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Effects of postweaning social isolation and re-socialization on the expression of vasoactive intestinal peptide (VIP) and dendritic development in the medial prefrontal cortex of the rat

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Abstract. In the present study we evaluated the effects of early social isolation and re-socialization on dendritic development and the expression of the vasoactive intestinal peptide (VIP) in the medial prefrontal cortex (mPFC) of the rat. Sprague-Dawley male rats were reared either in isolation (IC) or social (SC) conditions from postnatal day 18 to 32. Rats were then behaviorally evaluated in the open field test, and approximately half of the animals were sacrificed. Their brains were processed either for immunocytochemical labeling against VIP or for the Golgi-Cox-Sholl staining. The remaining IC rats were re-socialized during 30 additional days. The results demonstrated that early social isolation impair neuronal dendritic arborization and increase the number of VIP-immunoreactive neurons. Furthermore, animals displayed hyperlocomotion in the open-field test. According to our structural, immunocytochemical and open-field data, the re-socialization experience was unable to reverse neuronal and behavioral abnormalities.

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INTRODUCTION

It is well known that early social experiences may alter neuronal and behavioral development. For example, isolated-reared animals show impairments in prefrontal dopaminergic innervation (Neddens et al. 2001, Winterfeld et al. 1998), dendritic complexity and spine density (Pascual et al. 2003, Silva-Gómez et al. 2003). In addition, isolated animals exhibit behavioral deficits such as altered response inhibition, poor exploratory behavior and locomotor hyperactivity (Einon and Morgan 1978, Gentsch et al. 1981, Jones et al. 1991, 1992), all mediated by frontal cortex and related circuits (Dawirs et al. 1996). These data suggest that early adverse conditions may alter the development of the prefrontal cortex (PFC) microarchitecture and function.

Several studies have demonstrated that neuropeptides have regulatory, neurotrophic and morphogenic actions, mainly in early stages of brain development (Hayashi 1992). An example of such kind of trophic peptides is the vasoactive intestinal polypeptide (VIP), originally isolated from porcine duodenum (Said and Mutt 1970). In the cerebral cortex VIP is expressed in a defined subpopulation of vertically oriented GABAergic bipolar interneurons, with higher density in superficial layers (Hendry et al. 1984, Morrison et al. 1984). It is well known that VIP exerts neurotrophic actions promoting neuronal differentiation, neurite remodeling, and cell survival (Alleaume et al. 2004, Fukuchi et al. 2004, Héraud et al. 2004). Since VIPergic cells establish synaptic contacts with dendrites of adjacent pyramidal cells (Hajós et al. 1988), it is possible that this peptide exerts trophic/neuroprotective actions on post-synaptic target neurons.

Taking into account that social isolation alter the structural development of prefrontocortical pyramidal cells (Pascual et al. 2003, Silva-Gómez et al. 2003), and that VIP may exert putative neurotrophic functions on these cells, the objective of the present study was to determine if the dendritic impairment induced by post-weaning social isolation on prefrontal pyramidal neurons is related with changes in VIP immunoreactivity. In addition, we studied the reversibility of these effects after animal re-socialization. Finally, considering that mPFC has been also implicated in the process of behavioral inhibition (Bubser and Schmidt 1990) and that mPFC dysfunction could remove this inhibitory control (Sokolowski and Salamonte 1994), we analyzed the spontaneous locomotor activity in the open-field test.

METHODS

Subjects and experimental conditions

Ninety-eight male Sprague-Dawley rats were weaned at 18 postnatal day (P18) and randomly assigned to the isolated (IC, n=46) or social (SC, n=52) condition groups. IC rats were kept individually in small opaque plastic cages (20 × 20 × 30 cm), in which they could smell and hear their congeners, but were unable to touch, see or physically interact with them. SC rats were housed in groups of 4 animals per cage (20 × 20 × 50 cm). Pups were weaned at P18, because VIP's ontogenic expression in the cerebral cortex of the rat is mainly upregulated between P0-P28 (Emson et al. 1979). Both animals groups had free access to food and water, and were kept under regulated environmental conditions (21 ± 2°C, 65%–70% relative humidity and 12 h light-dark cycle). At P32, all animals were behaviorally evaluated and approximately half of them (IC, n=24 and SC, n=28) were euthanized under deep anesthesia (Pentobarbital 50 mg/kg, i.p.) for further functional and histological analysis.

In order to evaluate the potential therapeutic effects of re-socialization, the remaining IC animals (n=22) were housed in groups (3–4 animals per cage) until P62, and followed in parallel with the remaining SC age-matched controls (n=24). At the end of this period the animals were euthanized and their brains were removed and analyzed.

All animal procedures followed guidelines given at "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at Universidad Católica del Maule.

Behavioral analysis

Novelty-induced locomotor activity was evaluated at P32 and P62 in the open-field apparatus, as described elsewhere (Pascual et al. 1996). Briefly, each rat was placed in the center of 80 × 80 × 20 cm plywood surface, divided in 15 cm squares. The number of squares crossed by all four limbs during 90 seconds was quantified. The open-field apparatus was wiped between each test with a clean alcohol dipped cloth to

eliminate olfactory cues left by previously tested animals. Behavioral evaluation was performed at night (10:00 P.M., 20 ± 5°C). In order to mask the investigator's presence, the room was dimly illuminated with a red light and a white noise generated by the ventilator system. In order to exclude observer's bias, animals were assigned with code numbers and two blind observers carried out the evaluation.

Neuronal evaluations

IMMUNOCYTOCHEMICAL ANALYSIS

In order to study the impact of early social isolation on the number of VIP-ir mPFC neurons, randomly selected brains from P32 and P62 animals (SC, n=20, IC, n=17) were processed for immunocytochemistry analysis at both ontogenic stages. Rats were perfused transcardially with cold 0.9% saline for 1 minute followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS; pH 7.4) during 6 minutes. All the procedure was performed on rats under deep anesthesia (sodium pentobarbital, 50 mg/kg, i.p.). Brains were removed from skull, postfixed in 4% paraformaldehyde for 24 hours, cryoprotected in 0.1 M PBS containing 20% sucrose and stored at 4°C. Frontal coronal sections were cut with a freezing microtome (40 μm thickness), collected and washed free-floating in buffer 1 [0.1 M PBS with 0.3% Triton-X 100 (Fluka, Buchs, Switzerland) and 1% fetal calf serum (FCS, ICN, Costa Mesa, USA)] for 60 minutes. Sections were incubated with rabbit anti-vasoactive intestinal peptide polyclonal antibody (1:1000; Chemicon) for 24 hours. After 3 washes in buffer 1, prefrontal cerebral sections were incubated with goat anti-rabbit cyanine 3.18-conjugated IgG (1:2000; Jackson Immunoresearch, West Grove, USA). After 3 additional washes, sections were mounted on gelatin-coated slides, dehydrated in ethanol (96% and 100%), cleared with methylsalicilate and coverslipped with DPX (Fluka). To establish the specificity of the VIP immunostaining, a negative control with no primary antibody was performed.

Prefrontal VIP-ir neurons were analyzed under an Olympus BX-40 microscope equipped for epifluorescence. Selected images from mPFC sections (2.2 mm and 3.2 mm anterior to bregma) (Paxinos and Watson 1998) were taken with a digital camera (Olympus DP-20) attached to the microscope and analyzed using freeshare software from NIH (Image 1.62). The number of VIP-ir neurons/cortical field (cells/600 μm^2) was quantified in 137 prefrontal sections (SC, n=66; IC, n=71), which corresponded to 3–4 cortical fields per animal.

ANALYSIS OF DENDRITIC DEVELOPMENT

In order to study the impact of early social isolation on the dendritic phenotype of mPFC neurons, the brains of the remaining SC (n=32) and IC (n=29) animals were sacrified and immediately dissected out and immersed in the Golgi-Cox-Sholl solution (Sholl 1953). After 60 days of slow metallic mercury impregnation, brains were dehydrated in ethanol-acetone and ethanol-ether solutions (50%:50%), embedded in celoidin, hardened in chloroform vapors and cut coronally in a sliding microtome (120 μm thickness). Sections were mounted on slides with coverslips. A total of 594 pyramidal neurons were sampled from mPFC (SC=306; IC=288). This region was identified according to the stereotaxic atlas of Paxinos and Watson (1998).

In order to qualify the morphometrical evaluations, pyramidal cells should fulfill the following criteria: (i) have a well-defined pyramidal shape, (ii) show an adequate staining of the soma and dendrites, (iii) have no extensive processes overlapping neighboring neurons, and (iv) layer II/III pyramidal cells located in a cortical strip between 200 and 650 μm under the pial surface (2.2 mm and 3.2 mm anterior to bregma). The first 8–11 cells in each brain sections meeting the above criteria were traced with the aid of a camera lucida (Olympus, model BH-DA-LB, 400× magnification). The ramification of every dendritic tree was determined according to the method of Coleman and Riesen (1968). Dendrites coming out directly from the cell body were considered as first order; direct branches from first order dendrites were considered as second order, and so on. The complexity of the dendritic arborization was quantified using the Sholl ring analysis (number of intersections per neuron found in concentric rings) (Sholl 1953). All cells were drawn and analyzed by the same person in a blind manner to maximize reliability.

Statistical analysis

Data presented correspond to the mean ± standard deviation. Unpaired two-tailed t-tests were used for

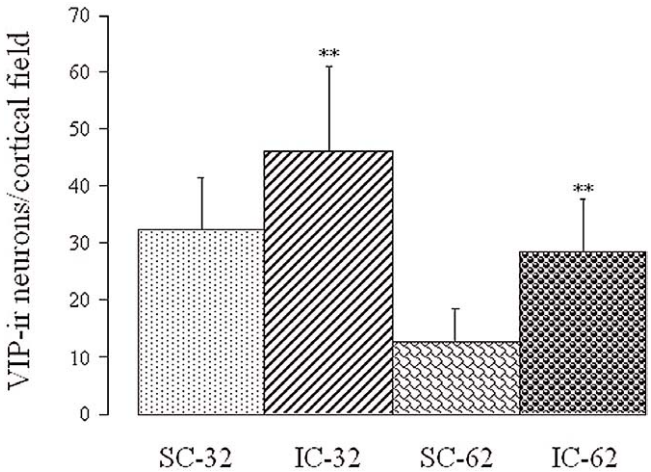

Fig. 1. Neuronal VIP expression in mPFC. (SC-32), (IC-32) socially and isolated-reared rats at postnatal day 32; (SC-62) socially-reared rats at postnatal day 62; (IC-62) initially isolated-reared animals and then re-socialized from P32 to P62. $^{**}P<0.01$ (unpaired *t*-test). Values are expressed as the mean number of VIP-ir cells per cortical field (600 μm^2).

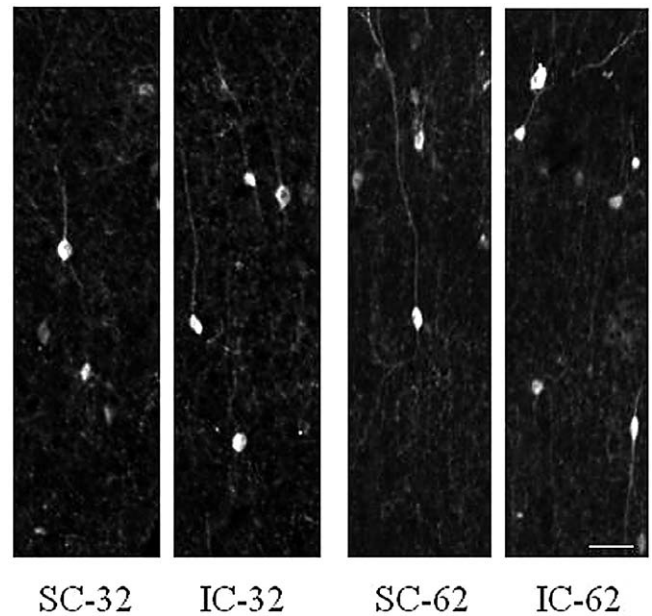

Fig. 2. VIP-ir neurons from the mPFC of the rat. Representative prefrontocortical strips (layers II/III) showing VIP-ir cells in animals reared in social (SC-32) and isolated (IC-32) conditions at postnatal day 32; and social (SC-62) and isolated and later re-socialized (IC-62) at postnatal day 62. Note the higher number of VIP-ir cells in IC-32 respect to SC-32 animals and SC-62 to IC-62 animals. Scale bar is 40 μm.

comparison between groups. Differences were considered significant when $P<0.05$.

RESULTS

Immunocytochemical data

Rats reared in isolation (IC) exhibited a significant increment in the number of VIP-ir neurons per cortical field compared to their age-matched SC controls ($P<0.01$) (Fig.1). Furthermore, the number of neurons expressing this peptide remained significantly higher among IC animals ($P<0.01$) even after 30 days of resocialization (IC-62 vs. SC-62). Representative immunofluorescent images are shown in Fig. 2 for both postnatal stages.

Table I

Mean number of dendritic segments per dendritc order quantified in mPFC neurons

Dendritic order	SC-32 *n*=165	IC-32 *n*=159	*P* value	SC-62 *n*=141	IC-62 *n*=129	*P* value
1st	6.3 ± 1.2	6.1 ± 0.9	ns	4.6 ± 1.5	4.1 ± 1.8	ns
2nd	8.1 ± 1.6	7.4 ± 1.2	ns	7.8 ± 2.1	5.7 ± 1.9	$P<0.05$
3rd	10.9 ± 3.3	6.8 ± 1.6	$P<0.05$	8.6 ± 2.7	5.1 ± 1.1	$P<0.01$
4th	9.7 ± 2.8	4.6 ± 0.7	$P<0.01$	6.3 ± 1.9	3.6 ± 0.7	$P<0.01$
5th	7.4 ± 1.8	2.9 ± 0.2	$P<0.01$	4.7 ± 1.1	1.9 ± 0.3	$P<0.01$
6th	3.5 ± 0.8	2.3 ± 0.3	$P<0.05$	2.8 ± 0.8	0.8 ± 0.06	$P<0.05$

(SC-32) socially-reared animals at P32; (IC-P32) isolated-reared animals at P32; (SC-62) socially-reared animals from P32 to P62; (IC-62) initially isolated-reared animals re-socialized after P32 until P62. *P*-value according to *t*-test; (ns) non significant.

Table II

Mean number of dendritic intersections per concentric rings quantified in mPFC neurons

Ring number	SC-32 *n*:165	IC-32 *n*:159	*P* value	SC-62 *n*:141	IC-62 *n*:129	*P* value
1	6.1 ± 1.7	6.0 ± 1.2	ns	6.2 ± 1.8	5.0 ± 2.1	ns
2	9.3 ± 2.1	8.5 ± 2.7	ns	11.7 ± 3.9	7.0 ± 2.5	$P<0.05$
3	11.3 ± 3.8	7.0 ± 2.5	$P<0.05$	11.3 ± 3.1	6.2 ± 2.8	$P<0.01$
4	8.9 ± 2.6	4.9 ± 1.7	$P<0.01$	8.1 ± 2.2	4.6 ± 1.9	$P<0.01$
5	6.5 ± 2.1	1.9 ± 0.7	$P<0.01$	5.2 ± 1.3	2.1 ± 0.8	$P<0.01$
6	2.7 ± 1.3	1.4 ± 0.7	$P<0.05$	2.5 ± 0.7	0.9 ± 0.08	$P<0.05$

(SC-32) socially-reared animals; (IC-32) isolated-reared animals at P32; (SC-62) socially-reared animals from P32 to P62; (IC-62) initially isolated-reared animals re-socialized after P32 until P62. *P*-value according to *t*-test; (ns) non significant.

Dendritic arborization

Pyramidal neurons from IC-32 rats showed less processes than their aged-matched control subjects (SC-32). As shown in Table I, the number of ramifications between the third and the sixth dendritic order was significantly reduced in IC rats compared to SC animals ($P<0.05$ and $P<0.01$, respectively). This dendritic impairment did not improve when isolated animals were re-exposed to a normal social environment for 30 additional days, between P32 and P62 ($P<0.05$, $P<0.01$). Furthermore, the dendritic complexity evaluated by the Sholl ring analysis confirmed the decreased dendritic arborization complexity of mPFC neurons from IC rats compared to their age-matched SC rats, at both P32 and P62 ($P<0.05$, $P<0.01$) (Table II).

Locomotor activity

The early social environment significantly affected the locomotor response to novelty observed in the open-field test. As displayed in Fig.3, IC rats exhibited a remarkable locomotor hyperactivity at both P32 and P62, showing a significantly higher number of squares crossed by all four legs ($P<0.05$ and $P<0.01$, respectively).

Body weight

The body weight of rat pups reared under isolation did not show to be significantly different from control rats at neither P32 (SC: 168 ± 20 g; IC: 173 ± 15 g) nor P62 (SC-SC: 257 ± 15 g; IC-SC: 246 ± 16 g).

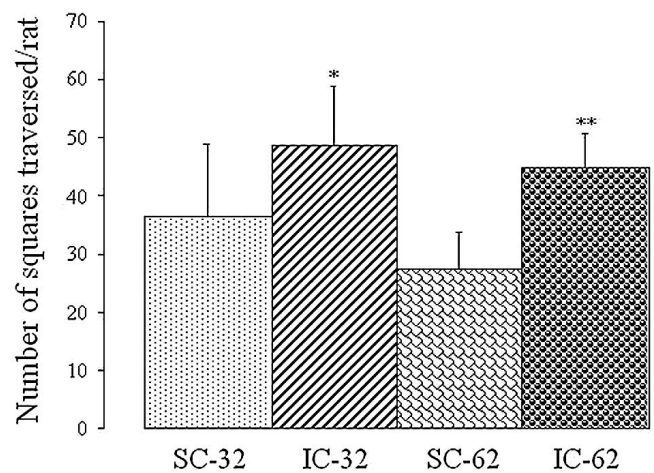

Fig. 3. Locomotor activity recorded in the open-field apparatus. (SC-32), (IC-32) socially and isolated-reared rats at P32, respectively; (SC-62) socially-reared rats at P62; (IC-62) initially isolated-reared animals re-socialized from P32 to P62. *$P<0.05$, **$P<0.01$ (unpaired *t*-test). Values are expressed as the mean number of squares crossed per rat during 90 seconds.

DISCUSSION

In this study we have demonstrated that early postweaning social isolation increased the density of mPFC neurons expressing VIP and decreased the dendritic complexity of pyramidal cells, in close association with locomotor hyperactivity. In addition, re-socialization was unable to offset any of the immunocytochemical, structural and behavioral alterations.

To our knowledge, this is the first study demonstrating that rodent social isolation induced an overexpres-

sion of VIP in cortical neurons. Although it has been demonstrated that VIP can stimulate neurite outgrowth and neuronal survival (Alleaume et al. 2004, Héraud et al. 2004), VIP's overexpression did not prevent the observed dendritic impairment of pyramidal cells. It is possible that the significant increment in VIP-ir neurons detected at P32 and P62 could be the result of a compensatory but insufficient protective mechanism induced to minimize the deleterious impact of social isolation. In addition, considering that VIPergic neurons directly innervate intraparenchymal microvessels, it is possible that the increment in VIP-ir reported in the present study may be due to a local cortical hemodynamic adjustment. In fact, there is an intimate association between VIP axonal or dendrites varicosities and cortical microvessels (Chédotal et al. 1994), whose direct activation is sufficient to either increase or decrease the diameter of neighboring microvessels (Cauli et al. 2004).

We observed that isolated-reared animals showed less dendritic ramification in mPFC pyramidal neurons compared with their age-matched socially-reared controls. Our results disagree with the report of Silva-Gómez and coauthors (2003), discrepancy that could be related to the onset time and duration of the isolation period, which in Silva-Gómez and coauthors (2003) was longer (60 days) and started later (P21) than ours. Those differences suggest that an earlier onset time of postweaning isolation is a critical variable that might result into a completely different scenario of neuronal changes. Although the mechanism involved in the dendritic impairment remains unknown, it is possible that the observed dendritic underdevelopment could be related with dysfunctions of the mesocorticolimbic dopaminergic system (Poeggel et al. 2003, Winterfeld et al. 1998). This suggestion is supported by the fact that DA fibers, that are vulnerable to isolation (Neddens et al. 2001, Winterfeld et al. 1998), exert neurotrophic effects on the maturation of pyramidal target cells (Kalsbeek et al. 1989, Reinoso et al. 1996). Correlation analysis between the activation of dopamine (DA) mesocortical pathways and the assessment of dendritic branching in mPFC neurons have to be done in order to prove the veracity of those links, which for the moment remain just as working hypotheses.

The locomotor hyperactivity observed in the present work agrees with previous studies in isolated rats exposed to novel environments (Gentsch et al. 1981, Hall 1998, Heidbreder et al. 2000). Whether this hyperactivity reflects an alteration in exploratory behavior or the preference for a novel environment is controversial since the effects of social isolation on motor activity seem to depend on several factors such as age, strain, housing, and testing environment. Furthermore, motor hyperactivity could be a consequence of the mPFC dendritic impairment, since it has been demonstrated that lesions in the prefrontal cortex can induce a similar behavioral disorder (Lacroix et al. 2002). Additionally, the hyperlocomotion detected in our work may be related with increments in prefrontocortical VIP expression. This suggestion is supported by two facts: (i) locomotor activity has been associated with increased VIP immunoreactivity in the frontal cortex (Eilam et al. 1999), and (ii) the intracerebroventricular injection of pituitary adenylate cyclase-activating polypeptide (PACAP), a VIP-related peptide, induce behavioral hyperactivity (Masuo et al. 1995).

Finally, we demonstrated that VIPergic, structural and behavioral abnormalities were not reversed after animal re-socialization, suggesting permanent neuronal sequelae. The failure in morphofunctional recovery may be related with the isolation period manipulated in the present study. Although many authors start animal isolation at P21, we decided to begin earlier (P18) since neuronal tissue appear to be more vulnerable to environmental cues (Hol et al. 1998, Pascual et al. 1996). Finally, we cannot discard that other types of environmental manipulations could be able to reverse our results, since it has been shown that complex environments diminished locomotor hyperactivity (Neugebauer et al. 2004), increase neuronal cytodifferentiation (Venable et al. 1989), spine density (Kolb et al. 2003) and synaptogenesis (Turner and Greenough 1985).

CONCLUSION

The present study demonstrates that early adverse experiences induced by the social isolation paradigm, increase the expression of VIP-ir neurons and impair the dendritic arborization of pyramidal cells in the mPFC of the rat. These alterations appear to be permanent since re-socialization was unable to recover any of the parameters tested in our work. Future studies need to be performed in order to determine whether enriched environments, rather than simple re-socialization, might be effective in overcoming the postnatally acquired deficits.

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