Behavioural Brain Research 200 (2009) 150–159

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Colocalisation of c-Fos and glucocorticoid receptor as well as of 5-HT$_{1A}$ and glucocorticoid receptor immunoreactivity-expressing cells in the brain structures of low and high anxiety rats

Małgorzata Lehner[a,*], Ewa Taracha[a], Piotr Maciejak[a,b], Janusz Szyndler[b], Anna Skórzewska[a], Danuta Turzyńska[a], Alicja Sobolewska[a], Aleksandra Wisłowska-Stanek[b], Adam Hamed[b], Andrzej Bidziński[a], Adam Płaźnik[a,b]

[a] Department of Neurochemistry, Institute of Psychiatry and Neurology, 02-957 Warsaw, 9 Sobieskiego Street, Poland
[b] Department of Experimental and Clinical Pharmacology, Medical University, 00-927 Warsaw, 26/28 Krakowskie Przedmieście Street, Poland

ARTICLE INFO

Article history:
Received 31 October 2008
Received in revised form 30 December 2008
Accepted 7 January 2009
Available online 15 January 2009

Keywords:
Conditioned freezing
Ultrasonic vocalisation
Colocalisation c-Fos/GRs-ir
Colocalisation GRs/5-HT$_{1A}$-ir
Prefrontal cortex
Dentate gyrus
Basolateral amygdala

ABSTRACT

We sought to determine the colocalisation of c-Fos (a marker of neuronal activation) and glucocorticoid receptors (GRs) as well as of 5-HT$_{1A}$ and glucocorticoid receptor immunoreactivity-expressing cells (ir) in the dorsomedial prefrontal cortex (M2), dentate gyrus of the hippocampus (DG), and basolateral nucleus of the amygdala (BLA) in low and high anxiety rats (i.e., rats with duration of a freezing response in the conditioned fear test one standard error or more below or above the mean value: low responders (LR) and high responders (HR), respectively). It was found that 1.5 h after a testing session of the conditioned fear test, the LR animals had a higher activity of the cortical M2 area and DG (c-Fos), a higher expression of GRs-ir, as well as an increased number of cells co-expressing c-Fos and GRs-ir in the same brain areas. In the case of HR rats, they had similar expression of c-Fos in the BLA, but a significantly higher concentration of GRs-ir and c-Fos/GR colocalised neurons in the same amygdala nucleus. The pattern of distribution of 5-HT$_{1A}$ and GR receptor-ir in LR and HR animals was similar to the c-Fos and GRs-ir expression. LR animals showed a higher density of 5-HT$_{1A}$ and GRs-ir in the cortical M2 area and DG as well as an increased number of cells co-expressing 5-HT$_{1A}$ and GR-ir in the same brain areas. HR rats had a significantly higher concentration of 5-HT$_{1A}$ and GR-ir as well as a greater number of c-Fos/GR protein colocalised neurons in the BLA. The present data add to the arguments for the neurobiological background of differences in individual responses to aversive conditioned stimuli.

1. Introduction

The aim of this paper was to further explore the neurobiological background of differences in individual responses to aversive conditioned stimuli, using rat conditioned fear response as a discriminating variable. The individual differences in responses to affective stimuli have important clinical implications as they are responsible for predisposition to affective disorders such as depression and anxiety (e.g., post-traumatic stress disorder, PTSD) [10,11,15]. We have recently found that when the strength of a rat conditioned freezing response (the contextual fear test) was selected as a discriminating variable, low responders (LR: i.e., rats with duration of a freezing response one standard error or more below the mean value) had higher activity of the dorsomedial section (M2) of the prefrontal cortex area, (PFCX, according to the criterion proposed by Uylings et al. [57], the dentate gyrus of the hippocampus, and median raphe nucleus (c-Fos expression) in comparison to the high responders (HR: i.e., rats with the duration of the freezing response one standard error or more above the mean value) [30]. These animals also had stronger 5-HT (serotonin)- and CRF (corticotropin releasing factor)-related immunostaining in the dorsomedial section (M2) of the PFCX and increased concentration of GABA (γ-aminobutyric acid) in the basolateral nucleus of the amygdala (*in vivo* microdialysis). The low responders group vocalised more during test session in the aversive band and had higher serum levels of corticosterone. It has been concluded that animals more vulnerable to stress (HR) might have innate deficits in the activity of brain systems control the hypothalamic–pituitary–adrenal (HPA) axis, a system that would normally allow them to cope with stress.

The prefrontal cortex, hippocampus and amygdaloid complex are considered to mediate a stressor-specific influence on the HPA axis function and on emotional processes [12,28]. The entire prefrontal cortical region and amygdala contain high levels of

* Corresponding author. Tel.: +48 22 4582771; fax: +48 22 4582771/4582595.
E-mail address: mlehner@ipin.edu.pl (M. Lehner).

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doi:10.1016/j.bbr.2009.01.012

glucocorticoid and serotonergic receptors [6,42,59]. Exposure to stress potently activates the immediate early gene, *c-fos*, in neurons within the prefrontal cortex and limbic areas, but the phenotype of these neurons is unknown [41]. The findings suggest that exposure to conditioned fear activates the brain areas responsible for integration of hormonal and behavioural responses to stress and suggest the potential for AP-1-glucocorticoid cross-talk in these cell populations (AP-1, activator protein-1) [1,24]. Translocation of glucocorticoid receptor (GR), from cytosol to nucleus occurs under conditions of elevated corticosterone such as stress or exogenous corticosterone administration, and increased nuclear GR levels in the stressed group likely represent such translocation of glucocorticoid receptors [34,50]. Fos and Jun proteins are able to form stable complexes with GR and repress its transactivating capacity [22,31]. Conversely, GRs are capable of impairing the Fos-mediated transactivation of AP-1-dependent transcription [22,31]. Colocalised Fos and GR proteins may interact within cortical and limbic neurons to provide transcriptional regulation of specific target genes, i.e., repress or stimulate the neurotransmitter and neurotransmitter receptor gene expression and thereby modulate stress-related autonomic mechanisms and behaviour.

There are also numerous data indicating an interaction between 5-HT_{1A} and glucocorticoid receptors in the control of the hormonal and emotional effects of stressors [18,19,35,36,43]. Repression by corticosteroids of transcription factors coexpressed in the hippocampus with the 5-HT_{1A} receptor was found to be mediated by both mineralo- and glucocorticoid-receptors (MRs and GRs) [36]. In SN-48 neuronal cells that express MRs, GRs and 5-HT_{1A} receptors, co-activation of MRs and GRs was required for maximal inhibitory action by corticosteroids on 5-HT_{1A} receptor level, and bacterially expressed recombinant MRs and GRs preferentially bound to the glucocorticoid response element as a heterodimer [36,43]. Thus, heterodimerisation of MRs and GRs mediates direct corticosteroid-induced transrepression of the 5-HT_{1A} receptor promoter [36,43]. Repeated administration of corticosterone (10 mg/kg, twice daily for 7 days) attenuated 8-OH-DPAT-induced (a 5-HT_{1A} receptor agonist) inhibition of population spikes and hyperpolarisation in rat CA1 (cornus Ammoni) hippocampal neurons [8]. Chronic treatment with corticosterone also decreased 5-HT_{1A} receptor binding in the CA1 region and the dentate gyrus [8]. On the other hand, exposure of the raphe cells to 8-OH-DPAT or citalopram (a serotonin uptake inhibitor) produced a significant decrease in the number of GR binding sites [18]. Flesinoxan (a 5-HT_{1A} receptor agonist)-treated rats exhibited higher plasma corticosterone levels than vehicle-treated animals [6]. After double immunolabelling (Fos/CRF) every CRF (corticotropin releasing factor) neuron detected in the paraventricular hypothalamic nucleus (PVN) also contained Fos 1 h after flesinoxan treatment [6]. Moreover, a significant correlation existed between the number of Fos-immunoreactive (ir) neurons in the PVN and the plasma corticosterone level. It was concluded that flesinoxan exerted its activating effects on the HPA axis via CRF neurons in the PVN. These effects are likely trans-synaptically mediated by other brain areas, such as the CeA (central nucleus of the amygdala) and BNSTdl (bed nucleus stria terminalis, dorsal section), which also show increased Fos-ir [6].

These and other data indicate that stressor-induced stimulation of c-Fos and corticosterone activates some cortical and limbic regions responsible for integration of responses to fear-evoking stimuli and suggests the potential for AP-1-GR-5-HT_{1A} receptor cross-talk. In view of all these facts, we sought to determine the colocalisation of c-Fos and glucocorticoid receptor as well as of 5-HT_{1A} and glucocorticoid receptor immunoreactivity-expressing (ir) cells in the prefrontal cortex, dentate gyrus of the hippocampus, and basolateral nucleus of the amygdala in low and high anxiety rats. It is noteworthy that in this paper, we have followed the criterion of the dorsomedial section of the prefrontal cortex proposed by Uylings et al. [57], and this area was defined as the M2 (Fr2) – secondary motor cortex and Cg1 and Cg2 (cingulate cortex) areas, according to the atlas of the rat brain of Paxinos and Watson [46]. Moreover, because well-habituated animals show very little if any enhancement of c-Fos production in basal conditions, all animals were trained in the conditioned fear test and examined 1.5 h after the final session of the test. It is noteworthy that we have used tissue samples collected in the previous studies on the neurobiological correlates of differences in emotional behaviour between LR and HR rats [30].

2. Materials and methods

2.1. Animals

The experiment was performed in a cohort of 75 male Wistar rats. The rats (180–200 g of body weight), bought from a licensed breeder, were housed in standard laboratory conditions under a 12-h light/dark cycle (lights on at 7 a.m.), in a constant temperature (21 ± 2 °C) and 70% humidity. The animals were kept in the translucent polycarbonate cages (43 cm × 27 cm × 19 cm) with standard bedding. The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). The Local Committee for Animal Care and Use at Warsaw Medical University, Poland, approved all experimental procedures using animal subjects.

2.2. Contextual fear-conditioning test and ultrasonic vocalisation

The fear-conditioning experiment was performed using a computerized fear-conditioning system, (TSE, Bad Homburg Germany), as described previously [32]. Fear conditioning was performed in the experimental cage (36 cm × 21 cm × 20 cm, *w*/*l*/*h*) under constant white noise condition (65 dB). The experiment was performed during three consecutive days. On the first day, the animals were placed separately for 2 min in a training box, for adaptation to the experimental conditions. The following day, during a 10 min long session, after 2 min of habituation, the animal received three footshocks (stimulus: 0.7 mA, 1 s, repeated every 60 s). The shock intensity was selected according to our previous experiments using this animal model of a conditioned fear response, and the moderate shock intensity effective enough to evoke a fear response, was used. A stimulus too strong could cause the 'ceiling response', for example a panic-like behaviour [30]. On the third day, the freezing response of rats was examined for a 10 min-long period in the testing box without any further stimulation. The conditioned response i.e., the freezing response, was recorded and analyzed by the fear-conditioning system. The freezing behaviour was measured by photo beams (10 Hz detection rate) controlled by the fear conditioning PC-program. Photo beams were spaced 1.3 and 2.5 cm in the direction of the *x*-axis and the *y*-axis, respectively. The absolute duration of inactivity was calculated by the fear-conditioning system, defined as no interruption of any photo beam over 5 s long periods, and then summarized for the whole 10 min long experimental session (total time of freezing). The fear-conditioning system has been validated previously in our laboratory [32,52]. The method of automated measurement of a freezing response has been used in our and other laboratories for years, and it has been validated pharmacologically using many clinically effective and experimental anxiolytic and anxiogenic agents [32,49]. Accordingly, computerized method based on latency between photobeam interruption measures is a reliable scoring criterion in rodents, and computer measures obtained during contextual fear-conditioning tests showed high correlation with hand-scored freezing, "*r*" values ranged from 0.87 to 0.94 [54,58].

Ultrasonic vocalisations were recorded simultaneously by an ultrasonic microphone built in the Mini-3 bat detector (Ultra Sound Advice, London, England), attached to the ceiling of the chamber and processed by an Ultravox interface (Noldus Information Technology) to an IBM compatible PC. The bat detector was set at 22 kHz (the range 17–27 kHz, with minimum duration of an individual acoustic signal accepted by the Ultravox apparatus as a vocalisation event – 300 ms). The vocalisation calls around 22 ± 5 kHz, were selected as this frequency calls are considered to be selectively induced by aversive stimulation [4,5,21]. The duration of ultrasonic vocalisation (s) during 10 min long session was recorded (total time of vocalisation, i.e., cumulated time of individual calls in 10 min) [29,30].

After 10 days of adaptation to the housing conditions, the animals were subjected to the conditioned fear test (S group, $n = 45$), while the control group (C, $n = 15$) was placed in the conditioning box only. Next, S animals were divided into three experimental groups according to the following criterion: LR: low responders, i.e., animals with the duration of a freezing response one standard error or more below 205 s ($n = 15$); IR: intermediate responders, i.e., rats with the duration of a freezing response between 205 and 236 s ($n = 15$), and HR: high responders, i.e., rats with the duration of a freezing response one standard error or more above 236 s ($n = 12$). This criterion was established according to the mean time of a freezing response in all fear-conditioned animals (group S, mean 220.6 s, ±15.54 s, SEM). The criterion for LR group was <205 s, i.e., 220 s − 15.54, for IR group 205–236 s, for HR group >236 s, i.e., 220 s + 15.54. The data from three animals were eliminated from the study because of