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ScienceDirect
Behavioural Brain Research 184 (2007) 91–100
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Transcription factor expression, RNA synthesis and NADPH-diaphorase across the rat brain and exposure to spatial novelty

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Received 7 June 2007; accepted 26 June 2007
Available online 30 June 2007

Abstract

The molecular hypothesis of learning and memory processes is based on changes in synaptic weights in neural networks. Aim of this study was to map neural traces of exposure to a spatial novelty were mapped by (i) the transcription factors (TFs) *c-fos*, *c-jun* and *jun-B* using Northern blot and immunocytochemistry (ICC), (ii) RNA synthesis by ^{3}H-uridine autoradiography and RNA level, (iii) NADPH-diaphorase (NADPH-d) expression by histochemistry. Thus, adult male albino rats were exposed to a Làt-maze and sacrificed at different times. Non-exposed rats served as controls. The latter showed a low constitutive expression of TF, RNA synthesis and NADPH-d across the brain. Northern blots showed a three-fold increase in TFs in exposed versus non-exposed rats in the cerebral cortex. ICC showed in exposed rats several TFs positive cells in the granular and pyramidal layers of the hippocampus and later in all layers of the somatosensory cortex, in the granular layer of the cerebellar cortex. The TF-positivity was stronger in rats exposed for the first time, and was time and NMDA-dependent. Autoradiography for RNA synthesis showed positive cells in the ependyma, hippocampus and cerebellum 6 h after testing, and in the somatosensory cortex 24 h later. In addition, exposure to novelty induced NADPH-d in the dorsal hippocampus, the caudate-putamen, all the layers of the somatosensory cortex. and the cerebellum. The positivity was absent immediately after exposure, appeared within 2 h and disappeared 24 h later. A strong neuronal discharge by the convulsant pentylenetetrazol, strongly induced TFs but not din not affect NADPH-d 2 h later. Thus, data suggest that the processing of spatial and emotional components of experience activates neural networks across different organization levels of the CNS.

Keywords: Model systems; Nitric oxide synthase; Brain ribonucleic acid; ^{3}H-Uridine incorporation; Behavioural habituation

1. Introduction

The molecular hypothesis of information processing, storage and retrieval in the CNS has been tested in different model systems [2,6,25,38]. It implies biochemical processes at the membrane level, with changes of first messengers, which in turn are transduced through second and third messengers into transcription regulation of gene expression [18,66], and eventually DNA remodelling [5,46].

The third messengers comprise a heterogeneous class of transcription factors (TFs), such as *c-fos*, *c-jun*, *jun-B* and others, also referred to as immediate-early genes (IEG) acting as protein synthesis switches [32,52]. The activation of TFs occurs in different conditions including various forms of learning and memory (see for a rev., e.g. refs. [51,73]). However, the synthesis of new proteins requires newly synthesized messenger RNAs (mRNAs) as shown in several model systems [12,23,37] or pre-existing mRNAs [31] which are stabilized by covalent modifications or protein–nucleic acid interactions [11,42].

Nitric oxide (NO) is an intracellular messenger or neuromediator in the CNS [9], the synthesis of which is triggered from L-arginine by the nitric oxide synthase enzyme (NOS) [63]. This occurs in a NMDA receptor and Ca^{2+}-dependent manner [74]. There are different NOS isoforms [28]: the inducible form is localized in the macrophages (iNOS) and is Ca^{2+}-independent, whereas the constitutive form is calcium-dependent and is expressed in the endothelium (eNOS) and in neurons (nNOS),

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doi:10.1016/j.bbr.2007.06.021

with several splicing variants [30]. NO is involved in a variety of processes including synaptic transmission, cerebral blood flow and excitotoxicity [17,21,39,45]. Neuronal nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is a NO syntase isoform that as been shown to be responsive to exposure to different behavioural tasks [7,8,16,27,70].

The behavioural paradigm used in this study was exposure to a spatial novelty to monitor arousal and habituation, a relatively simple form of non-associative learning [13,56,58]. Behavioural habituation requires (i) an intact neocortex, (ii) a balance between slow wave and paradoxical sleep during the post-trial period [13,40], (iii) an increased hippocampal cholinergic activity [67,68], (iv) an intact dorsal NE bundle [57], (v) the expression of the transcription factors *c-fos* and *c-jun* [46,48], (vi) the polysome aggregation and protein synthesis in the brain [13]. In addition it can be modulated by NMDA receptors [20,50] and endogenous vasopressin but not opioid peptides [13,61].

The aim of these studies was to map by molecular biology and light microscope imaging techniques the neural traces of information processing and storage in the rat brain in relation to exposure to spatial novelty. This was done using different markers such as the TFs *c-fos*, *c-jun*, *jun-B*, RNA syntesis or NADPH-diaphorase.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats of a random-bred stock (NRB) from our animal colony were used throughout. Rats were 60–80 days of age and 250–280 g body weight. They were kept in standard Makrolon cages (40 cm long, 24 cm wide and 15 cm high), two per cage, with water and food pellets (Mucedola, France) ad libitum. The animal room was lighted from 06:00 a.m. to 06:00 p.m. All other parameters fulfilled the requirements of the "Guide for the Care and Use of Laboratory Animals" by the National Research Council, implemented by EU and local rules.

2.2. Apparatus

The experimental box for spatial novelty was a Làt-maze described in detail elsewhere [58]. Briefly, it consisted of a 60 cm × 60 cm × 40 cm wooden cage with a 30 cm × 30 cm × 40 cm transparent plastic box inserted in the middle. Rats were allowed to explore the resulting corridor (60 cm long, 15 cm wide and 40 cm high). Each box was illuminated by a white, cold 4 W lamp placed 60 cm above the floor in the center of the cover, providing 0.1–0.2 $\mu W/cm^2$.

2.3. Experimental procedure

Rats underwent daily handling prior to the experiment for 1 week. Experimental rats were exposed to a Làt-maze for a 10 min period. Testing was carried out at the beginning of the light phase and the two members of the same cage were tested simultaneously in different boxes to minimize the interference with the post-trial period. The behaviour was monitored by a high-resolution CCD camera and tape recorded for off-line analysis. The dependent variables were the frequency of corner-crossings (horizontal activity: HA) and the frequency and duration of rearings on hindlimbs (vertical activity: VA) per 1 min blocks. At the end of testing, rats were put back in the home cage and left undisturbed until sacrifice. The number of fecal boluses was also counted as an emotionality index [10].

2.4. Perfusion

Rats were perfused through left ventricle with 50 ml of saline solution followed by 250 ml of 4% paraformaldehyde at pH 7.4, 0.1 M phosphate buffer at 4 °C at different times following testing (0.0, 2.0, 6.0 and 24 h) for the TFs ICC and (0.0, 2.0, 24 h) for the NADPH-d HC. For both markers the brains were removed from the skull and postfixed in the same fixative for 2 h, then rinsed in PB three times (10 min each) at +4 °C, then soaked in a 30% sucrose buffer solution for 48 h, frozen in 2-*N*-methylbutane (2-MB) on dry ice and stored at −80 °C. For RNA NB analysis brains were collected 2 h following testing, whereas for RNA experiment rats were sacrificed 6 or 24 h following ^{3}H-uridine injection. The brains were quickly frozen in 2-MB on dry ice and stored at −80 °C. The control groups were handled rats left undisturbed in their home cage for experiments on TFs, RNA synthesis and NADPH-d. Rats exposed for three times to the box at 24 h interval and sacrificed 2 h after exposure were used as additional control group for TFs ICC only.

2.5. Substances

The polyclonal rabbit antibodies were obtained from Santa Cruz Biotechnologies, USA. The secondary antibody, and the ABC system were from Vector Laboratories, USA. The NMDA receptor antagonist (3-(+)-2-carboxypiperazin-4-yl)-propyl-1-phosphate (CPP); [34], was obtained from RBI, Natick, USA. The 5,6-[^{3}H]-uridine 5′-triphosphate (specific activity: 40–60 Ci/mM) dissolved in 5 ml of saline was obtained from Amersham International, UK. NADPH-d diaphorase, nicotinamide adenine dinucleotide phosphate (NADPH), nitroblue tetrazolium and the NOS inhibitor L-nitro-arginine-methylester (L-NAME) were purchased from Sigma Chemical Co. (USA). Triton X-100 was obtained from ICN Biomedicals (USA).

2.6. Northern blot analysis for TFs

RNA was extracted from brain areas according to Chirgwin et al. [14].

Briefly, the samples were homogenized in guanidine thiocyanate, 0.1 M sodium acetate (pH 5.2) and 5 mM EDTA. *N*-Lauroyl sarcosine and CsCl were added to the homogenate up to a final concentration of 4% and 0.15 g/ml, respectively. After centrifugation at 15,000 × *g* the supernatant was stratified over 5.7 M CsCl and centrifuged at 130,000 × *g*. The harvested RNA was fractionated by electrophoresis on a 1% agarose gel containing 6% formaldehyde, and then blotted onto a Hybond N membrane. Ethidium bromide staining was performed on gels and filters to control the amount of RNA in each lane. UV-irradiated filters were then hybridised with a random primed P^{32} *c-fos*, *c-jun* or *jun-B* [41] or poly(ADP-ribose) polymerase (PARP) [43] DNA probe (1–2 × 10^9 c.p.m./mcg) at 42 °C in a buffer containing 50% formamide, 5× Denhardt's solution, 5× SSC, 0.1% sodium dodecyl sulphate (SDS) and 50 mcg/ml salmon sperm DNA. Filters were washed in 2× SSC/0.1% SDS at room temperature then in 0.5× SSC/0.1% SDS at 68 °C for 20 min. Membranes were exposed to X-ray film (Kodak XAR) for 5–7 days and the intensity of the bands was quantified using a LKB 2202 Ultrascan Laser densitometer.

2.7. Immunocytochemistry for TFs

It was performed as described in detail elsewhere [49]. Briefly, 40 μm thick cryostat coronal sections were collected in ice-cold PBS containing 0.1% sodium azide. Sections were free-floating immunostained by rabbit polyclonal antibodies for *c-fos*, *c-jun* and *jun-B* at a dilution of 1:1000 in 0.1 M, pH 7.4 PBS containing 0.15 mM NaN_3, 5% normal goat serum, 2.5% bovine serum albumine and 0.3% Triton X-100. To test specificity of immunostaining in some wells the corresponding peptides were added in excess. Adjacent sections were incubated in a humid chamber for 48 h with either antiserum at 4 °C, followed by a secondary biotinilated antibody (1:200). The immunoreaction was revealed by the avidin–biotin method and visualized using diaminobenzidine as chromogen (0.05%) in 0.01% H_2O_2 0.05 M Tris–HCl buffer. Immunohistochemically stained tissue sections were examined at light microscope.

2.8. Brain RNA synthesis

2.8.1. Implantation of cannulae

One week before the experiment rats were anaesthetized by Pentothal (40 mg/kg^{-1}, i.p.). Under stereotaxic guidance (Stoelting, USA) a stainless steel guide cannula (gauge 22, 16 mm long, 0.41 mm internal diameter, 0.71 mm outer