Chronic Stress Effects on NPY Neuronal Population During Rat Development

Efectos del Stress Crónico sobre la Población Neuronal NPY Durante el Desarrollo de Ratas

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BULJUBACICH, K. A.; MUGNAINI, M. T.; SOÑEZ, C. A.; ROLANDO, A. N.; ROMANINI, M. C.; BOZZO, A. A.; SOÑEZ, M. C. & GAUNA, H. F. Chronic stress effects on NPY neuronal population during rat development. *Int. J. Morphol.*, 27(3):879-889, 2009.

SUMMARY: The aim of this work was to determine the chronic stress effects on the encephalic NPY neurons population during the fetal Central nervous system development. Immunocytochemical techniques were used for this purpose: NPY neurons presented a similar morphology during the gestation days studied but their distribution varied in the anterior, medium and posterior brain. Statistical Highly significant differences in number of NPY positive neurons ($p < 0.01$) among anterior, medium and posterior brain of stressed fetus (SF) were determined depending on the gestation period and the brain area. The NPY neurons were increased in ARC (Arcuate Hypothalamic Nucleus), PH (Posterior Hypothalamic Area) and DM (Dorsomedial Hypothalamic Nucleus) in stressed fetuses (SF) of 17 days, and in ARC of 19 days SF ($p < 0.01$) were detected in the different brain nucleus. The NPY population increased in PnO (Pontine Reticular Nu, Oral Part) and RITg (Reticulotegmental Nu of the Pons) of 17 days SF, while they were detected in posterior brain at Pyx (Pyramidal Decussation), Rob (Raphe Obscurus Nucleus) and RPA (Raphe Pallidus Nucleus) in SF of 19 days. They also increased in number ($p < 0.05$) in DPGI (Dorsal Paragigantocellular Nu), CGPn (Central Gray of Pons) and PrH (Prepositus Hypoglossal Nucleus) of 17 days SF. Finally, any statistical differences were found among CF and SF in the following nuclei: anterior brain, AH (Anterior Hypothalamic Nucleus), DM (Dorsomedial L Hypothalamic Nucleus) of 17 days; ME (Median Eminence), VMH (Ventromedial Hypothalamic Nucleus) of 19 days; medium brain in CG (Central Periaqueductal Gray), DR (Dorsal Raphe Nucleus) of 17 days and posterior brain in PnC (Pontine Reticular Nu, Caudal Part), PrH (Prepositus Hypoglossal Nucleus), RMgG (Raphe Magnus Nucleus), IO (Inferior Olive) of 17 days. The increase number of NPY neurons found in the stressed rat fetuses in all periods studied would indicate the participation of the NPY System in the regulation of H.H.A axis.

KEY WORDS: NPY; Prenatal chronic stress; CNS; Rats.

INTRODUCTION

From the beginning of mammal life, the continuity of its different levels of organization depends on the capacity of the organism to support an internal dynamic equilibrium by homeostatic mechanisms. This complex dynamic equilibrium is constantly changed by intrinsic or extrinsic adverse forces which may be real or perceived and are called stressors or stressing agents (Pacák & Palkovits, 2001).

The neuropeptide Y molecule (NPY) is a neuromodulator of 36 aa with a characteristic aminotyrosine carboxile terminal group. It belongs to a peptide family structurally related: pancreatic polypeptide (PP), peptide YY

(PYY) from gastrointestinal tract and pancreatic peptide from fish (Py) (Tatemoto & Mutt, 1978; Wahlestedt & Heilig, 2002). It is widely distributed in the central and peripheral nervous systems during development and in adulthood of vertebrate species. It shows homologue sequences from fish to mammals. This neuropeptide has been also localized in sympathetic neurons that innervate the cardiovascular, respiratory, gastrointestinal, genitourinary systems and in the placenta (Tatemoto *et al.*, 1982; Thiele & Heilig, 2002)

The primary population of hypothalamic NPY neurons has been found in the arcuatum nucleus (ARC).

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These neurons projected towards the paraventricular nucleus (PVN), the pre-optic region and possibly the median eminence (EM) (Tatemoto & Mutt). They also coexist with gabaergic neurons in the brain cortex and in the PVN. In the striatum and the cortex they coexist with somatostatin and NADPH nitric diaphorase/oxide neurons (Holmes & Crawley, 1995). The localization of NPY was demonstrated in monoaminergic neurons such as noradrenergic neurons of A2 group of the ventro-lateral and dorsal spine; in the coeruleus locus, the adrenergic neurons of C1 group and C2 group of the solitary tract and serotonin neurons of the nucleus of the raphe (Wahlestedt & Heilig). NPY act in the regulation of brain functions and during physiologic and pathologic processes such as the neuroendocrine emotion regulation, alcoholism and energetic balance, modulation of GnRH release from hypothalamus, LH, ACTH, vasopressin and GH release from hypophysis (Thiele & Heilig; Urban *et al.*, 1993). The different biological responses are mediated by the sub-types of G-protein-coupled receptors (GPCRs) known as Y1-Y6 (Berglund *et al.*, 2003; Michel *et al.*, 1998).

NPY high concentrations in the hypothalamus would indicate its important role in the regulation of H.H.A. axis. The presence of NPY fibers tightly associated to cellular bodies of CRH in the PVN was demonstrated by immunocytochemistry (Wahlestedt & Heilig). Moreover, the central administration of NPY in this nucleus produced increase in plasma levels of the CRH, ACTH and CORT (Campbell *et al.*, 2003). It was proposed that the receptor sub-type Y_{ACTH} that mediates the ACTH increase in plasma requires the entire NPY molecule for the activation of H.H.A. axis (Small *et al.*, 1997; Wahlestedt *et al.*, 1989). Small *et al.* suggested that Y_{ACTH} receptor is similar to Y5 but different to Y1 – Y4 or Y6. Another study demonstrated that the intra-ventricular-brain injection (icv) imitates the action of NA stimulating RAm-CRH biosynthesis and release in the hypothalamus of rats; this effect seems to be mediated by β -adrenoreceptors (Holmes & Crawley).

Some histological evidences have established that the noradrenergic neurons of the coeruleus locus co-exist with two neuropeptides: galanin (GAL) and NPY. This co-existence would possibly act as modulator mechanism in a negative feedback for NA neurons of coeruleus locus (Holmes & Crawley). The CRH produced in the PVN parvocellular neurons function as an anxiogenic agent involved in the stress neuroendocrine and behavioral response while NPY exerts its anxiolytic action under stressful situations (Heilig, 2004). These neuropeptides are involved in the response to stress and experimental evidences suggest that the balance between the systems H.H.A and H.S.A. (Hypothalamus Sympatic Adrenal) would play an important role in stress regulation, anxiety, sleep and depression (Engler

et al., 1999; Sajdyk *et al.*, 2004). These effects were observed in other brain areas such as hippocampus, hypothalamus, locus coeruleus (LC), periaqueductal gray matter (PAG) and the septal nucleus. Even though all these brain regions participate in the beginning, maintenance and progression of anxiety, the amigdala is the principal structure integrating the interrelation between CRH and NPY in the regulation of anxiety and stress. Besides, different studies have suggested that in the basolateral/lateral complex of the amigdala, the NPY mediates the anti-stress effects (Sajdyk *et al.*; Thiele & Heilig).

The localization and distribution of the NPY neurons and their speculated functions as neuromodulator showed in stressing conditions, determine that chronic stress during gestation would modify the number of NPY neurons in development of different areas and brain nuclei of the CNS. The aims of this work were: 1) to identify NPY neurons by immunocytochemistry during the development of CNS on days 15th, 17th and 19th of the gestational stage in fetuses of control and stressed IMO groups of rats; 2) to quantify and statistical analyze these populations of NPY neurons, comparing them qualitatively in the brain areas and nuclei of control and stressed fetuses by stereological images analysis.

MATERIAL AND METHOD

Animals Laboratory Room Conditions. Groups of young Wistar female albino rats (80-130 days of age), about 300 g body weight were used. They were kept under standard laboratory conditions, fed with Cargill balanced food and water “ad-libitum” under controlled photoperiod (12 L, 7:30 am-7:30 pm) maintained at $20^{\circ} \pm 2^{\circ}$ C. The pro-estrous was determined by in fresh colpo-cytograms during 9:00-11:00 hs am. Mating took place for 12 hours in the day of proestrous. The pregnancy was checked by the presence of spermatozooids in vaginal fluid considering it the zero day to obtain the samples. These females were kept physically isolated in groups of control rats (CR) and experimental rats (ER).

Experimental Treatment. Rats were stressed by plate immobilization (IMO) 45 min each day from 4th day of gestation, on alternate days according to the method described by Michajloskij *et al.* (1988).

On 15th, 17th and 19th days of gestation, the animals were decapitated at 10:00 - 11:00 hs am., without anesthesia. The fetal heads from control rat fetuses (CRF) and stressed rat fetuses (SRF) were fixed “in toto” in 1:50 v/v Bouin

solution during 24 - 48 hs for fetuses of 15 - 17 days and 19 days respectively. Then, they were washed with water to eliminate picric acid, keeping in alcohol 70 %, 1 h. They were dehydrated and included in paraplast-paraffin. There were obtained 12 serial sagittal and parasagittal sections of 5mm from each head fetus using a Reichert-Young 2065 microtome. 2-4 sections were mounted in each slide previously treated with Vectabond (Vector Lab.)

Immunocytochemical Technique. An indirect immunocytochemistry method (ABC, Vectastain-ABC Kit, Vector Labs.) was applied on one hundred and eight sections, 54 from stressed rats (SR) and 54 from control rats (CR), 6 sections for slide. The sections were deparafinized and hydrated in phosphate buffer saline (PBS) pH 7.4. Unspecified sites were blocked using horse normal serum during 30 min. Rabbit anti NPY (Sigma Labs.) was used as primary antibody (Ab) diluted 1:2000 in PBS pH 7.4, incubating the sections at 4°C in a humidified chamber, during 20hs. The second and third antibodies (Elite PK rabbit IgG) were incubated for 1 h in wet-chamber at room temperature. The antigen-antibody complex was revealed using 0.003% 3.3' diaminobenzidine tetrahydrochlorure solution (DAB Substrate Kit for peroxidase, Vector Labs.) 2-5 min., in the same conditions as above. After lightly counterstained with Mayers' haematoxylin, the sections were dehydrated, cleared and mounted with Entellan Merck. ICC controls, performed on sections in each slide, consisted in omission of the 1st Ab. A minimum distance of 8 µm among sections assured that the immuno-reactive cells with 5-6 µm in diameter would be different for the quantification and the stereological images analysis.

Qualitative results of NPY immuno-reactive neurons were documented through microphotographies obtained by Axiophot Zeiss photomicroscope.

The nomenclature of different nuclei and brain areas was obtained from Pakinos *et al.*, 1994 (Table I).

Stereological Analysis of Images. The number of neurons containing NPY was obtained by applying microscopic stereological analysis (VIDAS-Kontron-system, Germany). A macro-program was used for the digitalization of each one of the images sections for NPY neurons recount in fetuses of 15th, 17th and 19th days of development. The images were processed with a Zeis Axiophot microscope provided with a digitalize camera of only one capture channel and a computational system with PC AT 486 and Kontron software VIDAS 2.5. Data bases were converted to Microsoft Excel format using the Converta program of VIDAS 2.5.

Statistical Analysis. Statistical analysis was performed by

STATA version 8 and the non parametrical tests of Kruskal-Wallis and Mann-Whitney. $p < 0.05$ was established as significant difference and $p \leq 0.01$ as highly significant one. Two different quantitative analysis inter and intragroup were applied of NPY neurons on brain areas and brain nuclei of CNS.

RESULTS

Qualitative Analysis of NPY Neurons. The NPY neurons in control and stressed rat fetuses of 15th gestation day were scarce and mainly distributed in the anterior, medium and posterior brain, showing slight immuno-reactivity.

In all fetuses of 17th and 19th gestation day, the NPY neurons were abundant with a strong expression of this neuropeptide. They were distributed in the anterior, medium and posterior brain areas. They were mainly clustered in the anterior, medium and posterior hypothalamus, and forming clusters in the mesencephalic and rhombencephalic areas. They were bipolar cells which extensive axons projected themselves from the hypothalamus to the medium eminence (Figs. 1, 2, 3 and 4).

Quantitative Analysis of NPY Neurons.

Brain Areas of CNS. Highly statistically significant differences were found among the brain areas (anterior vs medium and medium vs posterior) of the CNS ($p < 0.01$) in the intragroup of control and stressed rat fetuses of the periods studied.

Fetuses of 15th gestation day. Highly statistically significant differences in the anterior and medium brain ($p < 0.01$) were determined in the intergroup analysis while no statistically significant differences were found in posterior brain of control and stressed rat fetuses (Fig. 5). The brain nuclei in fetuses of this period were scarce and not well developed so that the quantification was not applied.

Fetuses of 17th gestation day. Highly statistically significant differences in the anterior brain ($p < 0.01$) were determined in the intergroup analysis while no statistically significant differences were found in medium and posterior brain in control and stressed fetuses (Fig. 6).

Fetuses of 19th gestation day. Statistically significant differences were determined in the anterior brain ($p < 0.05$) and highly statistically significant ones in medium and posterior brain ($p < 0.01$) among control and stressed rat fetuses (Fig. 7).

Table I. Nomenclature of fetus brain nuclei of 17th and 19th days of gestational age (Pakinos *et al.*, 1994).

DAYS	BRAIN AREAS	ABBREVIATION	NAME
17 th	ANTERIOR	SPT	SEPTAL NUCLEUS.
		VBD	NU VERTICAL LIMB DIAGONAL BAND.
		POA	PREOPTIC AREA NEUROEPI
		PH	POSTERIOR HYPOTHALAMIC AREA.
		AH	ANTERIOR HYPOTHALAMIC NUCLEUS.
		DM	DORSOMEDIA L HYPOTHALAMIC NUCLEUS
		ARC	ARCUATE HYPOTHALAMIC NUCLEUS
	MEDIUM	VTa	VENTRAL TEGMENTAL AREA.
		R	RED NUCLEUS.
		CG	CENTRAL PERIAQUEDUCTAL GRAY
		DR	DORSAL RAPHE NUCLEUS.
	POSTERIOR	PnC	PONTINE RETICULAR NU, CAUDAL PART.
		PnO	PONTINE RETICULAR NU, ORAL PART.
		RITg	RETICULOTEGMENTAL NU OF THE PONS
		DPGI	DORSAL PARAGIGANTOCELLULAR NU.
		PrH	PREPOSITUS HYPOGLOSSAL NUCLEUS
		RMg	RAPHE MAGNUS NUCLEUS
		IO	INFERIOR OLIVE.
19 th	ANTERIOR	TU	OLFACTORY TUBERCLE
		VBD	NU VERTICAL LIMB DIAGONAL BAND.
		MS	MEDIAL SEPTAL NUCLEUS.
		APMO	ANTERIOR MEDIAL PREOPTIC NUCLEUS.
		MPO	MEDIAL PREOPTIC NUCLEUS
		SCh	SUPRACHIASMATIC NUCLEUS.
		LA	LATEROANTERIOR HYPOTHALAMIC N U.
		AB	ANTEROBASAL NUCLEUS.
		AH	ANTERIOR HYPOTHALAMIC NUCLEUS.
		Pa	PARAVENTRICULAR HYPOTHALAMIC NUCLEUS
		DA	DORSAL HYPOTHALAMIC NUCLEUS
		VMH	VENTROMEDIAL HYPOTHALAMIC NUCLEUS
		ME	MEDIAN EMINENCE
		DM	DORDOMEDIAL HYPOTHALAMIC NUCLEUS
		PH	POSTERIOR HYPOTHALAMIC AREA.
		ARC	ARCUATE HYPOTHALAMIC NUCLEUS.
	MEDIUM	MCPC	MAGNOCELULAR NU OF THE POSTERIOR COMMISURE
		IMLF	INTERSTICIAL UN OF FASCICULUS
		DR	DORSAL RAPHE NUCLEUS
		PN	PARANIGRAL NUCLEUS
		PMR	PARAMEDIAL RAPHE NUCLEUS
	POSTERIOR	PnO	PONTINE RETICULAR NU, ORAL PART
		Pn	PONTINE NUCLEUS
		CGPN	CENTRAL GRAY OF PONS
		GIA	GIANTOCELLULAR RETICULAR NU, ALPHA.
		RMG	RAPHE MAGNUS NUCLEUS.
		PrH	PREPOSITUS HYPOGLOSSAL NUCLEUS
		ROb	RAPHE OBSCURUS NUCLEUS
		RPa	RAPHE PALLIDUS NUCLEUS.
		Pyx	PYRAMIDAL DECUSSATION

Fig. 1. NPY Neurons in the Preoptic area of controls fetus of 17 days of gestations (400x).

Fig. 2. NPY Neurons in the Hypothalamic area of stressed fetus of 17 days of gestation (630x).

Fig. 3. NPY Neurons in the Romboencephalic area of stressed fetus of 19 days of gestation (400x).

Fig. 4. NPY Neurons in the Mesencephalic area of control fetus of 17 days of gestation (630x).

CNS brain nuclei

Fetuses of 17th gestation day. NPY neurons quantification was done in all brain nuclei mentioned in Table I of the 17th days fetuses. Highly statistically significant differences were determined among the control and stressed rat fetuses in the following brain nuclei of the anterior brain: arcuate nucleus (ARC) and posterior hypothalamic area (PH) ($p < 0.01$) with an increase of NPY neurons in SRF (Fig. 8).

No statistically significant differences were found among CRF and SRF in Anterior Hypothalamic Nucleus (AH), Dorsomedial Hypothalamic Nucleus (DM) of anterior brain and Periaqueductal Gray Matter (CG), Dorsal Raphe nucleus (DR) of medium brain (Fig. 9).

Highly statistically significant increases were determined in the oral part of the pontine reticular nucleus (PnO) of posterior brain and in Reticulo Tegmental

Protuberance nucleus (RITg) in SRF of 17th day gestation. Statistically significant increases ($p < 0.05$) were also found in Dorsal Paragiganto Cellular (DPGI) in SRF of 17th day (Fig. 10). Finally, no statistically significant differences were detected among CRF and SRF in Pontine Reticular Nucleus, Caudal part (PnC), Prepositus Hypoglossal Nucleus (PrH), Raphe Magnus Nucleus (RMgGI) or in the inferior olive (IO) of the posterior brain (Fig. 10).

Fetuses of 19th gestation day. Quantification of NPY neurons was done in all the brain nuclei mentioned in Table I of 19th day fetuses. In the anterior brain, highly statistically significant differences were determined among CRF and SRF with increase of NPY neurons in SRF in the following brain nuclei: Dorsomedial hypothalamic nuclei (DM) and ARC ($p < 0.01$) (Fig. 11)

Highly statistically significant increases ($p < 0.01$) were found in the Dorsal Raphe nucleus (DR) of the medium

Fig. 5. Counting of NPY neurons in 3 areas of S.N.C. in fetus of 15 days. 1. anterior brain; 2. medium brain; 3. posterior brain. F.C.: control fetus; F.E. stressed fetus. ** $p < 0.01$. There are no significant differences between F.C. and F.E. in (3).

Fig. 8. Counting of NPY neurons in the brain nuclei of the anterior brain in control and stressed fetus of 17 days. F.C: control fetus; F.E: stressed fetus. ** $p < 0.01$. 1. Anterior Hypothalamic Nucleus (AH); 2. Dorsomedial Hypothalamic Nucleus (DM); 3. Posterior Hypothalamic Area (PH); 4. Arcuate Hypothalamic Nucleus (ARC). There are no significant differences between F.C. and F.E. in (1 and 2).

Fig. 6. Counting of NPY neurons in 3 areas of S.N.C. in fetus of 17 days. 1. anterior brain; 2. medium brain; 3. posterior brain. F.C.: control fetus; F.E. stressed fetus. ** $p < 0.01$. There are no significant differences between F.C. and F.E. in (3).

Fig. 9. Counting of NPY neurons in the brain nuclei of the anterior brain in control and stressed fetus of 17 days. F.C: control fetus; F.E: stressed fetus. 1. Periaqueductal Gray Matter (CG); 2. Dorsal Raphe Nucleus (DR). There are no significant differences between F.C. and F.E. in (1 and 2).

Fig. 7. Counting of NPY neurons in 3 areas of S.N.C. in fetus of 19 days. 1. anterior brain; 2. medium brain; 3. posterior brain. F.C.: control fetus; F.E. stressed fetus. * $p < 0.05$, ** $p < 0.01$.

brain while significant increases were observed in the Paranigral nucleus (PN) ($p < 0.05$) in SRF of 19th days (Fig.12).

In the posterior brain, there were determined highly statistically significant increases in the pyramidal decussation (Pyx), Raphe Obscure nucleus (Rob) and Raphe Pallidus nucleus (RPA) of SRF. Statistically significant increases ($p < 0.05$) were also found in the the protuberance Periaqueductal Gray matter (CGPn) and in the Prepositus Hypoglossal nucleus (PrH) in SRF (Fig. 13).

Fig. 10. Counting of NPY neurons in the brain nuclei of the posterior brain in fetus of 17 days. 1. Pontine Reticular Nucleus Oral Part (PnO); 2. Tegmental Reticular Nucleus of the Protuberance (RITg); 3. Dorsal Paragigantocellular Nucleus (DPGI); 4. Pontine Reticular Nucleus, Caudal Part (PnC); 5. Prepositus Hypoglossal Nucleus (PrH); 6. Raphe Magnus Nucleus (RMgGI); 7. Inferior Olive (IO). F.C: control fetus; F.E: stressed fetus. There are no significant differences between F.C and F.E. * $p < 0.05$; ** $p < 0.01$ in (4, 5, 6 and 7).

Fig. 12. Counting of NPY neurons in the brain nuclei of the medium brain in control and stressed fetus of 19 days. 1. Raphe Dorsal nucleus (DR); 2. Paragigantocellular Nucleus (PN); F.C.: control fetus; stressed fetus. * $p < 0.05$; ** $p < 0.01$.

Fig. 11. Counting of NPY neurons in the brain nuclei of the anterior brain in control and stressed fetus of 19 days. 1. Ventromedial Hypothalamic Nucleus (VMH); 2. Arcuate Hypothalamic Nucleus (ARC); 3. Dorsomedial Hypothalamic Nucleus (DM); 4. Medium Eminence (EM). F.C: control fetus; F.E: stressed fetus. There are no significant differences between F.C and F.E. ** $p < 0.01$ in (1, 2 and 4).

Fig. 13. Counting of NPY neurons in the brain nuclei of the posterior brain in control and stressed fetus of 19 days. 1. Periaqueductal Gray Matter of the Protuberance (CGPn); 2. Prepositus Hypoglossal Nucleus (PrH); 3. Raphe Obscurus Nucleus (Rob); 4. Raphe Pallidus Nucleus (RPA); 5. Pyramidal Decussation (Pyx). F.C: control fetus; F.E: stressed fetus. * $p < 0.05$; ** $p < 0.01$.

DISCUSSION

In our knowledge, no data related to the effects of IMO chronic stress on the NPY neurons during the development of CNS in rats have been reported. The findings of our experimental model were analyzed by analogy with the wide contribution obtained from the adult rats during the last twenty years. Few works showed the development

pattern and the physiological normal differentiation of NPY neurons during CNS morphologic-histogenetic period (Traverso *et al.*, 2002). Then, for the first time, the present study describes the effects of gestational maternal exposition to immobilization (chronic stress) on NPY neuronal population during brain development in rats.

Our results about anterior brain with its respective brain nuclei could be correlated to other studies performed in adult animals about the localization of the primary population of immuno-reactive NPY neurons in the Arcuate Hypothalamic Nucleus (ARC) of adult rats. These studies had been demonstrated that cell bodies of the primary NPY population is localized with major abundance in the arcuate nucleus (ARC) and project towards several hypothalamic sites including PVN, preoptic area (POA) and possibly, the median eminence (ME) (Traverso *et al.*). The nervous terminals of these neurons get to some regions which had been involved in the anxious effects of the neuropeptide such as lateral septum, amigdala, periaqueductal gray matter of the mid brain (PAG) and coeruleus locus (LC). There are also, at least four, well shown groups of neurons containing mRNA-NPY (Morris, 1989). The presence of NPY fibers tightly associated to the cellular bodies of CRH of PVN neurons has been identified by immunocytochemistry. The central administration of NPY in this nucleus produced an increase of CRH, ACTH and GC levels (Chronwall *et al.*, 1985; Kask *et al.*, 2002; Morris; Wahlestedt *et al.*; Wahlestedt & Heilig). Li *et al.* (2000) applying the anterograde trace techniques combined with triple immunofluorescence have shown the projections of NPY neurons of the ARC were one of NPY system access to the CRH neurons of the parvocellular division of the hypothalamic paraventricular nucleus (PVNp).

Thus, the localization of nervous terminals coming from the ARC in synapsis with the cellular bodies of neurons CRH of PVNp would indicate the action of NPY system in the hypothalamic control of H.H.A. axis (Wahlestedt & Heilig). Also, this system exerts multiple actions at hypophysis and adrenal glands (Krysiak *et al.*; Thiele & Heilig; Wahlestedt & Heilig).

In our experimental model of immuno-reactive NPY neurons localization we can infer that the increase of these neurons in the Dorsomedial hypothalamic nucleus (DM) might be induced in response to changes in the energetic balance generated by the exposition of gestating rat mothers to IMO stress (Grove *et al.*, 2001). These changes might also be the cause of the prenatal continuation of stress and the results of the neuroproliferative role of the NPY in this area. Several studies have also confirmed the crucial role of NPY neurons population of the ARH in the regulation of the energy balance while it is not known in some other structures such as DMH and PRF (Naveilhan *et al.*, 2001). Grove *et al.* demonstrated the expression of mRNA-NPY by hybridization in situ in the anterior parvocellular division (PVNp) and also in the lateral hypothalamus (LH).

Nevertheless, future studies will be necessary to evaluate their neurophysiologic behavior in postnatal periods. Consistent with these findings, the anxious effects of NPY at hypothalamic level are not extensively known yet. However, anterior and medial hypothalamus are sites involved with the fear and it had been observed that mRNA-NPY was up-regulated under conditions of acute and repeated IMO chronic stress (Hansel *et al.*, 2001).

It has been suggested that these areas are important hypothalamic feeding centers and possibly, the NPY expression would be necessary for their normal development. The presence of NPY in PVN, DMH, PRH or LH could be promoting the neurogenesis in these areas in accordance with the proposal of Hansel *et al.* whom established that NPY functions as neuroepithelial factor in the olfactory bulb promoting the postnatal proliferation of the neuronal precursor cells.

Studies related to the lateral hypothalamic nucleus (LH) had been proved that this is an area involved in sedation. The expression mRNA-Y1 was demonstrated in the Lateral (LH) and posterior (PH) hypothalamus, receptor Y1 functioned as mediator of anxiolytic effects in PH (Heilig). Intrahypothalamic microinjections of NPY in PH potentiated the sedative effect of pentobarbital (GABA agonist) (Heilig).

Naveilhan *et al.* assured that NPY modulates sedation through the Gabaergic System by interaction with receptors Y1 in PH. Our results are in accordance with the data previously mentioned; assuming that the posterior hypothalamic nucleus (PH) could be an area involved in sedation judging the increase of immuno-reactive NPY neurons would indicate the activation of NPY Alarm System under prenatal stress conditions, probably showing neuroprotector effect against stressing noxas.

There are also some evidences about the importance of insuline concentration in the NPY regulation in ARC and of the glucocorticoids (GC) at hypothalamic level. Makino *et al.* (2000) demonstrated that after the exposition of rats to IMO acute stress for 2 hours, GC concentration increased significantly while insuline concentration decreased within the 4 to 4 hours after the initial stress. Hypothalamic NPY neurons are sensitive to GC. PVN medial part is a structure with high densities of glucocorticoid receptors and the NPY activity is conditioned by the endogenous and exogenous GC. The GC regulation of hypothalamic NPY is produced by means of direct and indirect way (Krysiak *et al.*). The increase of mRNA-NPY in ARC was supposed to be implied in compensatory role recovering the body weight due

orexigenic (no se si se describe así) NPY function, which we have been previously demonstrated in relation with the fetal weight loss and posterior weight profit at final of gestation (Soñez, 2001).

In accordance with our results about the increase of NPY immunoreactive neurons in the brain nuclei, another studies showed high NPY levels in the hypothalamus, septum, accumbens nucleus, periaqueductal gray matter (PAG) and coeruleus locus. There were evidences in adult rats showing that NPY neurons of the periaqueductal gray matter were involved in behavioural, anticonceptive autonomic changes and in the regulation of defense responses (Hansel *et al.*).

In posterior brain of 17th stressed rat fetuses (SRF), we found high density of immunoreactive NPY neurons in Dorsal Paragiganto cellular nucleus (DPGI), Pontine Reticular nucleus, oral part (PnO) and the Reticular tegmental Protuberance nucleus (RITg). We have also shown high amount of NPY neurons in SRF of 19th gestational day in Pyramidal decussation (Pyx), Raphe obscurus nucleus (ROb), Raphe Pallidus nucleus (RPA), Gray matter of Protuberance Periaqueductal (CGPn) and Prepositus Hipoglossal nucleus (PrH). However, no statistically significant differences were detected in the posterior brain of SRF vs CRF at 15th and 17th gestational days, in the following brain nucleus localized at day 17: Pontine Reticular nucleus, caudal part (PnC), Prepositus Hipoglossal nucleus (PrH), Raphe Magnum nucleus (RMgGI) and Inferious Olive (IO). We estimate that in the posterior brain the area with immunoreactive NPY neurons corresponds to the coeruleus locus, coeruleus sub-locus and ventrolateral medulla judging. The increase number of NPY neurons in the Tegmental Reticular nucleus (RITg) and in the Protuberance Periaqueductal Gray Matter (CGPn) of SRF at 17th and 19th gestation days, where coeruleus locus is well developed.

It is well known that NPY by its receptor Y2 would exert its anxiolytic actions through the NA inhibition. The Hypoglossal Prepositus (PrH) corresponds to the group C3 in which NPY coexists with A; it is supposed that NPY would exert anxiolytic effects in this nucleus (Heilig; Kask *et al.*; Thiele & Heilig).

There were evidences showing that noradrenergic neurons of the coeruleus locus coexist with two neuropeptides: galanine (GAL) and NPY (Holmes & Crawely). It had been demonstrated that approximately 20-40% of the noradrenergic neurons of the coeruleus locus expressed NPY and were exclusively found in immuno-reactive tyrosine-hidroxilase neurons (Holmes

& Crawely). The coexistence of GAL and NPY with NA in coeruleus locus would possible function as inhibitor modulators, acting in negative feedback mechanism for noradrenergic neurons in coeruleus locus (Holmes & Crawely). Adrenergic projections arrive from the cortical cellular groups C1, C2 and C3, where NPY coexists with Adrenergic neurons extending up to PVNp. As been demonstrated that NPY depresses the postsynaptic potentials of the coeruleus locus neurons, probable due to the activation of Y2 receptor localized in the cellular bodies of the noradrenergic neurons. These authors assumed that NPY modulates the shot of the coeruleus locus neurons by Y2 receptor via.

In stressed conditions, NPY would act as an endogenous alarm system, exerting a neuroprotector effect against stressing noxas (Pacák & Palkovits). It would also participate in response to energetic balance changes generated by the exposition of the gestating rat mothers to IMO chronical stress. It was supposed that the NPY endogenous alarm system would be emphasized in the anterior and posterior brain of stressed rat fetuses of 17th gestation day while at the beginning and at the end of the gestation its activation was necessary in the three brain areas studied for contra resting the stressing conditions and facilitating the continuity of development and gestation.

CONCLUSIONS

The maternal IMO stress exert effects on fetal NPY neurons population from mid-gestation in rats which probably modulate sedative/anxiolytic effects and participate in response to energetic balance changes generate by the exposition of gestating mothers to IMO chronic stress. In this way, the increase of NPY neurons population would be correlated with major expression of NPY, assuring that the gestation can be completed with correct fetal development during maternal stress conditions.

ACKNOWLEDGEMENTS

The authors are grateful to Ms.Sc. María Inés Rodriguez for her contribution in the statistical analysis and to Mr. Mario Lazarte for his technical support in computation. This work was granted by SECYT-U.N.R.C. (PICTO-U.N.R.C.) and CONICET, Argentina.

BULJUBACICH, K. A.; MUGNAINI, M. T.; SOÑEZ, C. A.; ROLANDO, A. N.; ROMANINI, M. C.; BOZZO, A. A.; SOÑEZ, M. C. & GAUNA, H. F. Efectos del stress crónico sobre la población neuronal NPY durante el desarrollo de ratas. *Int. J. Morphol.*, 27(3):879-889, 2009.

RESUMEN: El propósito del presente estudio fue determinar los efectos del estrés crónico en la población de neuronas NPY encefálicas durante el desarrollo del S.N.C. fetal mediante técnicas inmunocitoquímicas. Se demostró que las neuronas NPY presentan un morfología similar en los días de gestación estudiados, pero su distribución varía en el cerebro anterior, medio y posterior. Se comprobaron diferencias altamente significativas entre el cerebro anterior, medio y posterior ($p < 0,01$) de fetos estresados (FE), variando dicha significación dependiendo del día de la gestación y del área estudiada. En los diferentes núcleos cerebrales del cerebro anterior se detectaron aumentos en ARC (Arcuate Hypothalamic Nucleus), PH (Posterior Hypothalamic Area) de 17° días y DM (Dorsomedial L Hypothalamic Nucleus) y en ARC (Arcuate Hypothalamic Nucleus) de 19° días ($p < 0,01$) de F.E. En el cerebro medio se detectaron aumentos en DR (Dorsal Raphe Nucleus) ($p < 0,01$) y PN (Pontine Nucleus) ($p < 0,05$) de 19° F.E. En el cerebro posterior se detectaron aumentos en PnO (Pontine Reticular Nu, Oral Part) y RITg (Reticulotegmental Nu of the Pons) de 17° F.E. y Pyx, (Pyramidal Decussation), Rob (Raphe Obscurus Nucleus) y RPA (Raphe Pallidus Nucleus) de 19° F.E. Asimismo se comprobaron aumentos ($p < 0,05$) en DPGI (Dorsal Paragigantocellular Nu.) de 17 F.E, CGPn (Central Gray of Pons) y PrH (Prepositus Hypoglossal Nucleus), de 19° F.E. Finalmente, no se comprobaron diferencias entre F. C. (fetos controles) y F. E. en los siguientes núcleos del cerebro anterior: AH (Anterior Hypothalamic Nucleus), DM (Dorsomedial L Hypothalamic Nucleus), de 17° días; y EM, (Median Eminence), VMH (Ventromedial Hypothalamic Nucleus) de 19° días. En el cerebro medio CG, (Central Periaqueductal Gray), DR (Dorsal Raphe Nucleus) de 17° días. En el cerebro posterior el PnC, (Pontine Reticular Nu, Caudal Part), PrH (Prepositus Hypoglossal Nucleus), RMgG (Raphe Magnus Nucleus), IO (Inferior Olive) de 17° días del cerebro posterior. El incremento del número de neuronas NPY en todos los periodos estudiados en ratas estresadas, podría indicar la participación del sistema NPY en la regulación del eje adrenal hipotálamo - hipófisis.

PALABRAS CLAVE: NPY; Estrés crónico prenatal; S.N.C.; Ratas

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Received: 27-02-2009

Accepted: 27-06-2009