes (Thiele et al., 2002). Alcoholism is often under-diagnosed and untreated in family practice. Alcohol dementia causes serious cognitive problems in many alcoholics and up to 19% of patients diagnosed with dementia have a history of prolonged alcohol abuse (Zuccala et al., 2001). Additionally, alcohol abuse in older adults is common and the problems associated with it are often mistaken for other conditions related with the aging process, leading to misdiagnosis and undertreatment of alcohol abuse and alcoholism in this population (Loukissa, 2007).

ACKNOWLEDGEMENTS

This study was supported by Centro de Morfologia Experimental (Unit 121/94 – FCT). The author thanks Professors M.D. Madeira and M.M. Paula-Barbosa for the supervision of this work. The author is also grateful to Dr. Pedro Pereira for the continuous help in the elaboration of this work.

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FIGURE CAPTIONS

Fig. 1. Photomicrographs of adjacent coronal sections at mid-NAc levels of an adult rat, stained with Cresyl violet (A) and immunostained for NPY (B). ac, anterior commissure. CPu, caudate putamen. lv, lateral ventricle. NAc, nucleus accumbens. Scale bars = 125 μm.

Fig. 2. Photomicrograph of a NAc NPY-ir neuron to illustrate the criteria used for sampling neurons for quantitative estimations. Scale bar = 5 μm.

Fig. 3. Graphic representation of the total number of NPY-ir neurons in the NAc of Control, Adult+CET, Withdrawal, Withdrawal+NGF and Old+CET rats. Columns represent means and vertical bars 1 SD. The number of NPY-ir neurons is significantly increased in Adult+CET relative to Control and Withdrawal rats. In Withdrawal+NGF rats, the total number of NPY-ir neurons is significantly augmented relative to the Control and Withdrawal groups. The number of NPY-ir neurons is significantly reduced in Old+CET rats relative to Adult+CET animals. Tukey's post hoc tests: * $P < 0.05$, compared with Control and Withdrawal rats; ** $P < 0.05$, compared with Control and Withdrawal rats; Student`s t-test *** $P < 0.05$, compared with Adult+CET animals.

Fig. 4. Graphic representation of the estimates of mean somatic volumes of NAc NPY-ir neurons obtained from Control, Adult+CET, Withdrawal, Withdrawal+NGF and Old+CET rats. Columns r epresent means and vertical bars 1 SD. The somatic size of NPY neurons is similar in all groups analyzed.

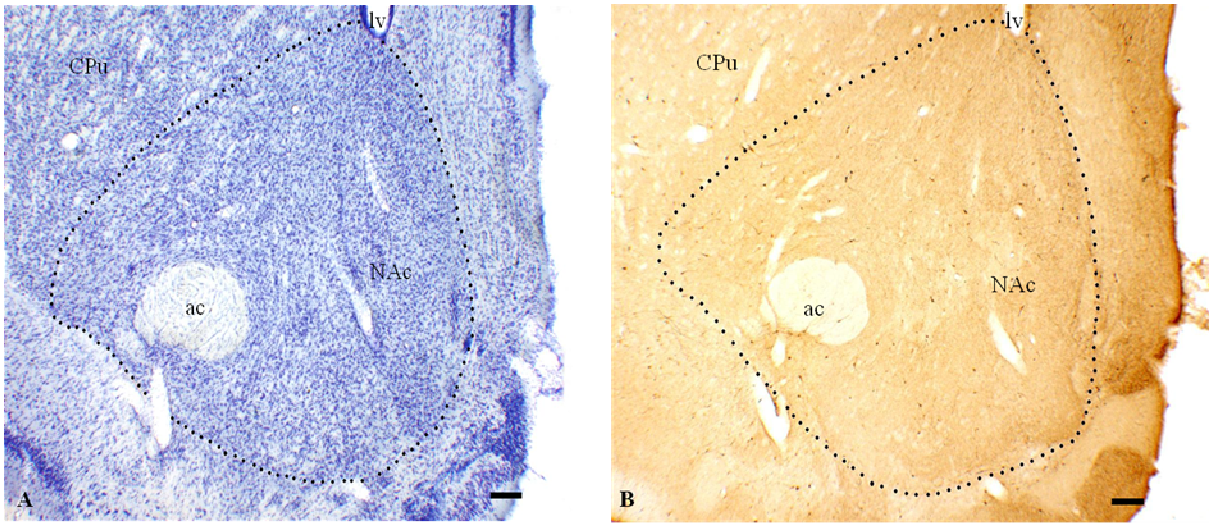

Figure 1 (A, B)

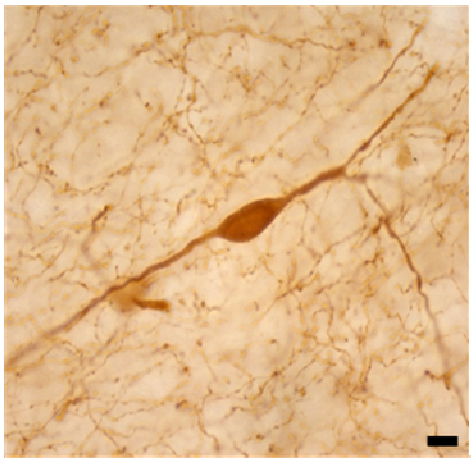

Figure 2

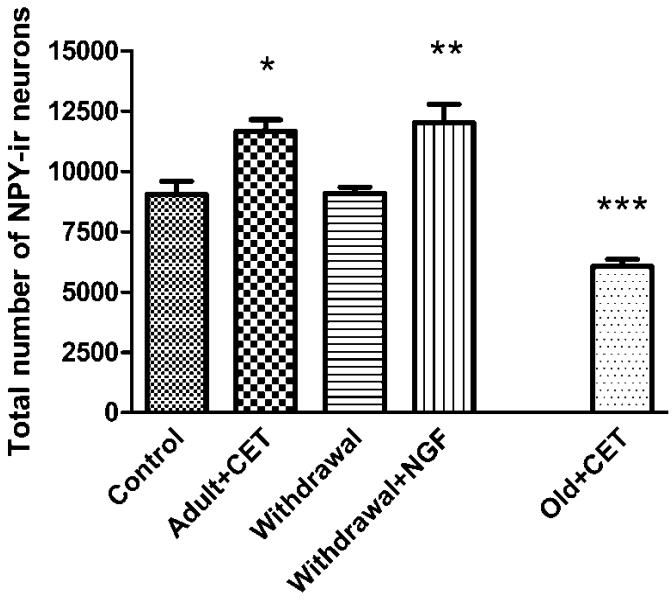
Total number of NPY-ir neurons
15000
12500
10000
7500
5000
2500
0
*
**
***
Control
Adult+CET
Withdrawal
Withdrawal+NGF
Old+CET

Figure 3

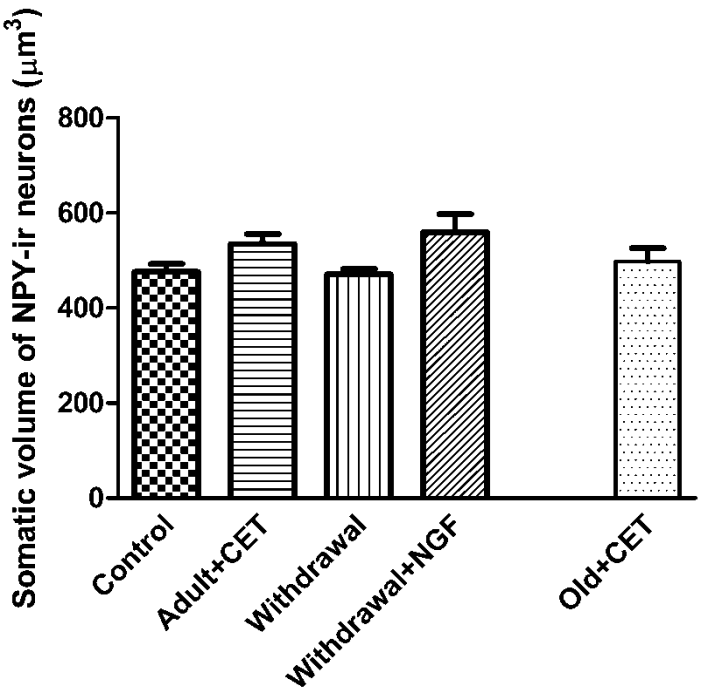
Somatic volume of NPY-ir neurons (μm3)
800
600
400
200
0
Control
Adult+CET
Withdrawal
Withdrawal+NGF
Old+CET

Figure 4

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