FINAL REPORT

on the project entitled

Trauma-induced behavioral deficits: the role of NMDA and AMPA receptor subtypes

(NKFI identification No. 101645)

1. Aims of the study

This project aimed at investigating the role of NMDA and AMPA receptors in traumatic stress-induced behavioral deficits. On the pharmacological side we exploited the functional diversity of NMDA and AMPA receptors, which results from their subunit compositions, and the recent development of subunit-specific antagonists. On the behavioral side we made use of our recently developed test battery that enables the detection of multiple behavioral deficits, which are analogous with, and model multiple symptoms of post-traumatic stress disorder (PTSD). In addition, we tested compounds at different time-points, as PTSD has a slow onset and is durable, for which delayed effects are highly relevant to the disorder.

The ultimate aim of the project was to describe the role of NMDA and AMPA receptors with various subunit compositions on distinct symptoms of PTSD, i.e. our analysis aimed at reaching beyond the usual practice where the conditioned fear paradigm is used as the sole preclinical indication of drug efficacy in models of PTSD. We also hoped that the study would identify new leads to the treatment of this disorder.

The aims of the project were reached and the planned experiments were performed. The study led to interesting and potentially important observations, and also identified one potential target for drug development as shown below.

2. Behavioral findings

2.1. Behavioral deficits

Rats exposed to electric shocks showed multiple behavioral dysfunctions as follows:

- conditioned fear, a commonly observed phenomenon, that is believed to model symptom B4 of PTSD ("psychological distress by trauma-related cues")

- enhanced heart rate responses during the conditioned fear testing, i.e. when subjects were faced with trauma-related cues. This dysfunction is proposed to model symptom B5 ("physiological reactions by trauma-related cues").

- decreased behavioral activity in various tests, including the elevated plus-maze, open-field, social interaction and resident-intruder tests. We propose this dysfunction being analogous to symptom C4 ("diminished participation in significant activities").

- decreased interest towards social partners in the social avoidance test developed by us, which appears analogous to symptom C5 of PTSD (" detachment or estrangement from others").

- the flattening of the day-night heart rate rhythm, which is proposed to model symptom D2 i.e. "difficulty falling or staying asleep".

- subjects exposed to electric shocks also buried inoffensive objects - a behavior rarely seen in controls, which "played" with objects rather than burying them. This symptom was found to be analogous with symptom D4 "hyper-vigilance".

2.2. Temporal changes in behavioral deficits

Behavioral dysfunctions - similar to symptoms of PTSD - did not vanish; moreover, intensified over time. Three types of temporal changes were observed:

1) The intensity of some dysfunctions was constant, i.e. no temporal changes were observed. This was the case of conditioned fear. As this is a common observation, findings were not shown[1].

2) Some dysfunctions, e.g. social avoidance (Fig. 1[2]) were expressed shortly after electric shock exposure, disappeared within approximately one week, but reoccurred strongly by the end of the investigation period i.e. one month after shock exposure. This pattern of changes is reminiscent of the succession of symptoms in PTSD patients who show acute stress disorder first, a condition which last about a month only, seem to recover for about 2-4 month after which the symptoms of PTSD fully develop.

3) Finally, some dysfunctions developed slowly over time such that they became observable after about four weeks. The flattening of the day-night heart rate rhythm is among such dysfunctions (Fig.2.).

2.3. Conclusions and the selection of behavioral paradigms

We conclude that electric shocks elicit long-lasting behavioral dysfunctions, the nature of which and their temporal evolution resemble those seen in humans showing PTSD.

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[1] To remain within the size limits of such reports, behavioral changes are only exemplified rather than shown in extenso.

[2] Figures were placed at the end of this report.

For the testing of pharmacologic agents we selected the conditioned fear, the social avoidance and object burying tests, which model symptoms belonging to clusters B, C, and D, respectively. We chose to administer 3mA shocks to rats during the conditioning trial, as this current intensity had the strongest effects on behavior.

3. Pharmacological findings

In these studies we tested the effects of various subunit-specific blockers of glutamate receptors. The diversity of the NMDA receptor is ensured by their varying subunit composition. As shown in the proposal, the NR1 subunit is a "mandatory" component of all NMDA receptors; the N2a and NR2b subunits are primarily present in NMDA receptors located in the limbic system, whereas NR2c and NR2d subunits are primarily located in NMDA receptors outside the limbic system. By blocking one particular subunit, one selectively blocks those NMDA receptor populations which contain the subunit in question, i.e. various subpopulations of NMDA receptors are selectively blocked.

AMPA receptors are usually differentiated by their calcium permeability. The GluA2 subunit confers calcium permeability to the receptor, which is associated with functional changes relevant for trauma-induced behavioral deficits (see proposal). We tested here the antagonist IEM-1460 which selectively blocks calcium permeable AMPA receptors (CP-AMPA).

3.1. Effects observed in the conditioned fear test

The studies reported here started by a conditioning trial, followed by testing for conditioned fear 1 or 28 days after conditioning. Before testing trials, rats were treated with vehicle, or various doses of the general NMDA receptor blocker MK-801 or with the subunit-specific antagonists PEAQX (NR2a), Ro25-6981 (NR2b), and PPDA (NR2c/d). The level of conditioned fear did not change over time (Fig. 3A-D). MK-801 ameliorated the conditioned fear response at both time-points (Fig. 3A) in a dose-dependent manner. This effect was replicated by the NR2b subunit blocker only (Fig. 3B). The blockers of the subunits NR2a, and NR2c/d were ineffective.

The CP-AMPA receptor blocker IEM-1450 had effects on the expression of conditioned fear neither 1 nor 28 days after shock exposure (data were presented together with effects on fear extinction, see below).

3.2. Effects observed in the social avoidance test

The general NMDA blocker MK-801 effectively ameliorated social avoidance 1 but not 28 days after shock exposure, confirming earlier findings suggesting that the mechanisms underlying the early and late effects of traumatic stress exposure are different (Fig. 4A).

Surprisingly, the NR2b blocker had a rather different effect profile (Fig. 4B). While this blocker was without effect one day after shocks at doses which decreased conditioned fear, it *aggravated* social avoidance 28 days after shocks. We conducted a series of control experiments, which showed that the compound does not induce social avoidance per se, and does not affect locomotion in the absence of shock exposure. We had to conclude that the blockade of NR2b subunit aggravates shock-induced social avoidance despite the fact that it ameliorates conditioned fear.

A similar finding was obtained with the NR2a blocker, which also aggravated social avoidance, this time already 1 day after shocks (the magnitude of changes was similar to that seen with the NR2b blocker, data not shown).

3.3. The object burying test

None of the antagonists affected object burying.

3.4. Fear extinction

CP-AMPA receptor blockade was selected for this type of studies because a series receptor expression and trafficking studies as well as studies with the AMPA receptor enhancer PEPA confirm its role in fear extinction, but the specific blockade of this receptor subtype became possible just recently, and this blocker was not tested so far in fear conditioning paradigms.

First we established a fear extinction paradigm suitable for studying the CP-AMPA receptor blocker IEM-1450 (Fig. 5a,b). We have chosen a paradigm where extinction decreased but did not abolish conditioned fear. As the effects of IEM-1450 on extinction were unknown, this protocol enabled the detection of both decreasing and enhancing effects.

As indicated above, the CP-AMPAR antagonist IEM-1460 did not affect the expression of conditioned fear at either time-point (Fig. 5b and e).

Surprisingly (but at the same time confirming the validity of our approach) the effects of the compound on fear extinction were markedly different 1 and 28 days after shock exposure. One day after conditioning, no fear extinction was observed in rats treated with 1 mg/kg IEM-1460, but the 3 mg/kg dose accelerated extinction. One day after extinction learning, the decrease was

still observable, but a spontaneous recovery occurred 4 weeks later (Fig. 5c). No such recovery was observed in controls. Thus, CP-AMPAR blockade accelerated learning during extinction trials, but inhibited it on the long-run when training and extinction were administered one day after conditioning.

When IEM-1460 and extinction training were administered 28 days after fear conditioning, the 1 mg/kg dose accelerated learning, and *abolished* conditioned fear both one day and four weeks after extinction trials. The 3 mg/kg dose did not accelerate learning during extinction trials, but abolished conditioned fear both one day and 4 weeks later. Albeit larger effects were observed with the lower dose, both IEM-1460-treated groups were similar to non-conditioned rats in trials 6 and 7, showing that extinction training *abolished* conditioned fear in these rats, and the effect lasted at least a month.

3.5. Conclusions

- We tested for the first time many of the specific blockers in paradigms of trauma-induced behavioral deficits. The CP-AMPA receptor blocker IEM-1450 for instance has never been tested in such paradigms, despite the fact the involvement of these receptors in conditioned fear was amply demonstrated. The same applies to the NMDA subunit blockers except for the NR2b blocker; this was also studied for the first time by us, but this happened earlier, in a study that prepared this project.

- The general NMDA blocker MK-801 ameliorated both conditioned fear and social avoidance. These findings show that as expected, the blockade of NMDA receptors ameliorates PTSD symptom-like behavioral dysfunctions in animals. Unfortunately, general NMDA blockers cannot be used in the clinic for their strong side effects.

- It occurs that the only glutamate receptors involved in the expression of conditioned fear are those NMDA receptors which contain the NR2b subunit. Neither other NMDA receptors nor the CP-AMPA receptors had similar effects.

- We observed markedly different effects in shock-induced social avoidance which was aggravated by both NR2a and NR2b blockade.

-Object burying was not affected by the compounds tested in this study.

- The CP-AMPA receptor blocker IEM-1460 transiently facilitated extinction 1 day after conditioning, but learned fear spontaneously recovered 4 weeks later. When the extinction protocol was applied 28 days after training, IEM-1460 enhanced extinction memory; moreover,

abolished conditioned fear for at least a month, a unique finding as no similarly strong effects were obtained earlier with other compounds.

4. Changes in receptor expression

4.1. NMDA receptor subunits

We investigated the mRNA expression of the NR2a and NR2b subunits, as NMDA receptors containing these subunits seemed to be involved in the behavioral dysfunctions induced by shocks (see above). In addition, we also investigated the expression DNA methyltransferase 3a (DNMT3a), an epigenetic enzyme highly responsive to environmental influences (the latter study was not yet fully finished, see Fig. 7).

We selected for study the CA1 area of the hippocampus, the basolateral and central amygdala, as well as the prelimbic and infralimbic region of the prefrontal cortex, as all these regions were shown to be involved in conditioned fear. No changes were observed in the central amygdala, for which data obtained in this region were not shown.

The pattern of changes depended largely on the brain region and the gene investigated. It occurs that gene expression was also affected by social isolation for 28 days, to which rats were submitted when the effects of shocks were investigated 28 days after conditioning. Albeit these changes interfered to some extent with the effects of shocks per se, controls were also kept in isolation, which allowed the detection off shock-induced changes.

Overall, larger shock effects were obtained with the NR2b subunit as compared with the NR2a subunit, and changes observed in the medial prefrontal cortex appeared to be correlated with behavioral findings. In this brain region, the expression of the NR2b subunit increased at both time points, which suggests that neurotransmission mediated by NMDA receptors containing this subunit were enhanced on the long term, and as such the ameliorative effects of NR2b blockers on conditioned fear may have been mediated by this route. Albeit difficult to interpret at present stage, there were large changes in DNMT3a gene expression, which likely affected gene expression profiles beyond NMDA receptor subunits.

4.2. AMPA receptor subunits

Of particular interest in these studies was the expression of the GluR2 subunit, which confers calcium permeability to the AMPA receptor, and the GluA1/GluA2 mRNA expression ratio, which is indicative of the expression ratio of calcium permeable and non-permeable AMPA

receptors. Taken together, the findings presented in Fig. 8 show that fear conditioning durably increased the expression of both GluA1 and GluA2 subunit mRNAs in the medial prefrontal cortex without altering their ratio. In the amygdala, no global changes in expression levels were observed, but the relative expression of the two specific GluA subunits was altered one day but not four weeks after fear conditioning. To investigate the relationship between behavioral dysfunctions and gene expression profiles, we ran a Multiple Regression analysis, which suggested that the level of conditioned fear was associated in the medial prefrontal cortex with increased expression of the subunit that confers calcium permeability to the AMPA receptor (GluR2); by contrast it was associated with the relative decrease of the this subunit in the BLA (i.e. with the increase of the GluA1/GluA2 expression ratio; Fig. 9).

4.3. Conclusions

- Fear conditioning by exposure to electric shocks induced durable changes in the expression of glutamate receptor subunit mRNAs.
- Changes depended on the brain area and gene investigated.
- Marked changes in the expression of the gene of DNMT3a suggests the development of epigenetic changes that likely went beyond glutamate receptor subunits.
- Changes in the expression of glutamate receptor subunit genes can at least partly explain the behavioral effects of the antagonists of the very same receptor subtypes.

5. Overall conclusions

- Our behavioral findings confirmed that the approach developed in our lab is not only applicable but also necessary. By employing tests of several behavioral dysfunctions instead or relying on one (as in most of the studies published so far), we were able to show that dysfunctions that model relevant PTSD symptoms have dramatically different pharmacological responsiveness. E.g. agents that significantly ameliorate one dysfunction my aggravate another. Similarly, testing effects at various time-points proved to be fully justified, as the time elapsed from the traumatic experience had a large impact of the effects of pharmacologic agents. This adds to the growing body of evidence supporting that the mechanisms underlying recent and remote fear are different and agents that ameliorate the former may not do so in the case of the latter.
- The behavioral effects of NMDA receptor subunit antagonist reveal a surprisingly complex interaction between behavioral dysfunctions and glutamate neurotransmission mediated by this

receptor. It occurs that the same NMDA receptor populations have opposite roles in particular behavioral dysfunctions induced by fear learning, which seems to suggest that the NMDA subunit antagonists are not promising as targets for PTSD drug development.

- By contrast, our findings concerning CP-AMP receptor antagonism suggest clinical implications for CP-AMPAR blockers, particularly for acquired anxieties (e.g. post-traumatic stress disorder) which have a slow-onset and are durable.

Fig. 1. *The behavior of rats in the social avoidance test.* Rats were exposed either to 0.8 or to 3 mA shocks, and studied in the test 4h, as well as 1, 3, 14, and 28 days after exposure. The test consisted in exposing rats to a two-chamber apparatus separated by a door. One of the chambers was empty, whereas the other contained another rat ("opponent") confined behind a transparent and perforated partition. Entries into, and time spent in the chamber that contained the opponent ("opponent visits") were considered as indicating social interest (duration was expressed as % test time). Shortly after shock exposure, 0.8 shocks decreased opponent visits more strongly and lastingly than 3mA shocks. The late effect was considerably stronger after 3mA than after 0.8mA shocks.

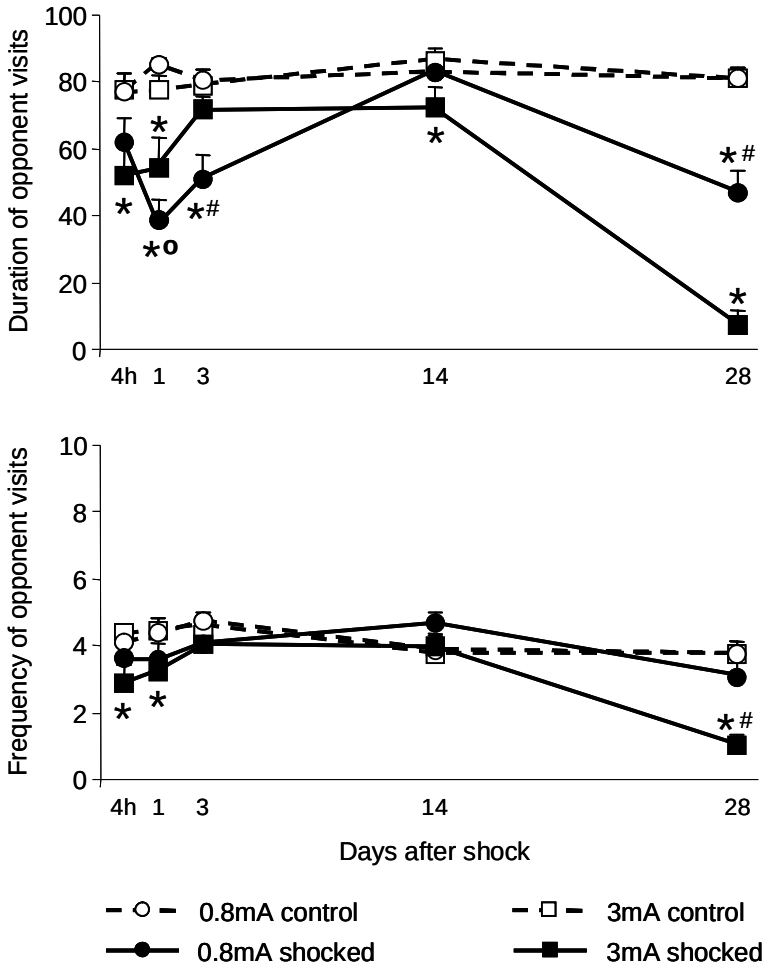

Fig. 2. . The day/night rhythm of heart rates (upper and middle left-hand panels) and home-cage locomotion (upper and middle right-hand panels). One day before shock application (Day -1) rats exhibited a clear day/night rhythm in both variables. 25 days after shocks the diurnal rhythm of heart rates was abolished in 3mA rats. No similar changes were seen in home-cage locomotion. The lower panel shows the dark-phase related increase in heart rates in control, 0.8, and 3mA rats, over the 25 post-shock days. A gradual decrease was noticed in 3mA rats, which reached significance on day 25. eD, early dark period (the first 4h); mD, mid dark period; lD, late dark period (the last 4h); eL, early light period (the first 4h); mD, mid light period; lD, late light period (the last 4h).

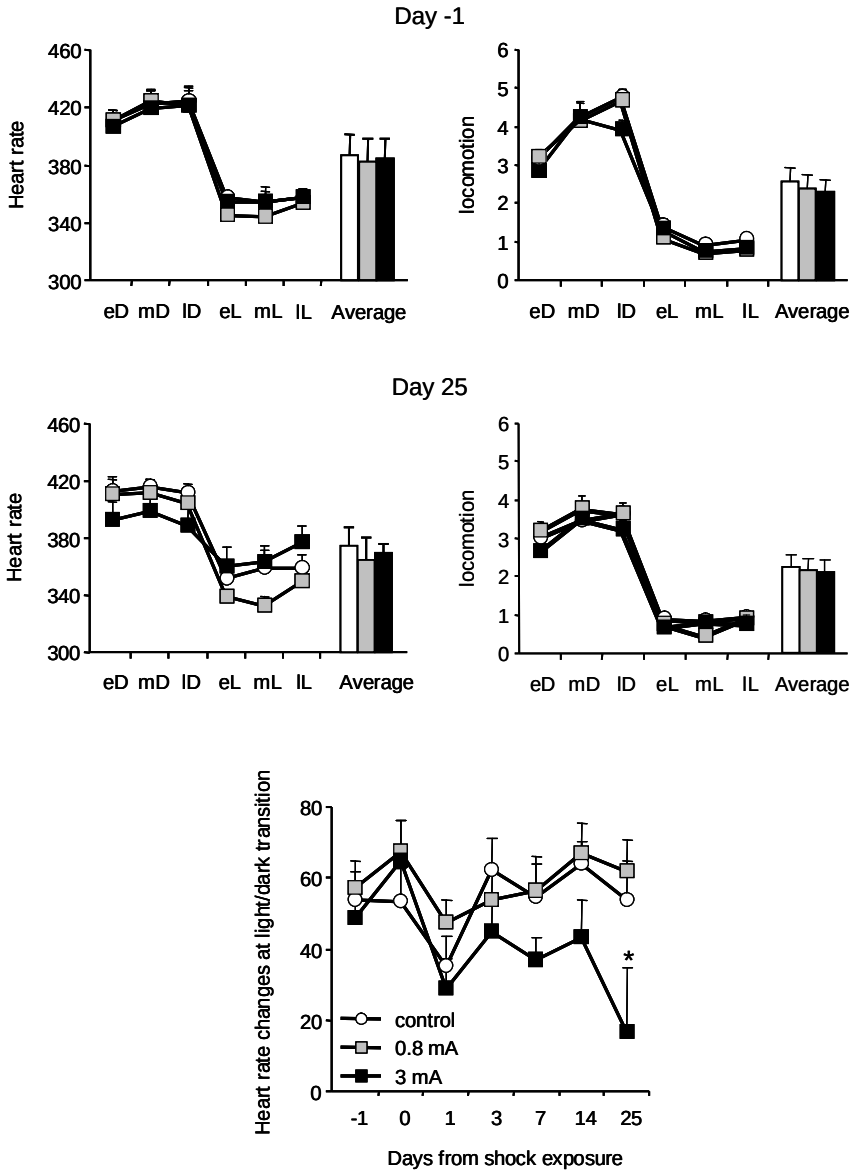

Fig. 3. The effects of specific NMDA receptor subunit antagonist in the conditioned fear test performed 1 or 28 days after shock exposure.

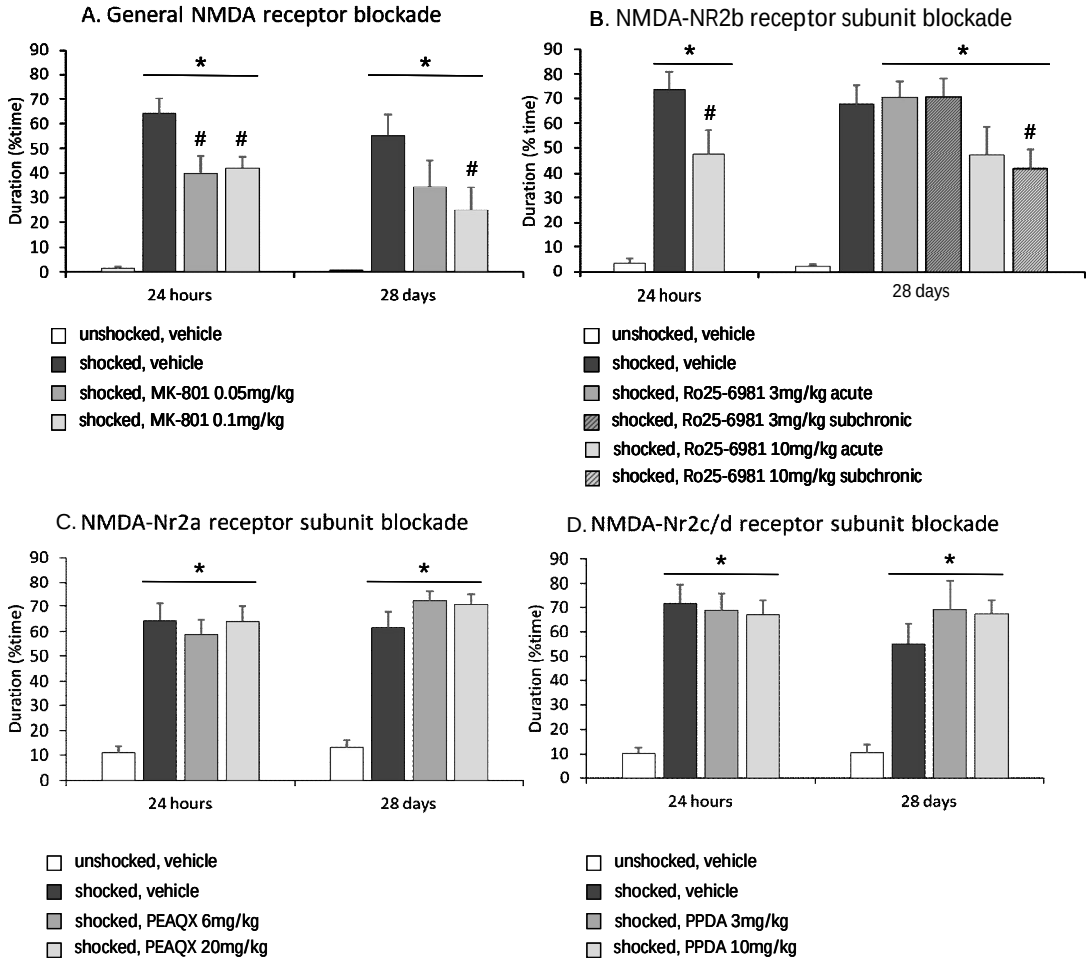

Fig. 4. The effects of the general NMDA receptor blocker MK-801 and of the selective NR2b subunit blocker RO25-6981 on shock-induced social avoidance 1 and 28 days after shock exposure.

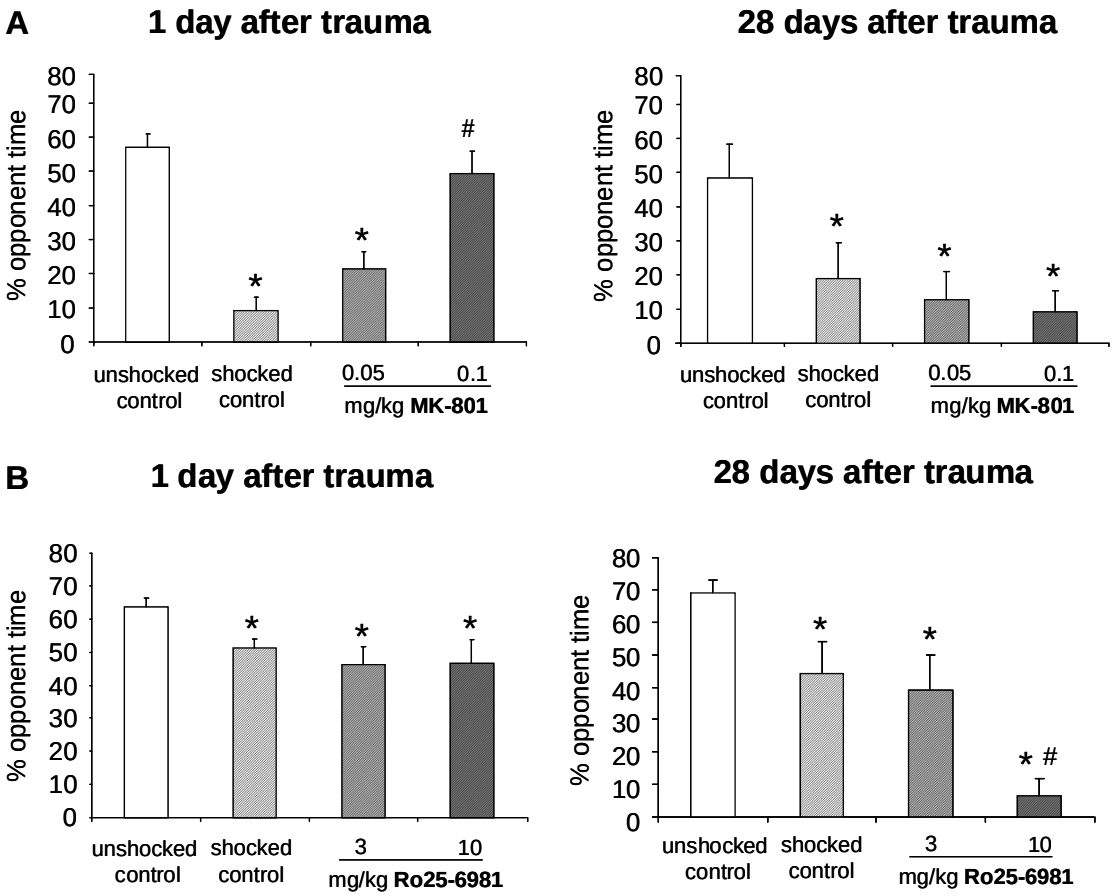

Fig. 5. Testing for a fear extinction paradigm suitable for the present project.

a. 7x1 protocol started 1 or 28 days after fear conditioning. **b**. 3x5 protocol started 1 or 28 day after fear conditioning. The precise experimental design is shown on graphs. The first day of the 3x5 protocol was chosen for testing the effects of IEM-1460, as after this day, conditioned fear was decreased but not abolished. This protocol allowed for the detection of both enhanced and inhibited extinction learning. FC. fear conditioning; CFT, conditioned fear test; Data are expressed as mean ± SEM. *. significant difference from day 1; #. significant difference from day 2 ($p < 0.05$ at least in all cases).

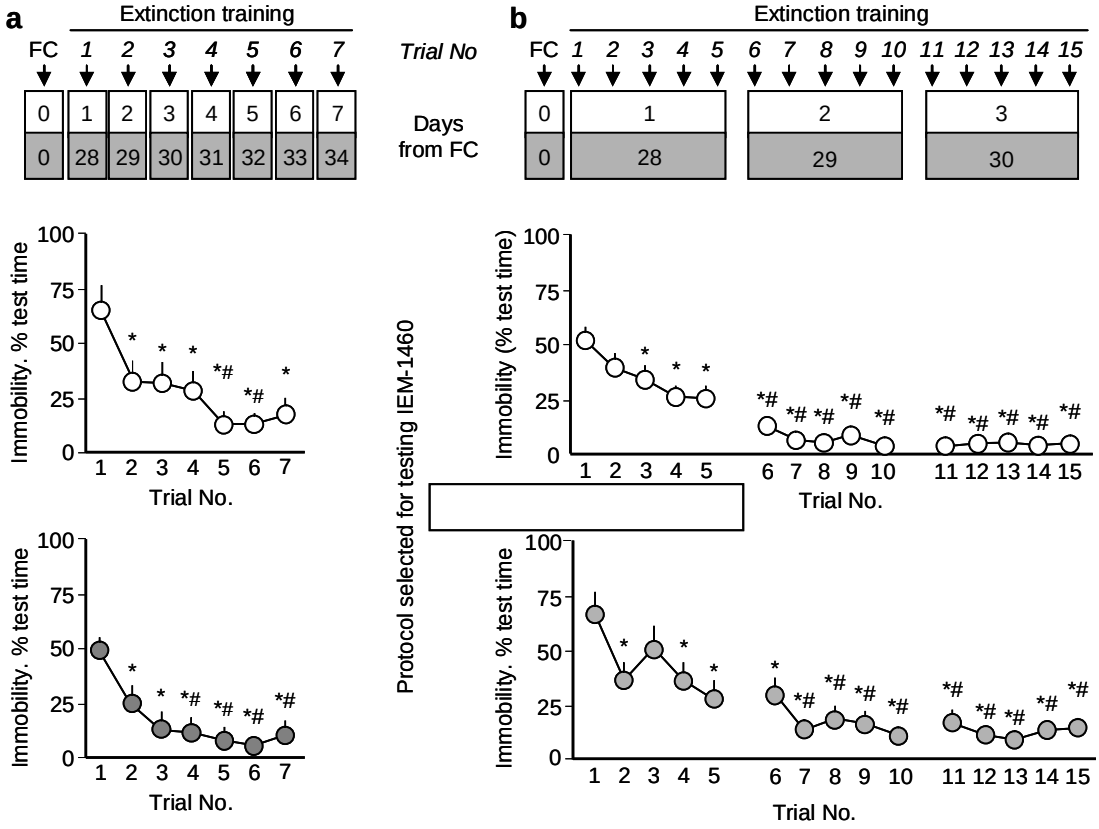

Fig. 6. The effects of the CP-AMPA receptor blocker IEM-1450 on the expression and extinction of conditioned fear. Immobility was expressed as percentage of values obtained in CFT in order to control for differences in behavioral baselines. For values expressed as %test time see Table 2. **a** and **d**. Experimental designs; **b** and **e**. Fear retrieval (conditioned fear test/first extinction trial); **c** and **f**. Behavior during extinction and in trials that tested extinction memory. FC. fear conditioning; CFT, conditioned fear test; gray horizontal lines with red horizontal columns, immobility in the first CFT of rats submitted to FC (mean ± SEM, respectively); gray horizontal lines with blue horizontal columns, the level of immobility in the first CFT of rats not submitted to FC (mean ± SEM, respectively); #, significant difference from non-conditioned controls (effect of fear conditioning); *, significant effect of extinction training (difference from trial 1); ‡, significant difference from vehicle-treated conditioned rats ($p < 0.05$ at least in all cases).

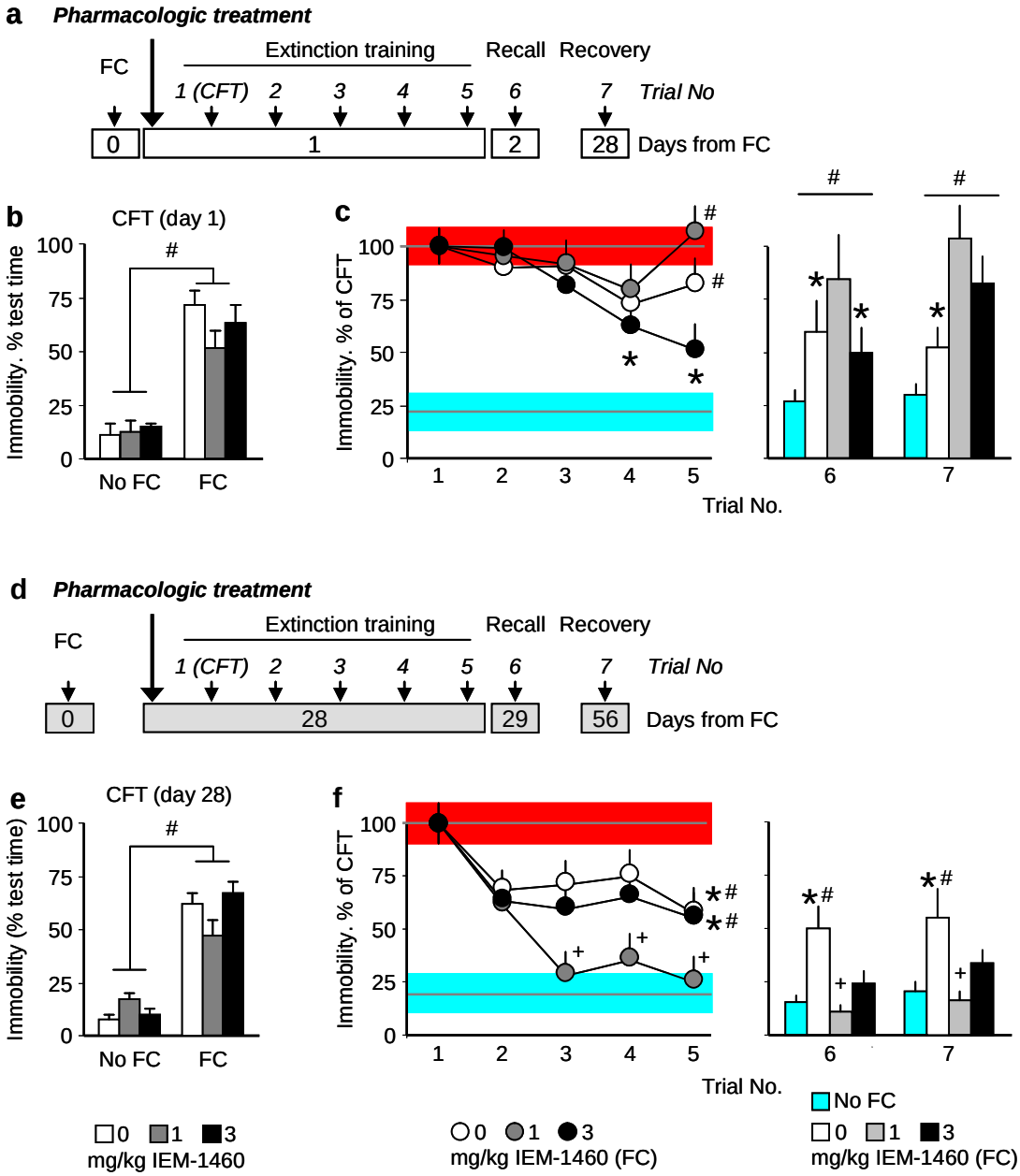

Fig. 7. Changes in the expression of genes encoding the NR2a and NR2b subunits of the NMDA receptor, together with changes in the expression of the gene for DNA methyltransferase 3a (DNMT3a).

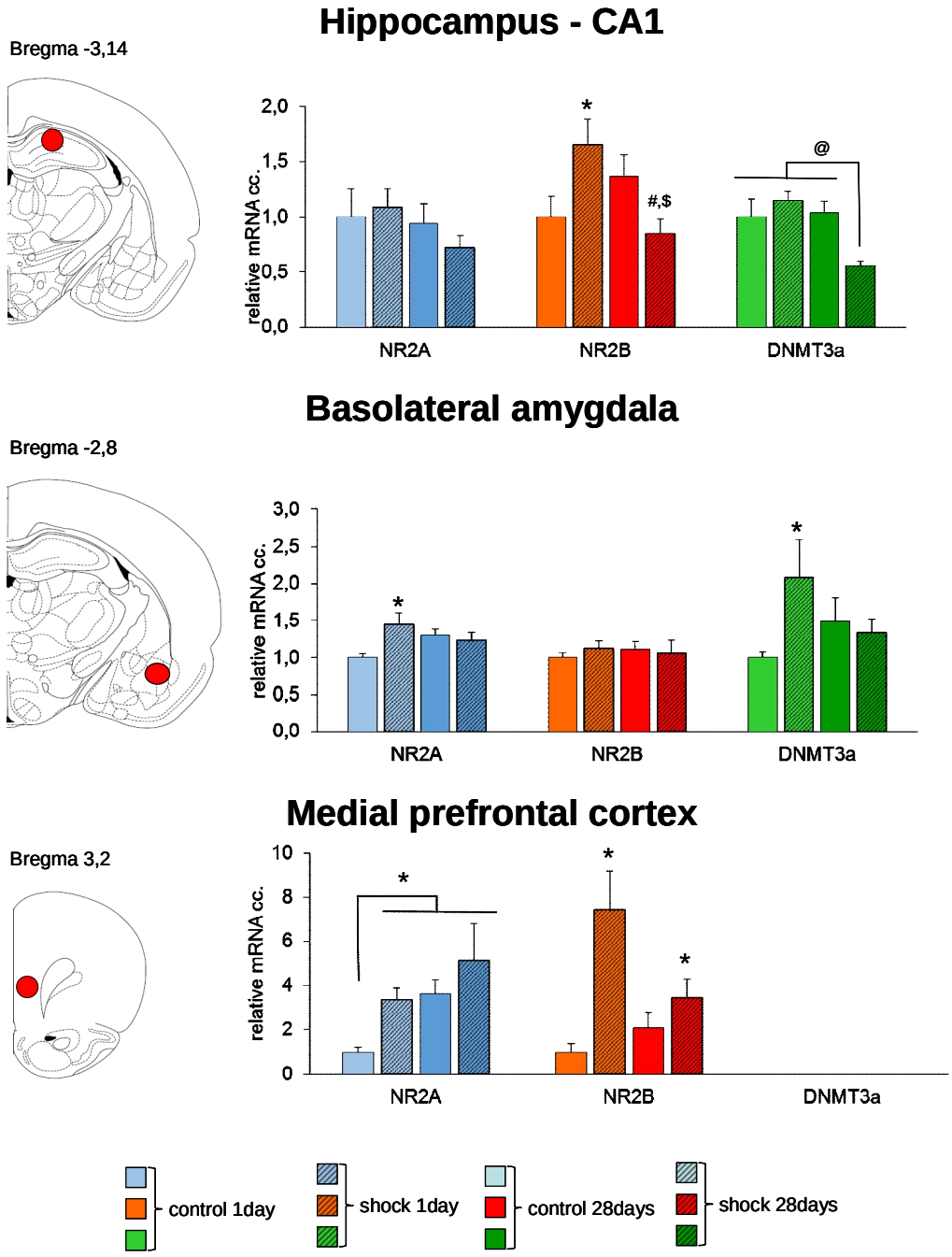

Fig. 8. Fear conditioning-induced changes in the expression of AMPA receptor subunits. Upper panels show data obtained in the mPFC; the middle and lower row show those obtained in the amygdala. **a** and **d**. The localization of brain punches in which gene expression was studied (schematics based on Paxinos and Watson. 2007); **b** and **e**. Subunit mRNA expression; **c** and **f**. GluA1 / GluA2 ratios; *FC*, fear conditioning; *vmPFC*, ventral medial prefrontal cortex; *BLA*, basolateral amygdala; *CeA*. central amygdala; *horizontal lines and gray bars on graphs*, mean ± SEM, respectively, of gene expression in non-conditioned controls; #, significant difference from not conditioned rats; *, significant within-group difference between GluA1 and GluA2 expression ($p < 0.05$ at least in both cases).

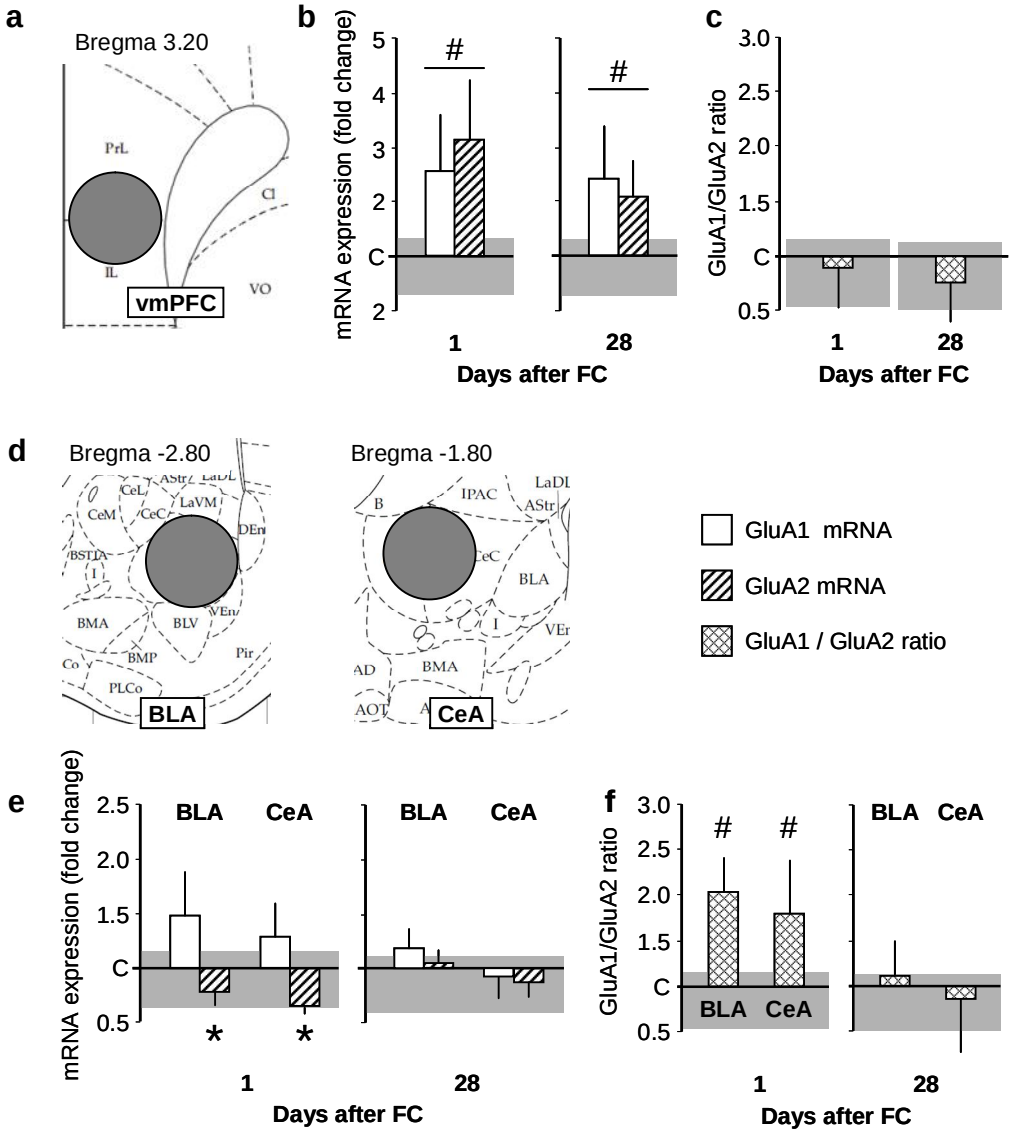

Fig. 9. *The contribution of gene expression profiles to conditioned fear*. **a**. Conditioned fear (immobility in the fear conditioning chamber) was best predicted by the prefrontal increase in GluA2 expression and the increase in GluA1/GluA2 ratios in the BLA and CeA (Multiple R= 0.69; F(3,19)= 5.16; p< 0.01). Partial correlations were significant for the vmPFC and BLA (see p values in brackets). **b**. A 3D graph that illustrates the results of Multiple Regression analysis. v*mPFC*, ventral medial prefrontal cortex; *BLA*, basolateral amygdala; *CeA*, central amygdala.