INTRODUCTION

It is well known that glutamate and GABA are intimately involved in emotional registration and that glutamate transmission through the N-methyl-D-aspartate receptor (NMDA(R)) is involved in learning and synaptic plasticity. Although anxiety symptoms can be relieved by NMDA(R) antagonists and partial agonists, the functions of NMDA(R) subunits in anxiety behaviour remain unclear (1, 2). NMDA receptors play a crucial role in behavioural and cellular processes related to aversive learning and memory (3). The distinct functional roles of NMDA(R) subunits in anxiety behaviour remain unclear (1, 2). NMDA receptors (GluN2A) and partial agonists, the functions of NMDA(R) subunits in anxiety behaviour remain unclear (1, 2). NMDA receptors play a crucial role in behavioural and cellular processes related to aversive learning and memory (3).

In this paper, we studied differences in the density of N-methyl-D-aspartate (NMDA) receptor GluN2B subunits in the brains of low (LR) and high (HR) anxiety rats subjected to extinction trials and re-learning of a conditioned fear response, modeling a natural course of anxiety disorders. Classifications of animals as LR or HR was determined by fear-induced freezing responses in the contextual fear test. Increased basal concentrations of GluN2B subunits were observed in the amygdala of HR rats as compared to the unconditioned control group by Western blot analysis. Re-exposure of HR animals to the fear-conditioned context resulted in elevated concentrations of GluN2B subunits in the amygdala, hippocampus and the prefrontal cortex compared to LR rats as well as in the hippocampus and prefrontal cortex vs. the control group. In addition, it was shown that re-test of a conditioned fear increased the number of cells expressing GluN2B subunits in the basolateral amygdala, dentate gyrus of the hippocampus and secondary motor cortex (M2) in the HR group relative to the LR group. Together, these data suggest that animals that are more anxious have altered patterns of GluN2B subunit expression in the frontal cortex and limbic structures, which control emotional behaviour.

**Key words:** conditioned fear, fear-extinction, immunocytochemistry, individual differences, N-methyl-D-aspartate receptor GluN2B subunits, brain, anxiety, re-learning
extinction sessions and re-test. For the immunocytochemical analysis, we selected brain regions that participate in the processing of emotional input to the brain and constitute an important part of the Papez circuit - one of the major pathways of the limbic system involved in the cortical control of emotion, i.e., the prefrontal cortex, the hippocampus and the amygdala (11). According to the recent concepts, the amygdala, together with the hippocampus and the medial prefrontal cortex, uses sensory information to evaluate an extinguished fear-conditioned stimulus and acts as a switching circuit that drives the appropriate behavioral response (12).

MATERIALS AND METHODS

Animals

Male Wistar 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±2°C) and 70% humidity. 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.

Fear conditioning (CFT-1)

The fear-conditioning experiment was performed using a computerised fear-conditioning system (TSE, Bad Homburg Germany), as described previously (13). On the first day, animals undergoing fear conditioning (group S) were individually placed for 2 min in a training box to allow them to adapt to the experimental conditions. The following day, after 2 min of habituation to the training box, animals underwent the fear conditioning procedure; each animal received three footshocks (stimulus: 0.7 mA, 1 s, repeated every 60 s) during a 10-min session. The conditioned fear was tested on the third day (test day, T) by examining the freezing response of rats during a 10-min context fear test involving re-exposure to the testing box. The control rats (group C) were exposed to the testing box but did not receive any footshock stimulation. Freezing behaviour was measured by photo beams (10 Hz detection rate) controlled by the fear-conditioning PC-program. The fear-conditioning system has been validated previously in our laboratory (13). Animals of group S (mean freezing duration: 272 s) were divided into two experimental groups according to the duration of context-induced freezing responses. Specifically, LR animals were considered low responders with the total duration of freezing responses one standard error or more below the mean (<248 s), and HR animals were considered high responders with the total duration of freezing responses one standard error or more above the mean (>296 s). Thus, the LR and HR animals did not overlap with respect to the duration of their conditioned fear responses.

Experiment scheme

1. Control experiments (Fig. 1A)

Animals were subjected to the conditioned fear test (S0, n=30), or were placed in the conditioning box only (C0, n=8). Next, S0 animals were divided into two groups, LR0 (n=9) and HR0 (n=10), according their behaviour in conditioned freezing test: HR0 had freezing durations above 268.5 s and LR0 had freezing durations below 216.2 s. Eight days after contextual fear conditioning, all rats were decapitated to assess basal levels of GluN2B subunits. The data from three animals were eliminated from all analyses due to technical problems.

2. Re-learning session (CFT-2) (Fig. 1B)

In the second part of the experiment, after two extinction sessions the animals were again subjected to the conditioned fear training. Group C (n=10) was placed in the conditioning box only. At 1.5 h after the re-test, all animals were decapitated; half of each animal’s brain was analysed by western blot, and the other half was analysed by immunocytochemistry (Fig. 2). The behavioural results have been submitted for publication see: Lehner et al. (10).

3. Western blot

We analysed the expression of GluN2B subunits under basal conditions (i.e., 8 days after contextual fear-conditioning test), and 1.5 h after the re-test. The prefrontal cortex, amygdala and hippocampus were located using the rat brain atlas (14) and

![Fig. 1. Experimental procedures. (A) Control experiment. CFT-1, first contextual fear-conditioning test, AD- adaptation/acclimatisation to the experimental box; T- context fear conditioning, training session (3x0.7 mA); decapitation. (B) Re-learning experiment. CFT-2, second contextual fear-conditioning test; AD- adaptation/acclimatisation to the experimental box; T- training session (3x0.7 mA); E1- context extinction session 1; E2- context extinction session 2; R- contextual fear test and final session during CFT-2; re-test - R. For more details, see Materials and Methods.](image-url)
dissected. Aliquots of tissue samples corresponding to 15 µg/well of total protein were heated at 100°C for 3 minutes with an equivalent volume of 2x sample buffer (containing 4% SDS and 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris HCl, pH 6.8) and loaded onto 10% SDS-polyacrylamide gels. The membranes were incubated in rabbit polyclonal immunoglobulin G specific for the GluN2B subunit of the NMDA receptor (1:1000, Thermo Scientific, Pierce Biotechnology) or a mouse monoclonal antibody directed against β-actin (1:10 000, Sigma) at 4-8°C for 72 hours in 2% non-fat dry milk in PBS supplemented with 0.1 % Tween-20 (PBST). The bands on the membrane were captured with the use of computerised image analysis system (Olympus DP-Soft version 3.2 software), and the optical density of each band of the GluN2B subunit of the NMDA receptor and the corresponding β-actin was measured and quantified. The results were normalised to the total protein level in each lane such that the values of the GluN2B subunit of the NMDA receptor are presented as a percentage of the control (β-actin optical density) (15).

4. Immunocytochemistry

The immunocytochemical reaction was performed on slide-mounted brain sections. The coronal 15-µm cryostat sections, identified using the rat brain atlas (14), were cut, mounted on silane-coated slides and fixed in methanol for 5 min. Three slices were taken from each section for immunolabelling of the GluN2B subunit of the NMDA receptor. Brain sections were incubated with rabbit polyclonal GluN2B subunit of the NMDA receptor (1:500, Thermo Scientific, Pierce Biotechnology) at 4-8°C for 72 h, washed in 0.01 M PBS solution (pH=7.4) three times for 15 min each, incubated with anti-rabbit IgG conjugated to Texas Red (Jackson Immunoresearch) for 2 hours and rinsed in 0.01 M PBS solution (pH=7.4) twice for 15 min. Finally, after washing in 0.01 M PBS solution (pH=7.4) twice for 15 min, slide-mounted brain sections were cover-slipped in a mixture containing glycerol and PBS (4:1). Immunoreactivity of the GluN2B subunit of the NMDA receptor was assessed by fluorescence microscopy (Olympus BX-51 with reflected fluorescence system, Olympus DP 70 digital camera) at a magnification of 100x (10x eyepiece and 10x objective lens). The number of cells was counted with the use of a computerised image analysis system (Olympus DP-Soft version 3.2 software) from three sections per rat in the following subregions: AP 1.20: secondary motor cortex (M2), AP 3.14: basolateral amygdala (BLA), and dentate gyrus (DG) area of the hippocampus (Fig. 2). It is noteworthy that localisation of the cortical area homologous to the prefrontal cortex in the rat brain remains a matter of debate. In this paper, we followed the criterion proposed by Uylings et al. (16), and the prefrontal cortex was defined as the M2 cortical area according to the rat brain atlas Paxinos & Watson (14) within the limits defined by Uylings et al. (16). The microscope field (892 µm x 676 µm), 0.60 mm², was placed within the appropriate region using anatomical landmarks (midline, corpus callosum, cingulum), and pictures were captured. The number of GluN2B-NMDA labelled cells within each region was determined by an experimenter who was blind to the animal group assignment. Only cells clearly identified as GluN2B-positive were counted (Fig. 3). The images were adjusted in brightness and contrast and small artefacts were retouched. The examined areas were sampled using a 0.2 x 0.2 mm frame, and co-expressed cells were counted and expressed as the number of positive nuclei per mm².

Fig. 2. Diagrams adapted from Paxinos and Watson (1989) showing regions of the brain analysed for expression of the GluN2B subunits of the NMDA receptor. Left side: structures used in Western blot analysis. Right side: areas analysed by immunocytochemistry. PFCX - prefrontal cortex, secondary motor cortex (according the criterion by Uylings et al., 2003); DG - dentate gyrus of the hippocampus; BLA - basolateral amygdala.
5. Control for Western blot and immunocytochemistry

Single labelling control experiments were performed without primary and secondary antibodies to detect nonspecific tissue binding of antibodies and endogenous peroxidase activity; these experiments yielded negative results. Control and experimental membranes for Western blot and section for immunocytochemistry method were incubated and processed for all steps in parallel. These experiments also yielded negative results.

6. Statistical data

The data are shown as means±S.E.M. The behavioural, western blot and immunocytochemical data were analysed using ANOVA followed by Newman-Keuls post-hoc test. For the correlation analysis, a Pearson’s coefficient was calculated. Statistical analyses were performed using Stat-Soft Statistica 8.0 for Windows (StatSoft Inc., USA).

RESULTS

Behavioural data from the control experiment in basal conditions and from the re-test experiment

These data were previously published in Lehner et al. (10) (see Table 1). HR rats showed a significant decrease in the conditioned fear response over the course of two extinction
session. Upon re-testing, the fear controlled behaviour of HR rats partially returned at levels below the preextinction value.

Western blot

1. Basal levels of the GluN2B subunit in the prefrontal cortex, hippocampus and amygdala (Fig. 4A)

The results of a one-way ANOVA revealed statistically significant differences between groups in the concentration of GluN2B subunits in the amygdala only (F(2,20)=3.51; P=0.04). Post-hoc analyses showed higher levels of GluN2B subunits in the HR0 group compared to the C group (P<0.05). For other structures, the differences were not statistically significant (the prefrontal cortex: F(2,18)=0.12; P=0.88; the hippocampus: F(2,18)=0.20; P=0.81).

2. Expression of the GluN2B subunit in the prefrontal cortex, hippocampus and amygdala after the re-test session (Fig. 4B)

The results of a one-way ANOVA revealed significant differences in the levels of GluN2B subunits in the following structures: prefrontal cortex (F(2,19)=6.88; P<0.01); amygdala (F(2,22)=4.69; P<0.05); and hippocampus (F(2,22)=5.92; P<0.01). Higher levels of GluN2B subunit expression in the HR group (compared to the LR and C groups) were observed in the prefrontal cortex and hippocampus (P<0.05 and P<0.01, respectively). In the amygdala of HR rats, a higher density of GluN2B subunit expression was observed compared to that in the LR group (P<0.05). A trend for higher GluN2B subunit expression in HR rats as compared to group C (P=0.05) was also observed. Correlational analyses revealed a significant negative relationship between freezing time during re-test (R) and expression of GluN2B subunits in the prefrontal cortex of the HR group r=(-)0.71; P=0.02 (Fig. 5). A positive correlation between the expression of GluN2B subunits in the prefrontal cortex and the density of alpha2 subunits of GABA-A receptors r=(+)0.73; P=0.01 was also observed in the HR group (10).

3. Immunocytochemistry (cell number/mm²) (Table 2, Fig. 3)

The results of a one-way ANOVA revealed statistically significant differences between groups in the density of cells expressing GluN2B subunits in the following brain structures: M2 area (F(2,23)=14.78; P<0.01); the BLA (F(2,20)=10.98; P<0.01); and the DG (F(2,21)=14.31; P<0.01). In animals in the HR group, the density of GluN2B subunits in the BLA, DG and M2 was significantly higher than in animals of the LR group (P<0.01), and the C group (P<0.01).

DISCUSSION

The role of the GluN2B subunit in learning processes

The major finding of the present study is that re-exposure of HR animals to the fear conditioned context resulted in elevated concentration of GluN2B subunit in the amygdala, hippocampus and prefrontal cortex compared to LR rats. Although it is well-established that fear extinction depends on NMDA(R) activation (17, 18) only recently has a role for GluN2B subunit-containing NMDA receptors in fear extinction been proposed (19) In vivo studies have shown that

<table>
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<tr>
<th>Brain region</th>
<th>Control (n=8)</th>
<th>LR (n=9)</th>
<th>HR (n=9)</th>
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<tr>
<td>M2</td>
<td>85.75±5.91</td>
<td>91.33±4.77</td>
<td>165.33±18.01</td>
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<tr>
<td>BLA</td>
<td>88.28±6.45</td>
<td>110.25±6.76</td>
<td>152.25±13.56</td>
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<tr>
<td>DG</td>
<td>80.6±5.04</td>
<td>90.38±6.6</td>
<td>135.51±11.33</td>
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Data are shown as means ± S.E.M. C - control group, rats exposed to the conditioning box only (not subjected to fear conditioning procedure); LR - low responders, HR - high responders. * - differs from C, & - differs from LR. *, & P<0.05; **,&& P<0.01. For more details see Materials and Methods.

Table 2. The freezing behaviour in the experimental groups.

<table>
<thead>
<tr>
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<th>T</th>
<th>E1</th>
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<tr>
<td>C (n=8)</td>
<td>101.12±20.82</td>
<td>x</td>
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<tr>
<td>LR (n=9)</td>
<td>104.87±22.75</td>
<td>x</td>
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<tr>
<td>HR (n=10)</td>
<td>338.2±16.54**</td>
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The data are shown as means±S.E.M. (seconds). The experiment was performed in two parts; the C0, LR0 and HR0 groups were used for the determination of basal levels of the NMDA GluN2B subunits in experimental groups, and the C, LR and HR groups were used to analyse changes in NMDA GluN2B subunits after fear conditioning, extinction sessions and a re-test. T- context fear test session; E1- first extinction session; E2- second extinction session; R- re-test; test session performed 24 h after the re-training. C-, C0- unconditioned control rats; LR, LR0- low responders; HR, HR0- high responders; n- number of rats. *- differs from C or C0; &- differs from LR or LR0; *no-differs from HR during the context fear test session (T, within group comparisons). *,&& P<0.01; **,&&,## P<0.001. (x)- not tested. See Introduction, Materials and Methods and Fig. 1 for more details.
incorporation of the GluN2B subunit into the NMDA receptor complex prolongs the duration of receptor channel opening and results in stronger voltage-dependency during memory formation, particularly in the cortex and hippocampus (20, 21). Furthermore, tyrosine phosphorylation (activation) of GluN2B has been correlated with synaptic plasticity in the amygdala, hippocampus and insular cortex (22). GluN2B subunits are expressed in adult cerebral cortical and hippocampal excitatory neurons, and their increased expression in the forebrain improves learning and memory function in the aged brain (23). It has been shown that receptor-mediated coincidence-detection function of the NMDA(R) is enhanced in cortex of the GluN2B overexpressing transgenic mice, which leads to superior learning and memory in a variety of behavioural tests (24). It was also recently found that administration of NMDA antagonist (ifenprodil) more selective for NMDA receptors containing GluN2B subunits induced alterations of synchronous local field potentials in the prefrontal cortex and limbic structures, that were similar to those observed in the dorsal part of prefrontal cortex of rats exposed to chronic stress (25). The increase in GluN2B subunits in the prefrontal cortex, amygdala and hippocampus found by Western blot and immunocytochemistry in HR rats that underwent two extinction sessions and the re-test supports an essential role of GluN2B subunits in extinction and re-learning processes. These results correspond with behavioural data showing a decline in fear responses over extinction sessions and re-test in HR rats, but not in the LR rats. HR rats showed a marked decrease in the conditioned fear response in the course of two extinction sessions (16 days) in comparison with the control and LR groups. On re-testing, the fear-controlled behaviour of HR rats (a freezing response) only partially returned to the pre-extinction value, and was still significantly below the pre-extinction level. Moreover, HR rats showed increased expression of c-Fos, GR-ir and c-Fos/GR-ir colocalised neurons in the basolateral amygdala (8). Thus, the HR animals showed partial recovery of fear-conditioned behaviour on re-learning accompanied by a characteristic pattern of stimulation of brain structures (M2 area, DG and BLA). This means that an increase in the density of cortical GluN2B subunits could be an adaptive mechanism occurring in the course of extinction sessions (i.e., an activation of the cortico-amygdala output pathway), responsible for a diminution of fear responses in this group of animals on re-test of a conditioned fear. To answer this question, however, it would be necessary to perform additional experiments at different time points of the whole extinction procedure.

The interaction between prefrontal cortex and limbic structures

The extinction of fear in general is considered to be inhibitory learning, such that the expression of an intact association is prevented but not erased (26). There is evidence of postsynaptic receptors’ contribution to the enhanced inhibition of amygdalar neurons in extinction, for example, it was reported that extinction training causes an increase in the expression of alpha-2 GABA-A receptor subunits in the amygdala (10, 27). Fear extinction and memory is mediated by cross-talk between different brain structures, including the amygdala and prefrontal cortex.
cortex (especially the ventromedial part) (28-30). Indeed, it was reported that electrical stimulation of the prefrontal cortex activates GABAergic interneurons (intercalated) in the amygdala, causing an inhibition of principal cells and a reduction of their responses to a previously paired conditioned fear stimulus (12, 31, 32). According to the hypothesis put forward by Sotres-Bayon et al. (33), the processes mediated via GluN2B subunits in the prefrontal cortex after extinction training are particularly crucial in this respect (33). Local infusions of NMDA(R) antagonists: ifenprodil (selective for GluN2B subunit) or CPP ((±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid) into the ventromedial part of the prefrontal cortex immediately after extinction training impaired extinction consolidation (33, 34). The intra-amygdalar injections of ifenprodil administered before extinction training disrupted not only the learning of extinction but also the re-learning of an extinguished conditioned fear response as well as the acquisition and retrieval of fear extinction (19).

Consistent with these studies, our current data support the corollary that fear extinction requires activation of NMDA(R) containing the GluN2B subunit in the prefrontal cortex and, as a consequence, stimulates inhibitory processes within the BLA. In line with this reasoning, it has been previously reported that extinction evokes NMDA-dependent synaptic plasticity in the amygdala (35). Recently, we have found that the lower levels of anxious behaviour displayed by LR rats were accompanied by elevated basal concentration of glutamate in the BLA as compared to HR rats selected for high levels of anxious behaviour (9). These findings might suggest that animals more vulnerable to stress, such as HR rats, have innate deficits in the glutamate system that controls the activity of the BLA, which mediates the central effect of fear. In accordance with the evidence that the extinction and re-learning processes involve NMDA(R) trafficking, we found a higher concentration of GluN2B subunits in HR rats following the two extinction sessions and a re-test in the amygdala by Western blotting, and in the BLA by immunocytochemistry. Thus, it is possible that a relatively low level of glutamate in the basolateral amygdala of HR rats (in comparison with LR animals) could bring about a compensatory increase in the local GluN2B subunit densities upon re-test. Our data also indicate that the higher basal levels of glutamate in LR rats might be a necessary condition for the processing of fear responses and behavioural adaptation (9).

However, the LR rats were characterised by significantly lower densities of GluN2B subunits upon re-test. This paradox may be explained by a compensatory down-regulation of local glutamate-related post-synaptic mechanisms (i.e., a relative decrease in GluN2B subunit density in LR rats compared to HR after the re-test) secondary to elevated basal levels of this neurotransmitter in the amygdala of LR rats. Previous findings using c-Fos expression suggest that HR animals are particularly vulnerable to changes in brain activity evoked by extinction, as shown by increased c-Fos expression in the medial prefrontal cortex and DG of the hippocampus (8). The current data indicate an increased density of GluN2B subunits in the prefrontal cortex, amygdala and DG of HR rats subjected to extinction trials and re-learning of a conditioned fear response and, thus, strengthen the evidence in favour of a role for GluN2B subunits in these brain structures in the processing of fear responses.

Our data provide evidence for the hypothesis that prefrontal cortex might participate in fear extinction via feed-forward activation of inhibitory neurons of the BLA (12). Accordingly, reductions in anxiety symptoms of phobic patients following exposure therapy were associated with increased medial orbitofrontal cortex activity and decreased activation of the amygdala (36). We found also a significant positive correlation between the density of GluN2B subunits in the prefrontal cortex and the density of alpha-2 subunits of GABA(R) in the BLA of HR animals (current work and previous results) (10). Both the density of GluN2B subunits in the cortex and density of alpha-2 subunits in BLA correlated negatively with the duration of the freezing response upon re-test of conditioned fear in HR group.

**Fig. 5.** Scattered plots representing correlation analyses between freezing responses and expression of GluN2B subunits of the NMDA receptor in the prefrontal cortex in HR rats. A- basal conditions. B- the re-test session. C- relationship between expression of GluN2B subunits of the NMDA receptor in the prefrontal cortex and alpha2 of the GABA<sub>a</sub> receptor in the basolateral amygdala in the re-test session (10).
neurons that secrete the inhibitory neurotransmitter GABA (from NMDA-receptor-mediated changes in the amygdala, BLA neurons encode fear and extinction). Experimental data provide support for the idea that fear and fear extinction result from NMDA-receptor-mediated changes in the amygdala, possibly via a descending cortical excitatory innervation of local neurons that secrete the inhibitory neurotransmitter GABA (cf. Sah & Westbrook (12)). It has been suggested that activation by cortical neurons of the intercalated cell masses in the BLA, including neurons which inhibit the neurons in the CeA, inhibits the amygdala’s output during extinction (40). Our correlational data indicating a relationship between the expression of GluN2B subunits in the prefrontal cortex and alpha-2 subunits in the BLA add more arguments for a such conclusion (unfortunately, due to the limitation in the methodology, we could not localise this subunit expression on the inhibitory neurons in the intercalated cell masses in the BLA).

Previous papers from our laboratory indicate also a role of the hippocampus, especially the dentate gyrus (DG), in consolidation, extinction and re-learning processes. Significant increases of c-Fos and glucocorticoid receptor-immunoreactivity in the cortical M2 area and the DG of LR rats compared to HR rats after the first fear-conditioning trial have been reported (8). Moreover, after two extinction sessions and re-testing, c-Fos and glucocorticoid receptor immunoreactivity significantly increased in cortical areas and the DG of HR animals (8). Furthermore, hippocampal neurogenesis is proposed to play an essential role in contextual learning, and it has been demonstrated that an intact DG is required for the acquisition of new contextual memories (41). Our previous and current results on changes observed following re-learning and re-test (in the density of the GluN2B subunits, the expression of c-Fos and glucocorticoid receptors, and the colocalisation of alpha-2 GABA receptor subunit/gerphyn) emphasise the role of the DG-BLA interplay in the modulation of contextual fear-related behaviour, and indicate that HR animals are especially susceptible to factors affecting these processes (8, 10).

LIMITATIONS AND CONCLUSIONS

It should be noted that both groups of animals (HR and LR rats) have been selected using a strict behavioural criterion. Thus, these animals model clinical situations where patients are qualified as more or less anxious depending on the results of psychological tests (42). Taking into account that in the current study, biochemical analyses were performed after a history of fear conditioning, two extinction sessions, re-learning and a final extinction session (re-test), the observed results could be an aggregated effect of the influence of all contributing factors. This has been done to model a natural course of anxiety disorders in humans. It is also possible that during extinction trials, the individually shaped molecular processes, including expression of GluN2B receptors in the prefrontal cortex, can modify information storage or recovery in some brain circuits, such as the amygdala and hippocampus, and lead to the modulation of fear responses (33). Accordingly, IMRI studies revealed that hyperresponsivity of the amygdala and deficient cognitive control during the extinction of conditioned fear in anxious subjects reflect an increased resistance to extinguishing the fear response (43).

The immunochemical part of the study can be criticised on the ground that it does not allow for evaluation of the protein staining in the context of neuronal compartments where GluN2B subunits function might have the relevance for synaptic transmission and behavioural expression (e.g. synaptic, somatrical, or extra-synaptical localisation). However, given that the immunocytochemistry was performed 1.5 hours after the re-test, i.e. at the moment of an intensive nuclear protein production, the intracellular compartment is the likely site of GluN2B subunit expression. Although not all newly produced proteins are reaching synaptic compartments, these are the sites where they are functionally active. Thus, the results of the immunocytochemical study can be directly related to changes in the behavioural expression found among both experimental groups.

Another problem concerns the specificity of the immunological reaction of the antibodies with the GluN2B subunits. Watanabe et al. (44) showed, using GluRe2 (i.e. GluN2B, according to the current nomenclature) (+/-) mutant mouse, digestion by pepsin of the molecular matrix and confocal microscopy, that this receptor subunits are highly expressed in the neuritps, while neuronal cell bodies and thick dendrites were immunonegative or labeled very weakly. According to the authors, the pattern of conventional immunostaining (i.e. without the protease pretreatment), reported by many other authors, represent no authentic immunoreactivity. It should be however stressed, that Watanabe et al. (44) did not exclude the possibility that NMDA receptor subunits in cell bodies and thick dendrites are highly susceptible to protease digestion. Moreover, it is difficult to understand the absence of NMDA receptor proteins in the cell sites where these proteins are intensively produced. Furthermore, in the last decade several research reports have been published on a similar topic (i.e. changes in the expression of GluN2B subunits in response to different physiological and pharmacological stimuli), showing similar to the results presented by us pattern and distribution of the GluN2B subunits in the brain cortical and limbic structures.

Another issue that deserves comment concerns a negative correlation between the expression of GluN2B subunits in the prefrontal cortex and duration of freezing behaviour in HR rats, while both the expression of GluN2B and duration of freezing was higher in this group on the re-test in comparison to the control and LR groups. This suggests that the expression of GluN2B correlates positively with re-learning and/or extinction of conditioned fear. It should be noticed, however, that the HR group underwent a long procedure, with their conditioned fear response showing a significant decrease over the course of two extinction sessions, re-learning and the re-test. This significant behavioural effect (a relative decrease in freezing response) was accompanied by parallelly occurring increase in the prefrontal cortex density of GluN2B subunits, thus validating the negative correlation between both experimental variables.

The current and previous results indicate that the prefrontal cortex exercises decisive influence on the activation of inhibitory mechanisms in the amygdala. Additionally, the current results suggest that individual differences in the expression of GluN2B subunits might determine the pattern of animal behaviour and brain responses to fear-evoking stimuli, using an animal model of the clinical situation in which anxious patients undergoing exposure therapy are re-exposed to the fear-conditioned stimuli. The current results also indicate that GluN2B subunits are required for the initiation of neural changes in the prefrontal cortex, dentate gyrus of the hippocampus and amygdala underlying fear extinction and provide preclinical evidence in support of the clinical use of NMDA(R) modulators for the treatment of anxiety-related disorders (19, 33).
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Author’s address: Dr. Malgorzata Lehner, Department of Neurochemistry, Institute of Psychiatry and Neurology, 9 Sobieskiego Street, 02-957 Warsaw, Poland; Phone: (48) (22) 4582771; E-mail mlehner@ipin.edu.pl