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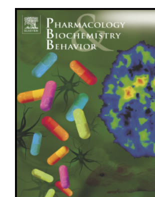

Midazolam treatment before re-exposure to contextual fear reduces freezing behavior and amygdala activity differentially in high- and low-anxiety rats

Anna Skórzewska [a,*], Małgorzata Lehner [a], Aleksandra Wisłowska-Stanek [b], Danuta Turzyńska [a], Alicja Sobolewska [a], Paweł Krząścik [b], Adam Płaźnik [a,b]

[a] Department of Neurochemistry, Institute of Psychiatry and Neurology, 9 Sobieskiego Street, 02-957 Warsaw, Poland
[b] Department of Experimental and Clinical Pharmacology, Medical University, 1B Banacha Street, 02-097 Warsaw, Poland

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ABSTRACT

The aim of this study was to examine the effects of benzodiazepine (midazolam) administration on rat conditioned fear responses and on local brain activity (c-Fos and CRF expressions) of low- (LR) and high- (HR)anxiety rats after the first and second contextual fear test sessions. The animals were divided into LR and HR groups based on the duration of their conditioned freezing response in the first contextual fear test. The fear-re-conditioned LR and HR animals (28 days later) had increased freezing durations compared with those durations during the first conditioned fear test. These behavioral effects were accompanied by increased c-Fos expression in the medial amygdala (MeA), the basolateral amygdala (BLA), and the paraventricular hypothalamic nuclei and elevated CRF expression in the MeA. All these behavioral and immunochemical effects of fear re-conditioning were stronger in the LR group compared with the effects in the HR group. Moreover, in the LR rats, the re-conditioning led to decreased CRF expression in the primary motor cortex (M1) and to increased CRF expression in the BLA. The pretreatment of rats with midazolam before the second exposure to the aversive context significantly attenuated the conditioned fear response, lowered the serum corticosterone concentration, decreased c-Fos and CRF expressions in the MeA and in the BLA, and increased CRF complex density in M1 area only in the LR group. These studies have demonstrated that LR rats are more sensitive to re-exposure to fear stimuli and that midazolam pretreatment was associated with modified brain activity in the amygdala and in the prefrontal cortex in this group of animals. The current data may facilitate a better understanding of the neurobiological mechanisms responsible for individual differences in the psychopathological processes accompanying some anxiety disorders characterized by stronger reactivity to re-exposure to stressful challenges, e.g., posttraumatic stress disorder.

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1. Introduction

Fear is an evolutionarily conserved behavioral response essential for survival that initiates avoidance or escape from predators and from harmful situation (Ohman and Mineka, 2001; Marek et al., 2013). Emotional learning processes, such as fear conditioning, are assumed to be important mechanisms in the etiology of anxiety disorders (Mineka and Oehlberg, 2008), and their impairment may lead to the development of anxiety in response to negative events or trauma (Hermann et al., 2014). The amygdala and the medial prefrontal cortex (mPFC) are involved in the modulation of fear conditioning (Marek et al., 2013). The amygdala is the key structure in the acquisition and expression of fear conditioning and extinction, whereas the mPFC is required for the expression of learned fear and the consolidation of extinction memory (Marek et al., 2013). Dysfunctions of this network may be involved in several forms of psychopathology, including posttraumatic stress disorder (PTSD) and phobia (Hermann et al., 2014).

The involvement of GABA neurotransmission in learning and memory processes, as well as in the mediation of defensive behavior, fear and anxiety, has been well-documented (Harris and Westbrook, 1995, 1999; Dielenberg et al., 1999; Pain et al., 2002; Gafford et al., 2005; Santos et al., 2005; Kroon and Carobrez, 2009). Benzodiazepines may interfere with both the acquisition and expression of learned fear by potentiating the inhibitory effects of GABA in the basolateral amygdala (BLA) (Harris and Westbrook, 1999). Microinjecting benzodiazepines into the BLA before conditioning impaired the freezing and avoidance responses to the context (Dickinson-Anson and McGaugh, 1993; Helmstetter, 1993; Harris and Westbrook, 1995). However, the intra-BLA infusion of bicuculline (a competitive antagonist of GABA-A receptors) blocks the effects of midazolam on the retention of inhibitory avoidance training (Dickinson-Anson et al., 1993).

* Corresponding author. Tel.: +48 22 4582732; fax: +48 22 4582771.
E-mail address: skorzews@ipin.edu.pl (A. Skórzewska).

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In recent years, we have studied the central mechanisms responsible for individual vulnerability to stressors by employing a model that divides rats into high- (HR) and low- (LR)anxiety groups based on the duration of their conditioned freezing response in a contextual fear test. We found that the LR rats exhibited higher activity in the prefrontal cortex, whereas HR rats had significantly higher concentrations of CRF, glucocorticoid receptors (GRs) and c-Fos/GR-expressing neurons in the BLA compared with those concentrations in the LR group (Lehner et al., 2008, 2009a). Additionally, HR rats displayed increased basal concentrations of GABA-A receptor alpha-2 subunits in the amygdala (Lehner et al., 2010a). Moreover, in the recent studies, we have demonstrated that HR rats are more susceptible to anxiogenic and depressive effects of chronic corticosterone administration or chronic restraint stress and that these effects were accompanied by changes in the expression of GABA-A receptor alpha-2 subunits in the mPFC and in the BLA (Wisłowska-Stanek et al., 2013; Skórzewska et al., 2014).

The aim of this paper was to model changes in the sensitivity to fear stimuli occurring over time and following re-exposure to a stressor in animals with different degrees of response to fear, i.e., in high- and low-anxiety rats. Thus, we aimed to model and to study the psychopathological processes accompanying some anxiety disorders, e.g., PTSD, at behavioral and immunocytochemical levels. Accordingly, some data indicate that PTSD patients are characterized by the occurrence of some biological predisposing factors, including varying sensitivity to stressors and changes in stress-induced cortisol release. An additional aim was to examine the role of the GABAergic system by administering midazolam, which is a benzodiazepine receptor agonist, before re-exposing rats to the contextual fear stimuli.

2. Materials and methods

2.1. Animals

The experiments were performed on 84 male Wistar rats (200–220 g body weight), purchased from a licensed breeder (Polish Academy of Sciences Medical Research Center, 5 Pawinskiego Str., Warsaw, Poland) and housed under standard laboratory conditions with a 12 h light/dark cycle (lights on at 7 a.m.) at a constant temperature (21 ± 2 ° C). 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 the Warsaw Medical University, Poland approved all experimental procedures using animals.

2.2. Experimental protocol

After four days of acclimatization to the vivarium, the animals were subjected to a contextual fear-conditioning test to assess individual responses to conditioned aversive stimuli (Lehner et al., 2010a, 2011). The rats were divided into low- (LR, n = 40) and high-anxiety (HR, n = 41) groups based on the duration of their conditioned freezing in a contextual fear test. Next, the HR and LR rats were divided into six experimental groups: HR_{CFC} — high-anxiety rats after the first contextual fear test (n = 14); LR_{CFC} — low-anxiety rats after the first contextual fear test (n = 15); $HR_{Re\text{-}CFC}$ — high-anxiety rats pretreated with vehicle solution and conditioned for the second time to the aversive context (n = 13); $LR_{Re\text{-}CFC}$ — low-anxiety rats pretreated with vehicle solution and conditioned for the second time to the aversive context (n = 12); HR_{mid} — high-anxiety rats given midazolam at the dose of 0.25 mg/kg, and conditioned for the second time to the aversive context (n = 14); and LR_{mid} — low-anxiety rats given midazolam at the dose of 0.25 mg/kg, and conditioned for the second time to the aversive context (n = 13). Then, the part of the animals (HR_{CFC} and LR_{CFC} groups) was decapitated: 10 min after the exposure to the first aversive context for corticosterone detection (HR_{CFC}, n = 8; LR_{CFC}, n = 8) and 90 min after the exposure to the first aversive context for c-Fos and CRF immunocytochemistry (HR_{CFC}, n = 6, LR_{CFC}, n = 7). The remaining animals ($HR_{Re\text{-}CFC}$, $LR_{Re\text{-}CFC}$, HR_{mid}, LR_{mid} groups) were housed in their home cage for 28 days. Next, the animals were subjected to the contextual fear training and test again. 30 min before the second contextual fear test, the rats received injections of midazolam or vehicle. Ten minutes after the second exposure to the aversive context (50 min after drug administration), twenty four animals ($HR_{Re\text{-}CFC}$, n = 6; $LR_{Re\text{-}CFC}$, n = 6; HR_{mid}, n = 6; LR_{mid}, n = 6) were decapitated in a different room and trunk blood samples were taken and stored at −20 °C for corticosterone assay. The remaining animals ($HR_{Re\text{-}CFC}$, n = 7; $LR_{Re\text{-}CFC}$, n = 6; HR_{mid}, n = 8; LR_{mid}, n = 7) were decapitated 90 min after the exposure to the aversive context (130 min after drug administration). Their brains were removed, frozen and stored at −70 °C for c-Fos and CRF immunocytochemistry (Fig. 1).

2.3. Contextual fear-conditioning test

The fear-conditioning experiment was performed using a computerized fear-conditioning system (TSE, Bad Homburg, Germany; FCS 04.11) in a Plexiglas cage (36 × 21 × 20 cm, w/l/h) with a steel foot-shock grid (the 38 floor bars are 0.4 cm in diameter and are spaced 0.5 cm apart), under constant white noise (65 dB) and constant illumination (12 V, 10 W halogen lamp ~150 lx). Freezing behavior was recorded by an infrared photobeam system (10 Hz detection rate) controlled by the fear-conditioning system. Photobeams were spaced 1.3 cm in the direction of the x-axis and 2.5 cm in the direction of the y-axis. This method and equipment have been used in our and other laboratories for years and have been validated pharmacologically using many clinically effective and experimental anxiolytic and anxiogenic agents (Stiedl et al., 2000; Maciejak et al., 2008).

The total duration of inactivity was calculated by the fear-conditioning system. Total duration was defined as no interruption of any photobeam over 5-s periods, which was then summarized for the whole experimental session to yield total time of freezing. The box was cleaned with 95% ethanol after each trial. The experiment was performed on three consecutive days in the same testing box and experimental chamber. On the first day, the animals were placed separately for 2 min in a training box without aversive stimulation to adapt to the experimental conditions. On the second day, a training day, animals were placed for 10 min in the training box. After a 5-min pause, the animals received four 1-s footshocks repeated every 59 s (each consisted of a train of stimuli: 0.7 mA, 150/300 ms) delivered through the stainless steel floor grid. The animals were removed from the testing boxes 1 min after the last shock was delivered. On the third experimental day, the freezing behavior of rats was observed for 10 min in the same box. The conditioned response (freezing reaction) was analyzed and recorded by the fear-conditioning system. The absolute duration of inactivity was calculated from the activity plots and expressed as total time during which the animals were inactive. The computerized method, based on the latency between photobeam interruption measures obtained during contextual fear-conditioning tests, is highly correlated with hand-scored freezing (r values ranged from 0.87 to 0.94) (Takahashi, 2004; Valentinuzzi et al., 1998). The rats were divided into two experimental groups according to the duration of context-induced freezing responses. The LR group had a total duration of freezing responses at least one S.E.M or more below the mean value (230.73–14.98, i.e., <215.75 s). The HR group had a total duration of freezing responses at least one S.E.M or more above the mean value (230.73 + 14.98, i.e., >245.71 s). The mean value of freezing for the LR group = 103.37 s, and for HR group = 348.95 s. Three rats did not meet either criterion.

2.4. Drug treatment

Midazolam hydrochloride (Polfa SA, Poland) was diluted in 0.9% saline, and injected intraperitoneally at a dose of 0.25 mg/kg in a volume of 2 ml/kg. The midazolam dose was selected on the basis of a pilot

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