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The exploration of a new environment leads to the modulation of gene expression for prolonged times in the rat

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A B S T R A C T

In the present study we performed a transcriptional analysis in order to evaluate changes in gene expression induced by exploration in prolonged times. The analysis was carried out 3, 10 and 20 days after exploration. We analyzed the modulation of the expression levels of Pfn2, Casp3, Pdrg1, Pea15, Ywhaz genes which previously were found not modulated 2 days after exploration. Our data show that the expression of Pfn2, Casp3, Pdrg1, Pea15, Ywhaz genes was modulated at 10 or 20 days. The transcript, whose expression had been evaluated with the qRT-PCR, code for proteins which belong to the following functional categories: synaptic modulation, apoptosis, signal transduction.
It is interesting to note that the modulation of the expression of these genes was evident some days after environmental exploration, and not previously at 2 days after conditioning as occurred after contextual fear conditioning (CFC). Hence it is possible to hypothesize that the spatial memory processes require a longer period of elaboration than the emotional ones, fundamental for the survival of the species.

Key words
Exploration • Gene expression • Spatial memory •

1. Introduction

All the prey animals (i.e. mice, rats), in the course of the evolution of the species, have improved their behavioral strategies in order to obtain more information about risk management in a new environment. In an exploration model (Archer 1973; Belzung 2001; Lister 1990; Treit 1985), the animal is exposed to a new situation which should create a conflict between a motivation to explore the new situation and an unconditioned fear of the unknown.

The behavioral parameters which "reflect" the exploration like locomotion or bringing-up the offspring are therefore used to evaluate the emotional reactivity or "anxiety" with the underlying idea that an animal that explores a new situation is emotionally involved.

In rodents, many studies have been conducted using paradigms of preference for new situations. The results showed that perirhinal cortex and the hippocampus are involved in recognizing new objects (Mendez 2015), and an active behavior, like the exploration of a new environment, induces

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the expression of the Arc gene, coding for a protein associated with the cytoskeleton regulated by the activity, in brain regions such as the hippocampus, neocortex and striatum (Vazdarjanova 2006). Moreover, Ramìrez-Amaya V et al (2005) have observed that the Arc gene is transcribed in the neurons that are part of stable neural networks active during spatial explorative behavior, and the ARC protein regulates the traffic of AMPA type receptors of glutamate selecting endosomal paths, suggesting a direct role in synaptic plasticity.

In the present work we evaluated the gene expression in rats which were left free to explore a particular cage which differed from the housing cage in the material that formed the sides. At 3, 10 and 20 days after exploration a transcriptional analysis was performed in order to evaluate changes in gene expression induced by exploration in prolonged times. We focused on the expression of Pfn2, Casp3, Pdrg1, Pea15, Ywhaz genes which did not show changes in the expression levels at 2 days after exploration as described in a previous paper (Federighi 2013).

Materials and Methods

Animals

Normal male Wistar rats (200-250 g body weight, Charles River, Calco, Italy), were employed. 15 rats were randomly assigned to 3 exploration groups (explor) and 9 rats which had never left the home cage were employed as controls. The exploration groups consisted of 5 rats sacrificed 3 days, 5 rats sacrificed 10 days and 5 rats sacrificed 20 days after exploration. The control rats were housed like the exploration rats and in threes were sacrificed 3, 10 and 20 days after the exploration session to which the experimental rats had been subjected.

The animals were housed under a natural daylight rhythm (12 hours light/12 hours dark cycle) at a constant temperature of 20 ± 1 °C. The rats had access to food and water *ad libitum* throughout the experiment.

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and to institutional rules for the care and handling of experimental animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Florence (Italy) (Prot. N. 77/2008).

Exploration procedure

The rats were transferred from the housing room to a soundproofed room. There they were placed inside a box module (dimensions 29 x 31 x 26 cm; Modular Operant Cage, Coulbourn Instruments L.L.C., Allentown, PA, USA) designed differently from the housing cage. It had the top and two opposite sides consisting of aluminum panels, the other two sides of transparent plastic and the floor made of stainless steel. The acoustically insulated room was kept at a constant temperature of 20 ± 1°C. Illumination inside the room was 60 lux. Herein, the rats were left undisturbed for 8 minutes and then gently put again in their housing cage. All behavioral testing were performed between 10:00 and 13:00 hours to minimize circadian influences.

Quantitative real-time RT-PCR

All animals were anesthetized with ether and then killed by decapitation to obtain samples of a mid-temporal region containing hippocampus, amygdala and the overlying cerebral cortex. Brains were quickly removed, dissected, frozen and stored at -80°C until use.

Total RNA was extracted in ClCs gradient and single strand cDNAs were synthesized using a iScript Select kit (Bio-Rad, Milan, Italy). Quantitative real-time PCR was performed with a SYBR Green kit on the MiniOpticon Two-Color Real-time PCR detection system (Bio-Rad, Milan, Italy). The primers used are listed in Federighi et al (2013). For the PCR reaction 15 µL master mix were prepared using 7.5 µL iQ Sybr Green Supermix (200 µM dNTP, 5 mM MgCl2, 3.75 U iTaqTM DNA polymerase), 1 µg / µl cDNA, 300 nM of each primer and RNase-free H2O. Cycling conditions included initial denaturation (3 minutes at 95 °C), amplification, and a quantification program repeated 40 times (10 seconds at 95°C, 60 seconds at 59 °C with a single fluorescent measurement at the end of each elongation step) and a dissociation protocol (60 °C to 95 °C by 1°C increments followed by 30 seconds hold and fluorescent measurement) (Federighi 2013).

According to the protocol indicated by Bio-Rad (Milan, Italy), the primers' efficiency had been previously assessed. After amplification the melting curve analysis confirmed that the signal obtained corresponded to a single amplicon. Each target gene was loaded with the glyceraldehyde 3-phosphate dehydrogenase (G3PDH), used as a constitutively expressed control gene.
Changes in the expression were assessed by the method of $2^{-\Delta\Delta CT}$ (Livak 2001): the expression levels of each target gene were normalized to the G3PDH gene expression level. This method assumes that both genes are amplified with an efficiency close to 100% and not less than 5%.

Statistical analysis

The modulation of the gene expression was determined by comparing the target gene in explor rats and control rats after normalization to G3PDH. All reactions were made in triplicate. Histograms in the figures represent mean ± E.S. of the data. A Two-way ANOVA was made to assess the significant differences between groups and post-exploration times. When significant differences were observed, the Bonferroni post-hoc test was performed.
GraphPad Prism software (version 4.0, Graph Pad Software Inc., San Diego, CA) was used to perform statistical analyses, and the results were regarded as not significant if $P > 0.05$.

Results

Exploration affected the Pfn2 expression.
In Figure 1, the expression levels of the Pfn2 gene are shown. 3 and 10 days after exploration no difference from control (constitutive expression) was detected, while at 20 days the Pfn expression resulted significantly increased with respect to 3 days and control ($F_{6,28} = 3.47$, $P = 0.005$, Bonferroni post-hoc test $P < 0.05$, in both cases). In this case and for all tested genes, the data reported refer to the expression level of the gene in the hippocampus, amygdala and overlying cerebral cortex as a whole.

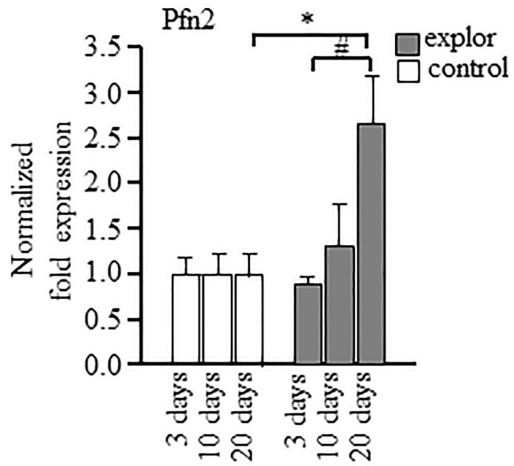

Fig. 1. - Gene expression in control and explor rats after conditioning. In this and in the following figures, transcript levels of each target gene were normalized to the G3PDH gene expression level and compared with the control sample. The levels of expression of the Pfn2 gene were significantly increased 20 days after exploration with respect to control and 10 days after exploration *indicates $P < 0.05$ vs control; # indicates $P < 0.05$ within exploration group.

Exploration modulated genes coding for proteins involved in signal transduction.
In Figure 2, we show the changes due to exploration in the expression of the genes coding for PEA15 (Figure 2A) and YWHAZ (Figure 2B), two proteins involved in the CREB-dependent activation of gene transcription. At 10 days from exploration both Pea15 and Ywhaz were significantly more expressed in the explor group than in control, and, in the explor group, with respect to 3 days (for Pea15: $F_{7,32} = 2.994$, $P = 0.015$, Bonferroni post-hoc test $P < 0.05$; for Ywhaz: $F_{6,32} = 5.341$, $P = 0.001$, Bonferroni post-hoc test $P < 0.05$).

Exploration modulated genes coding for proteins involved in different cellular functions.
In Figure 3A, the modulation of the Casp3 gene expression is plotted. CASP3 is a cysteine protease involved in the execution of apoptosis. 10 days after exploration the Casp3 gene expression was greater than at 3 and 20 days and with respect to control ($F_{7,28} = 4.220$, $P = 0.0007$, Bonferroni post-hoc test $P < 0.05$).
In Figure 3B, Pdrg1 gene expression is shown. PDRG1 is a nuclear phosphoprotein involved in DNA repair. The transcript levels increased progressively and 10 days after exploration were significantly higher than in the control ($F_{6,32} = 3.522$, $P = 0.003$, Bonferroni post-hoc test $P < 0.05$).

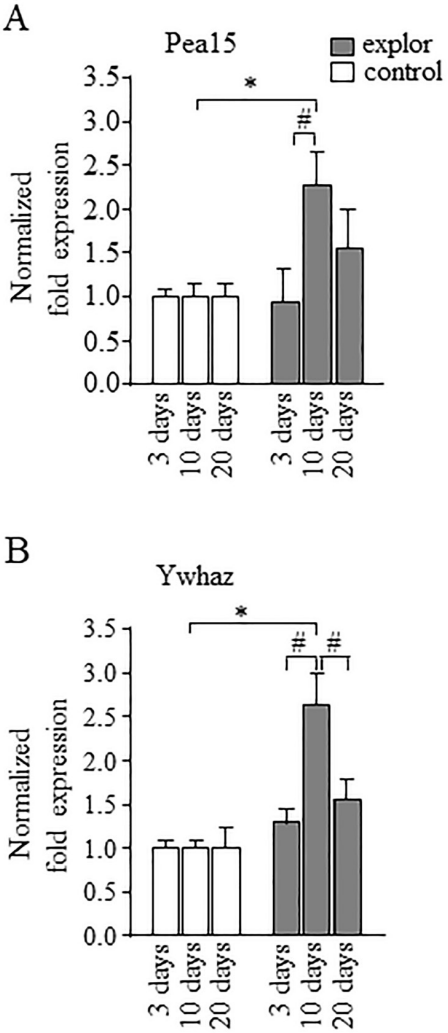

Fig. 2. - Expression of Pea15 and Ywhaz genes after exploration. Three days after exploration the genes Pea15 (A) and Ywhaz (B) kept the constitutive expression level, while, at 10 days the expression was significantly increased. Finally, at 20 days the expression of Pea15 and Ywhaz returned to the constitutive expression level. *indicates P < 0.05 vs control; # indicates P <0.05 within exploration group.

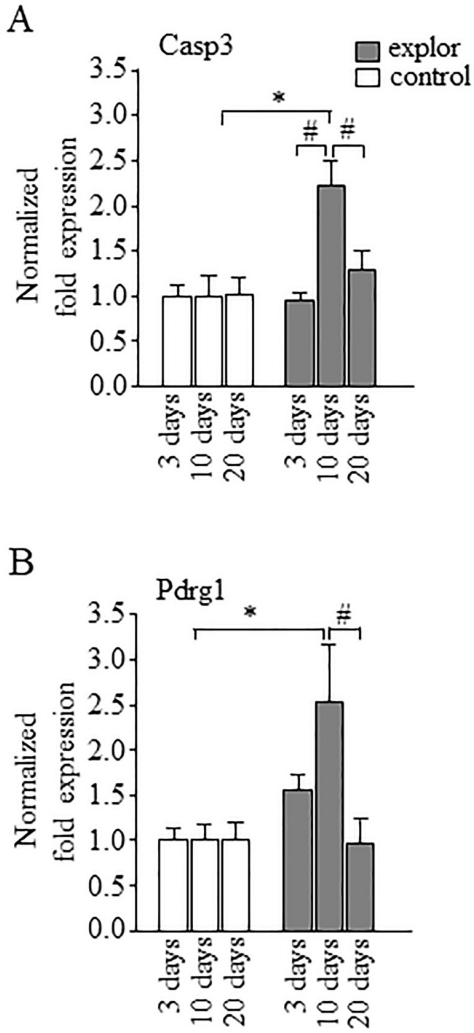

Fig. 3. - Expression of Casp3 and Pdgr1 genes after exploration. Three days after exploration the Casp3 (A) and Pdgr1 (B) expression remained at the constitutive expression level. At 10 days the expression of both genes was significantly increased with respect to the control. Within the exploration group, the expression levels detected at 10 days were significantly increased in comparison with those detected at 3 and 20 days for Casp3 gene, and at 20 days for Pdrg1 gene. * indicates P < 0.05 vs control; # indicates P < 0.05 within exploration group.

Discussion

In this study we evaluated the modulation of the gene expression in rats left to explore a new environment and sacrificed 3, 10 and 20 days after the exploration. Studies have shown that in rodents the hippocampus plays a fundamental role in spatial orientation and behavioral learning that permit the animal to move effectively and generate a "spatial memory" (Sutherland 1982; Irle 1985; Mundy 1988; Barnes 1988; Robinson 2008; Matsumoto 2011; Lieberwirth 2016; Rojic-Becker 2019). From a neural point of view, the information which is elaborated when a rodent explores an environment is essentially by a peak of activity in a particular group of hippocampal neurons consisting of specific pyramidal cells, called positional cells (O'Keefe 1971). It is thought that both the learning and the long-term memory entail changes temporarily involved in the gene expression that lead to the

reinforcing of the synaptic connections in specific cerebral regions (Robles 2003).

Previously, it has been demonstrated that a paradigm of contextual fear conditioning (CFC) induced a long-lasting potentiation of the neural electrical activity in the hippocampus persisting up to 7 days (Sacchetti 2001), and the same CFC paradigm produced a modulation of the expression levels of genes coding for proteins involved in cell growth and proliferation up to 20 days (Federighi 2018).

In the present study, the rats were removed from their own home-box and placed in a cage in which they were free to move about and explore the new environment for a total period of 8 minutes. Then, the rats were taken back to their own home-box and sacrificed 3, 10 and 20 days after.

qRT-PCR data we obtained show a long-term modulation of the expression levels of genes coding for proteins with different functions regarding synaptic modulation, signal transduction apoptosis and DNA repair mechanisms.

Our study showed that the exploration of a new environment led to the modulation of genes encoding the following proteins:

a) PFN2, a protein required for neuritogenesis, the first step of neuronal differentiation, a critical process for neurogenesis in the developing brain (Da Silva 2003). PFN2 is also necessary for axons and dendritic processes (Witke 1998; Carlier 2015; Pernier 2016).
b) PEA 15, a protein involved in the CREB-dependent activation of gene transcription. It can also adjust both ERK and RSK2 when activated it, can translocate into the nucleus where it triggers transcription factors including CREB (Twomey 2013; Lee 2016). Moreover, the involvement of PEA15 in forms of spatial learning has also been reported by Ramos et al. (2009), who observed a strong deficit in the acquisition of spatial tasks in PEA15 knock-out mice.
c) YWHAZ, a protein, which functions as molecular scaffolding by modulating the conformation of its binding partners (Fu 2000; Aitken 2006; Hermeking 2006; Kasinski 2014).
d) Caspase 3, which has been recently studied by Li et al. (2010) who ascribed to it a role in the induction of synaptic depression in rat hippocampal slices. Caspase 3 can be activated by NMDA stimulation without inducing cell death. The caspases are present in dendrites, axons and pre- and postsynaptic terminals, and there is evidence that caspases can be activated in dendrites, synaptosomes and growth cones (Campbell 2003; Gilman 2002; Kuo 2006; Williams 2006; Yuan 2006). It has been reported that the caspases play non-apoptotic roles in the structural remodeling of hippocampal neuron synapses (Gilman 2002), and in bird-song learning (Huesmann 2006).
e) PDGR1 protein, a nuclear phosphoprotein known to bind DNA in order to activate mechanisms of DNA repair (Ko 1996). Recently it has been shown that the presence of members of the Pdrg1 protein family prevents neurodegenerative and cognitive alterations (Wetzel 2008; Rivas-Arancibia 2010; Jazvinšćak 2019).

The interesting aspect that emerges from this work is the fact that after exploring a new environment, some genes coding for proteins with such a broad spectrum of action are modulated very late, starting from ten days after exploration therefore at a time far from the exploration phase, capable of determining a modulation of gene expression (Hejblum 2015; Woodruff 2016). These genes code for proteins involved in multiple functions, some of which currently cannot be included among the mechanisms that are classically recognized as being at the base of the induction and retention of learning and memory. Very few data describe events similar to those considered in our work. A study was conducted by Zalcman et al. (2019) who analyzed the temporal profile (1, 7 to 20 days) of the levels of RNA coding for the CAMKIIδ protein, observing an increase of the expression for distant times compared to the beginning of conditioning, as seen in the present study and in a previous our paper (Federighi 2013). Federighi et al., (2018) showed that an increase of the expression levels occurred in Tiprl (up to 20 days) and Trim32 (up to 10 days) genes in both CFC and explor rats, while Ran gene resulted positively modulated up to 20 days only in explor rats.

At the moment we are unable to explain the mechanisms that determine such a prolonged modulation of gene expression, therefore our data give results that enrich the database of information on the molecular mechanisms underlying learning and memory.

Further investigations will be performed to clarify this issue.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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