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The effects of midazolam and D-cycloserine on the release of glutamate and GABA in the basolateral amygdala of low and high anxiety rats during extinction trial of a conditioned fear test

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ABSTRACT

In this study, we investigated how midazolam and D-cycloserine regulate the tonic activity and/or phasic reactivity of brain neurotransmitter systems to fear-evoking stimuli in rats with varying intensities of a fear response. We used a new animal model composed of high (HR) and low (LR) anxiety rats, selected according to their behaviour in the contextual fear test (i.e., the duration of a freezing response was used as a discriminating variable). In these rats, we examined the effects of both drugs on the release of glutamate and GABA in the basolateral amygdala (BLA) during the first extinction trial of a conditioned fear test. The results showed that administration of D-cycloserine (15 mg/kg, i.p.) significantly enhanced the inhibition of an aversive context-induced freezing response observed during the extinction session in HR and LR rats. In contrast, midazolam (0.75 mg/kg, i.p.) accelerated the attenuation of fear responses only in HR rats. The less anxious behaviour of LR animals given saline was accompanied by elevated basal levels of glutamate in the BLA, in comparison with HR rats, and a stronger elevation of GABA in response to contextual fear. In HR animals, the pretreatment of rats with D-cycloserine and midazolam significantly increased the local concentration of GABA and inhibited the expression of contextual fear. These findings suggest that animals more vulnerable to stress have innate deficits in brain systems that control the activity of the BLA mediating the central effect of stress. These results contribute to our understanding of observed individual differences in the effects of anxiolytic drugs among patients with anxiety disorders.

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1. Introduction

Recently, our group proposed a new animal model using high (HR) and low (LR) anxiety rats to examine the neurobiological basis of individual responses to anxiety-causing stimuli. These rats, selected according to their behaviour in the contextual fear test, (i.e., the duration of a freezing response was used as a discriminating variable), could be distinguished based on the activation by conditioned fear of brain structures (c-Fos immunochemistry), expression of glucocorticoid receptors, and changes in local monoamine concentration (*in vivo* microdialysis) (Lehner et al., 2008a, 2008b). All of these changes indicated differences in the involvement of brain structures and dissimilar neurochemical profiles. In brief, LR animals displayed a higher activity in the cortical M2 area and hippocampal dentate gyrus (c-Fos), higher expression of glucocorticoid receptor-immunoreactivity (GRs-ir) and an increased number of cells co-expressing c-Fos and GRs-ir in the same brain areas. In contrast, HR rats exhibited a similar pattern of c-Fos expression in the basolateral amygdala (BLA) but a significantly higher concentration of GRs-ir and c-Fos/GR-positive neurons in this amygdala nucleus (Lehner et al., 2009a).

In our most recent study, we found that HR animals demonstrate a marked decrease in the conditioned fear response over the course of two extinction sessions (16 days), as compared to control and LR groups. Moreover, the recovery of context-related behaviour upon re-learning of contextual fear in HR animals was accompanied by a selective increase in c-Fos expression and GRs-ir in the DG area of the hippocampus, and in the BLA nucleus (Lehner et al., 2009b). This study was designed in such a way to mirror some aspects of clinical situations, in which anxious patients subjected to exposure therapy are again exposed in their environment to the aversive, contextual, conditioning stimulus.

Here, we examined the effects of midazolam and D-cycloserine on glutamate and GABA release in BLA, during the first extinction

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trial of a conditioned fear test. Thus, the local activity of amino acids in both groups of animals was studied in a non-stimulated condition and after a pharmacological challenge. We hypothesised that midazolam (a benzodiazepine that reinforces transmission at $GABA_A$ receptors) and D-cycloserine (a partial agonist of the glycine site of the NMDA receptor) have the potential to control the BLA-related emotional process. Brain GABA and glutamate regulate or modulate many of the same behavioural dimensions (e.g., negative or positive affect) that are disturbed in depression and anxiety disorders and are ameliorated by drug treatment (cf. Millan, 2003). Glutamate and GABA release in the amygdala is thought to be important for the acquisition and expression of fear responses, and the expression of conditioned fear to a context previously paired with shock can produce a rapid and transient increase of these amino acids in the basolateral and central nuclei of the amygdala (Reznikov et al., 2007; Skórzewska et al., 2009; Venton, Robinson, Kennedy, & Maren, 2006).

The extinction of classical fear conditioning is thought to involve activity-dependent synaptic transmission in the medial prefrontal cortex, resulting in the inhibition of amygdala-dependent fear responses (Herry & Mons, 2004; Lehner et al., 2009b; Quirk, Garcia, & Gonzales-Lima, 2006). Although the impaired extinction of traumatic memory is a hallmark of post-traumatic stress disorder (PTSD), the underlying mechanisms are unclear, and effective pharmacological interventions have not been developed. Animal studies have identified NMDA receptors to be crucial in fear memory acquisition and consolidation, as well as for extinction and reconsolidation. Indeed, the NMDA receptor partial agonist D-cycloserine, which facilitates fear extinction in rodents, has been shown to increase the effect of exposure therapy in psychiatric patients in conditions such as phobias, social anxiety and obsessive–compulsive disorder (Mathew, Price, & Charney, 2008). In this paper, we investigated how midazolam and D-cycloserine regulate the tonic activity and/or phasic reactivity of the BLA amino acid systems to fear-evoking stimuli in rats with varying intensities of a fear response. Our findings may help elucidate the mechanisms of observed individual differences in response to anxiolytic drugs among patients with anxiety disorders.

2. Materials and methods

2.1. Animals

The experiment was performed in a cohort of 55 male Wistar rats. The rats (180–200 g body weight) were bought from a licensed breeder and were housed in standard laboratory conditions under a 12 h light/dark cycle (lights on at 7 a.m.) at a constant temperature (21 ± 20 °C) and 70% humidity. The animals were kept in translucent polycarbonate cages (43 × 27 × 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. Experimental protocol

We aimed to assess the effect of midazolam and D-cycloserine on rat behaviour and amino acid levels (*in vivo*) in the basolateral nucleus of the amygdala during the extinction session on the 8th day after the contextual fear conditioning test. After 4 days of acclimatisation in the vivarium, the animals were subjected to the conditioned fear test. Afterwards, all animals remained undisturbed in their home cages for 7 days and then were subjected to the context extinction session (E). To reduce the stress of the drug injection, all of the fear-conditioned animals were handled prior to the context extinction session for 5 days by receiving injections of saline. Two days before the context extinction session (E), a microdialysis probe was stereotaxically implanted into the basolateral nucleus of amygdala (Table 1). The animals were divided into six groups: LR-s, low responders, rats given saline, $n = 7$; LR-mid, low responders, rats given midazolam at a dose of 0.75 mg/kg, $n = 7$; LR-Dcs, low responders, rats given D-cycloserine at a dose of 15 mg/kg, $n = 7$; HR-s, high responders, rats given saline, $n = 8$; HR-mid, high responders, rats given midazolam at a dose of 0.75 mg/kg, $n = 6$; HR-Dcs, high responders, rats given D-cycloserine at a dose of 15 mg/kg, $n = 7$ (for detailed information, see Section 2.5). Two days after the surgery, microdialysis assays (collection of dialysate samples) were performed during the context extinction session (E) in the fear conditioning box. The animals received drugs or saline 30 min before the context extinction session (E) (Table 1).

2.3. Microdialysis probe implantation

The rats were anesthetised by an intraperitoneal injection of ketamine (100 mg/kg) and fixed in a stereotaxic apparatus (Stoelting & Co., USA). The microdialysis probe (hand-made microdialysis probe, membrane loop 4 mm in length, U-shaped, 2 mm tip length, pore diameter 0.8–2.0 μm, 30-kDa cut-off) (Gołembiowska & Dziubina, 2000) was implanted into the right basolateral nucleus of the amygdala. The mean *in vitro* recovery rate for the amino acids was about 25%. Using the bregma as a reference point, the following coordinates for the basolateral amygdala (the tip of a microdialysis probe) were selected: AP = −2.5 mm, ML = 5 mm, DV = 8 mm (Paxinos & Watson, 1989). The microdialysis probe was fixed to the skull with jewellery screws and dental acrylic cement (Fig. 1).

2.4. Pharmacological treatment

Rats were injected intraperitoneally with either saline or midazolam (0.75 mg/kg) or D-cycloserine (15 mg/kg) freshly

Table 1
Treatment scheme.

Days	Procedure
1–4	Adaptation to the housing condition
5	Contextual fear conditioning test: adaptation/acclimation
6	Contextual fear conditioning: training session (3 × 0.7 mA); (S, C groups; S – fear conditioned group; C – control group exposed to the novel context only)
7	Contextual fear test, test session (T) S animals divided into groups according to the criterion of a freezing response: LR (low responders) < 240 s HR (high responders) > 279 s
8–12	Rats in home cages, handling to injection stress (saline)
13	Microdialysis probes implantation into the basolateral amygdala, right site (LR-s, LR-mid, LR-Dcs, HR-s, HR-mid, HR-Dcs groups)
14	Resting time
15	Context extinction session (E) and microdialysis, (the data are shown in Figs. 2–4) (LR-s, LR-mid, LR-Dcs, HR-s, HR-mid, HR-Dcs groups) 60′ Stabilisation of perfusion (rats in home cages in the experimental room, point '0' 2 × 10′ Sample I (averaged data: samples 1 plus 2, basal perfusion before drug injection) (rats in home cages in the experimental room) 3 × 10′ Sample II (averaged data: samples 3–5; midazolam, D-cycloserine or vehicle injection) (rats in home cages in the experimental room) 10′ sample III (sample 6), context extinction session (rats in test cages, in the experimental room) 10′ Sample IV (sample 7) after the context extinction session (rats in home cages, in the experimental room) 10′ Sample V (sample 8) after the context extinction session (rats in home cages, in the experimental room) 10′ sample VI (sample 9) after the context extinction session (rats in home cages, in the experimental room)

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