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Time-dependent effects of escitalopram on brain derived neurotrophic factor (BDNF) and neuroplasticity related targets in the central nervous system of rats

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ABSTRACT

Chronic treatment with antidepressants affects several proteins linked to neuroplasticity, particularly brain derived neurotrophic factor (BDNF): this leads eventually to their therapeutic effects. It is possible that also for putative early therapeutic onset, antidepressants may act by promoting cellular adaptations linked to neuroplasticity. Escitalopram, known to be already effective in preclinical models of depression after 7 days, allowed us to investigate whether two effective treatment regimens (7 and 21 days) may contribute to synaptic plasticity by acting on BDNF signalling. We focused our attention on two regulators of BDNF transcription, CREB and CaRF (calcium responsive factor), and on kinases, CaMKII, ERK1/2 and p38 MAPK, linked to BDNF that play a distinctive role in synaptic plasticity. We evaluated whether the effects of escitalopram on these targets may be different in brain areas involved in the depressive symptomatology (hippocampus, frontal and prefrontal cortex). Here we demonstrate that escitalopram regulates intracellular pathways linked to neuroplasticity at both the time points evaluated in an area-specific manner. While the two escitalopram-treatment regimens failed to affect gene expression in the rat frontal cortex, 7 days of treatment with escitalopram activated intracellular pathways linked to BDNF and increased the levels of Pro-BDNF in the rat prefrontal cortex. Moreover, 21 days of treatment with escitalopram decreased CREB/BDNF signalling while increasing p38 levels in the rat hippocampus. Even if further experiments with different antidepressant strategies will be needed, our data suggest that escitalopram efficacy may be mediated by early and late effects on synaptic plasticity in selective brain areas.

1. Introduction

In recent years more and more interest has focused on the molecular mechanisms involved in neuroplasticity for its possible implication in the treatment of major depression (Krishnan and Nestler, 2008). In particular, selective serotonin reuptake inhibitors (SSRIs), currently among the most used antidepressants (ADs) in clinical practice, trigger a complex biochemical cascade mediating the actions of the neurotrophin brain derived neurotrophic factor (BDNF) and leading ultimately to promote neuronal plasticity (Duman and Monteggia, 2006; Martinowich and Lu, 2008).

We used as a pharmacological tool the S-enantiomer of citalopram, escitalopram, the SSRI with the highest selectivity for the serotonin transporter (Owens et al., 1997, 2001) to gain further insights into antidepressant actions in the CNS and to explore molecular mechanisms that can be responsible for an earlier appearance of the therapeutic effect. In fact this drug has been shown to be effective as early as after 7 days of treatment in several behavioural models of depression (Capone et al., 2006; Montgomery et al., 2001; Sanchez et al., 2003; Reed et al., 2009).

Taking advantage of this peculiar feature we tested the hypothesis that different temporal molecular mechanisms may be involved in mediating escitalopram effects by using two treatment regimens. We treated healthy rats with escitalopram for 7 or 21 days at a dose that has been shown to be active (10 mg/kg) and examined the temporal effects on neuroplasticity related targets of this SSRI in key areas involved in the etiopatogenesis of depression and in mediating the outcome of the ADs: hippocampus, frontal and prefrontal cortex (Holmes and Wellman, 2009; Kennedy et al., 1997; Manji and Duman, 2001; Mayberg, 2003; Nestler et al., 2002). In doing so, we followed a four-step approach: first, we evaluated the effects of the two treatment regimens on gene expression of BDNF and of cAMP response element binding (CREB) protein that acts as a regulator of BDNF expression (Blendy, 2006; Martinowich and Lu, 2008).

Our attention was then focused in those areas where at least one of the two treatment regimens was able to affect gene expression. As

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second step, we evaluated the protein levels of BDNF (mature and Pro-BDNF) and CREB to assess whether changes in gene expression could be coupled to changes also in protein levels.

Third, protein kinases acting as downstream targets of BDNF signalling, participating at least in regulating CREB activation and that likely play a role in mediating antidepressant behavioural effects were investigated. In particular, calcium/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinases (ERKs) and p38 MAPK total and phosphorylated (activated) levels were measured to assess whether these kinases could be regulated by these two treatment regimens (Barbiero et al., 2007; Gourley et al., 2008; Huang and Reichardt, 2001; Miller and Raison, 2006). Fourth, we investigated the effects of escitalopram on another transcription factor, the calcium responsive transcription factor (CaRF), which regulates BDNF neuronal expression in a calcium selective manner (Tao et al., 2002), after 7 and 21 days of treatment with escitalopram. The present study was undertaken to test whether this complex interconnected pathway, may present a temporal and area-specific profile in response to escitalopram treatment.

2. Material and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley albino rats of 8 weeks of age at the beginning of the experimental procedure (Charles River, Calco, Italy). Animals were housed 3 per cage in polycarbonate cages (28 × 17 × 12 cm) in a temperature- and humidity-controlled environment on a 12-h light–dark cycle (lights on at 6.00 a.m.) with *ad libitum* access to food and tap water. The procedures used in this study were in strict accordance with the European legislation on the use and care of laboratory animals (EEC n. 86/609), with the guidelines of the National Institutes of Health on the use and care of laboratory animals, and had the approval of the Ministry of Health and of the local Ethical Committee. All efforts were made to minimise animal suffering and to reduce the number of animals used in this study.

2.2. Drugs

Escitalopram oxalate (kindly provided by H. Lundbeck A/S. Copenhagen-Valby, Denmark) solution was freshly prepared prior to use dissolving the drug in saline and was administered via an intraperitoneal route (i.p.) in a volume of 1 ml/kg body weight at a dose of 10 mg/kg body weight calculated from the free base.

2.3. Pharmacological treatments

Animals ($n = 8$ in all groups) were randomly subjected to either subchronic (7 days) or chronic (21 days) treatment with escitalopram (10 mg/kg free base) or saline. All animals were weighed daily throughout the experiment and received a single i.p. injection. Rats were sacrificed 18 h after the last injection; brain areas (hippocampus, frontal and prefrontal cortex) were dissected, immediately frozen on dry ice and stored at −80 °C until further analyses. Dissection was performed according to the rat brain atlas (Paxinos and Watson, 1998). In detail, the frontal cortex (defined as frontal association cortex (FrA), lateral orbital cortex (LO), medial orbital cortex (MO), dorsolateral orbital cortex (DLO) and ventral orbital cortex(VO)), average weight 10 mg, was obtained by dissecting 1 mm slices (approximately from bregma 5.50 mm to bregma 4.50 mm). Prefrontal cortex (defined as prelimbic cortex (PrL), medial orbital cortex (MO), dorsolateral orbital cortex (DLO), ventral orbital cortex (VO), cingualte cortex, area 1 (Cg1) and, secondary motor cortex (M2)), average weight 8 mg, was dissected from 1.5 mm thick slices (approximately from bregma 4.70 mm to bregma 3.20). The hippocampi (including both ventral and dorsal parts) were dissected from the whole brain after dissection of frontal and prefrontal cortex.

2.4. Probe preparation

The cDNA (530 bp EcoRI/StuI fragment) for CREB1 was subcloned into pBluescript sk-(Stratagene) (see Blom et al., 2002 for further details) and linearized by BamHI (Fermentas). Probe for rat BDNF was generated from plasmid pSK-rB(C1), containing rat BDNF coding region (nt 661-942, Genbank accession number: NM_012513), linearized with Xma I (Fermentas) (see Maisonpierre et al., 1991 for further details). The cDNA (nt 496-725, Genbank accession number: XM_217411) for CaRF, obtained by RT-PCR, was subcloned into p-Drive Cloning Vector® (Quiagen) and subsequently linearized by HindIII (Fermentas). The antisense cRNA probes were synthesized, using Maxiscript® (Ambion), by *in vitro* transcription at 37 °C for 1 hr from 1 μg of linearized plasmid with T7 RNA polymerase for CREB, BDNF and CaRF using ^{32}P-CTP as the radiolabeled nucleotide (Perkin Elmer Life Sciences). Rat cyclophilin (#AM7680; Ambion) was used as internal standard. The probe for cyclophilin was generated by a T3 RNA polymerase, had a 142 base length, and generated a protected fragment of 105 bases. All probes were digested with DNase I (2000 units/ml) at 37 °C for 15 min, extracted with phenol:chloroform:isoamyl alcohol (50:49:1) and chloroform, precipitated with ammonium acetate and ethanol, and washed with ethanol. The purified antisense RNA probes were dissolved in double distilled DEPC-H_2O.

2.5. RNA preparation

Total RNA from different brain structures was isolated using Trizol® method (Gibco). Briefly, the tissue was homogenized in Trizol® reagent using Polytron ("Ultra Turrax", Janke & Kunkel) and incubated at room temperature for 5 min. After this procedure, chloroform was added to the tube and the solution was shaken vigorously for 15 s, and incubated at room temperature for 3 min. The tube was then centrifuged at 12,000 ×g for 15 min. The aqueous phase was transferred to a clean tube, precipitated with isopropyl alcohol, and centrifuged at 12,000 × g for 15 min. The resulting pellet was washed in 75% cold ethanol, centrifuged for 7500 × g for 5 min. The pellet was dried at room temperature, resuspended in double distilled DEPC-H_2O, and stored at −70 °C. Quantification was carried out by absorption at 260 nm, A_{260}/A_{280} ratio was calculated for each RNA sample and had a value between 1.7 and 2.0 and RNA integrity was assessed after electrophoresis in 1.0% agarose gels by ethidium bromide staining. RNA was re-precipitated in ethanol for RNase protection assay.

2.6. Protein extraction

For protein extraction, hippocampus and prefrontal cortex were homogenized by potter (12 strokes at 600 rpm) in lysis buffer containing hepes 10 mM, EGTA 0.1 mM, sucrose 0.28 M, $Na_4P_2O_7$ 5 mM, NaF 20 mM, Na_3VO_4 1 mM, and phenylmethanesulfonyl fluoride 0.1 mg/ml. After homogenization a fraction of the lysate was collected (total extract) and the remaining was centrifuged at 1000 ×g for 5 min at 4 °C. The pellets obtained from centrifugation were resuspended in buffer (3 ml/g of tissue) containing NaCl 120 mM, hepes 20 mM, EGTA 0.1 mM, dithiothreitol 0.1 mM, sodium pyrophosphate 5 mM, NaF 20 mM, Na_3VO_4 1 mM, and phenylmethanesulfonyl fluoride 0.1 mg/ml (nuclear enriched extract). A fraction of the total extracts was centrifuged at 9000 ×g for 15 min, to obtain a clarified fraction of cytosolic proteins. Protein concentration of the extracts was determined using standard protocol Coomassie® reagent (Pierce).