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Selegiline (deprenyl) decreases calbindin-D28k expression in cortical neurons of rats socially deprived during the post-weaning period

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ABSTRACT

Preclinical studies indicate that selegiline (deprenyl), frequently used in some neurodegenerative diseases, exert protective effects on central nervous system neurons of individuals exposed to social isolation (SI). Furthermore, it has been suggested that SI produces neuronal dysfunction due in part to an excessive intracellular Ca^{2+} overload. Since the main intracellular Ca^{2+} buffering mechanism involves changes in the calcium-binding protein calbindin-D28k (CB), and that CB neuronal expression can increase in response to Ca^{2+} transients, we hypothesized that chronic selegiline administration in early socially isolated animals could minimize cell CB expression as an indirect indicator of protective mechanism against Ca^{2+} overload. In the present study male rats were weaned at postnatal day 21 (P21) and randomly assigned to social deprivation (SI) or control (SC) environments for 30 days (P21–51). SI animals were further subdivided in two experimental groups: socially deprived-saline (SI-SAL) and socially isolated-selegiline (SI-SEL) for additional 30 days (P52–82). Medial frontal CB immunoreactivity (CB-ir) neurons were quantitatively and qualitatively analyzed. The results obtained indicate that neocortical cells of adult rats submitted to early SI show a significant increase in the number of CB-ir neurons per cortical field, while selegiline treatment significantly reduces this parameter.

Several lines of evidence indicate that the level of brain calcium-binding protein calbindin-D28k (CB) expression can be elevated by social stressful experiences. In fact, hippocampal neurons of juvenile or adult animals submitted to maternal separation (Lephart and Watson, 1999), social challenges (Krugers et al., 1996) or treated directly with corticosterone (Iacopino and Christakos, 1990; Krugers et al., 1995) showed significant increases in CB expression. This CB up-regulation probably occurs because glucocorticoids (GCs), released by stress-induced activation of the hypothalamic–pituitary–adrenal axis, can promote Ca^{2+} influx through either voltage-dependent Ca^{2+} channels (Zhou et al., 2000) or *N*-methyl-D-aspartate (NMDA) glutamate receptors (Stein-Behrens et al., 1994). Therefore, it is likely that the increased expression of CB reported in these studies constitutes a cell mechanism directed to buffer the potential risk for Ca^{2+} toxicity (Zupanc and Zupanc, 2006).

On the other hand, preclinical studies have shown that selegiline (SEL), an irreversible monoamine-oxidase B (MAOB) inhibitor commonly used in Parkinson's disease, exerts protective effects on central nervous system neurons. For example, SEL reduces oxidative damage caused by catabolism of dopamine, enhances free radical elimination by increasing superoxide dismutase and catalase activity, protects neurons from 1-methyl-4-phenylpyridinium ion (MPP^+) toxicity, and inhibits apoptosis (Chetsawang et al., 2008; Heinonen and Lammintausta, 1991; Magyar and Szende, 2004). In addition, it has been shown that SEL promotes dendritic outgrowth in frontal, hippocampal, mesencephalic and spinal neurons (Iwasaki et al., 1994; Kontkanen and Castrén, 1999; Lakshmana et al., 1998; Shankaranarayana-Rao et al., 1999), suggesting trophic-like actions. Likewise, in a previous study we were able to demonstrate that SEL can partially recover cortical dendritic outgrowth impairments induced by post-weaning isolation stress (Pascual and Zamora-León, 2007).

It has been suggested that the underlying beneficial action of SEL is related with the reduction of Ca^{2+}-induced mitochondrial permeability and therefore neuroprotection through stabilization of the mitochondrial membrane potential (Czerniczyniec et al., 2007; Wadia et al., 1998). The suggested toxic action of Ca^{2+} overload in mitochondrial vulnerability is also supported by the fact that drugs that inhibit cellular Ca^{2+} influx protect mitochondria from oxidative damage (Starkov et al., 2004; Verweij et al., 2000). Consequently, the cellular mechanisms involved in regulating Ca^{2+} homeostasis may play a crucial role in neuronal safeguarding. Because the increased up-regulation of CB is related to elevated levels of intracellular Ca^{2+} and the neuroprotective action of SEL appears to be related to reductions in Ca^{2+} overload, we hypothesized that SEL

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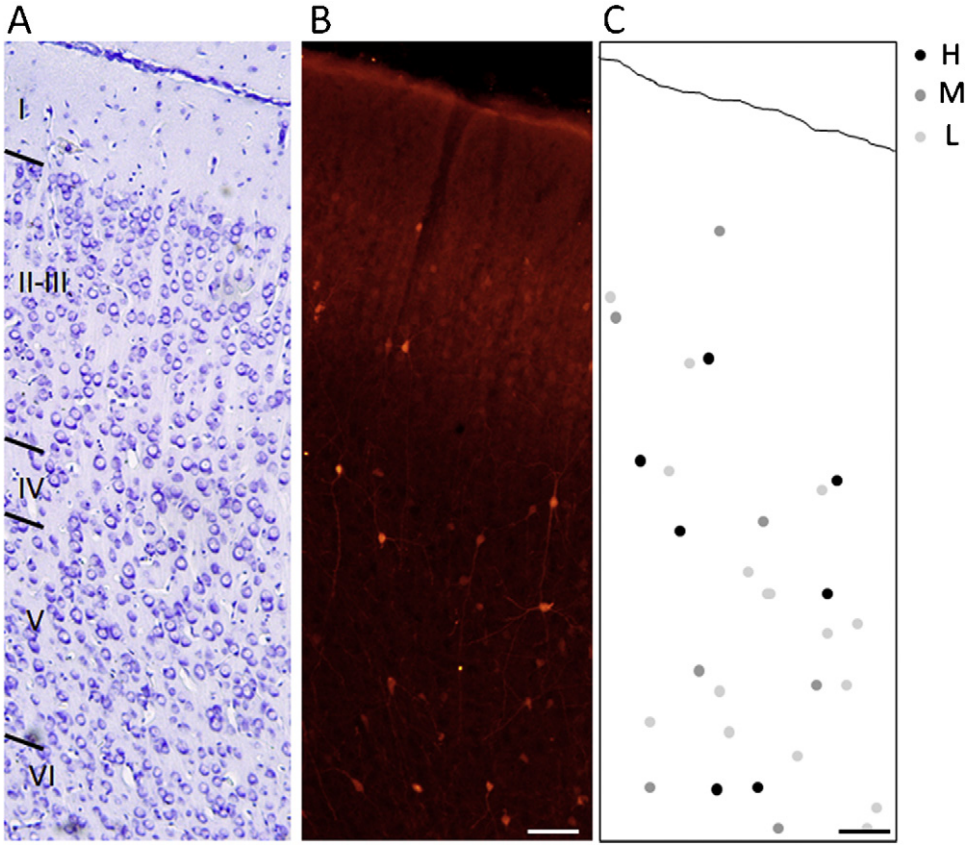

Fig. 1. Calbindin-immunoreactive cells (CB-ir) classified according to a qualitative criterion of fluorescent intensity. (A) Classical cresyl violet staining of the medial frontal cortex (as referential anatomical neocortical architecture). (B) Photomicrograph of a representative cortical field showed CB-ir cells. (C) Distribution of punctate profiles of CB-ir cells according to high (H), moderate (M), and low (L) CB-ir (bar B and C: 200 μm).

should decrease the number of CB immunoreactive cells/cortical (frontomedial) field in early socially stressed animals.

1. Experimental procedures

1.1. Experimental groups

Twenty-eight Sprague–Dawley male rats were purchased from National Institute of Health (Chile), weaned at postnatal day 21 (P21) and randomly assigned to social isolation (SI; $n = 17$) or control (SC, $n = 11$) for 30 days (P21–51). SI animals were kept individually in small, opaque, plastic cages (20 cm × 20 cm × 30 cm), and SC animals were housed in groups of 3–4 rats per cage (20 cm × 20 cm × 50 cm). Both groups were maintained in homogeneous environmental conditions (12:12 h light/dark cycle; 21 ± 2 °C) and had free access to food and tap water. After 30 days of differential social environments, SI animals were further subdivided in two experimental groups: (i) socially deprived-saline (SI-SAL, $n = 8$) and (ii) socially deprived-selegiline (SI-SEL, $n = 9$). SC animals were undisturbed except for daily vehicle (saline) administration (SC-SAL, $n = 11$).

1.2. Selegiline and immunohistochemical procedure

Between P52–82, SI-SEL animals received a daily dose of 0.2 mg kg^{-1} SEL i.p. (Sigma–Aldrich) dissolved in 2 ml saline (0.9% NaCl; 8:00 h p.m.). The dose of SEL and the treatment duration were selected based on neuroprotective and trophic effects reported in previous studies (Foley et al., 2000; Heinonen and Lammintausta, 1991; Pascual and Zamora-León, 2007). SI-SAL and SC-SAL animals received vehicle in a similar volume. At P83, animals were weighed and euthanized under deep pentobarbital anesthesia (90 mg kg^{-1}), then transcardially perfused for 6 min with cold 0.9% saline-heparin solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS; pH 7.4). Brains were removed from skulls and post-fixed in 4% paraformaldehyde for 24 h, cryoprotected in 0.1 M PBS–20% sucrose and stored at 4 °C, and frontal cortex was cut coronally with a freezing microtome (~20 μm thicknesses). Every twelve adjacent frontal coronal sections were collected and processed for calbindin-D28k immunohistochemistry. Free-floating sections were washed in 0.1 M PBS, 0.3% Triton-X 100 (Sigma) and 1% fetal calf serum for 2 h at room temperature, then incubated in primary anti-calbindin-D28k (anti-CB) monoclonal antibody (1:5000, Sigma) for 48 h. After 3 washes in PBS, frontal brain sections were incubated with secondary antibody goat anti-rabbit cyanine 3.18-conjugated IgG (1:200; Sigma). After 3 additional washes in PBS, sections were mounted on gelatin-coated slides, dehydrated in ethanol (96% and 100%), cleared in methyl salicylate, and cover-slipped with Canada balsam (Merck). To establish the specificity of the calbindin-D28k immunoreactivity, an omission test of primary antibody was performed. CB immunoreactivity (CB-ir) was analyzed under a Leitz microscope (model DMRB) equipped for epifluorescence. Medial frontal cortex sections were delimited by the coordinates provided in the Stereotaxic Atlas of the Rat Brain (Paxinos and Watson, 1998). Images were taken with a digital camera (Olympus) attached to the microscope. For each brain, the total number of CB-ir cells was counted in a rectangular band of 400 μm width × 1000 μm depths of medial frontal cortex (A: 3.2 mm; $L \pm 0.8$ mm; DV 2.3 mm with reference to the bregma), perpendicular to the pial surface. Moreover, because there are usually varying degrees of immunofluorescent intensity, we classified neurons in three groups according to fluorescence level: high, moderate and low (Fig. 1). Quantitative data were expressed in number of CB-ir cells/cortical field (average per animal). A total of 55 cortical frontal fields were sampled (2–3 per animal): SC-SAL = 20; SI-SAL = 14; SI-SEL = 21. During this process, all slides were coded so that neither the animal nor its condition was known by the evaluator.

Animals were treated and housed in accordance with the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1985), and experimental protocols received approval from the local animal ethics committee.

1.3. Statistical analysis

Data were statistically analyzed using one-way ANOVA, in combination with the Scheffé test for post hoc analysis (STATA 9.1 software).

2. Results

As shown in Fig. 2, the number of CB-ir cells per cortical field was significantly increased (~75%) in SI-SAL animals compared to the age-matched SC-SAL controls ($p < 0.01$, ANOVA). By contrast, SI-SEL animals showed a marked reduction in the number of CB-ir neurons, reaching values similar to those in the SC-SAL group.