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https://doi.org/10.1038/s41684-022-01054-4

Refinement of the stress-enhanced fear learning model of post-traumatic stress disorder: a behavioral and molecular analysis

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Post-traumatic stress disorder (PTSD) is a debilitating mental health condition for which current treatments have long-term efficacy in 50% of patients. There is a clear need for better understanding of the mechanisms underlying PTSD and the development of new treatment approaches. Analog trauma procedures in animals, such as the stress-enhanced fear learning (SEFL) procedure, can be used to produce behavioral and neurobiological changes that have validity in modeling PTSD. However, by necessity, the modeling of PTSD in animals requires them to potentially experience pain and suffering. Consistent with the '3Rs' (reduction, refinement and replacement) of animal research, this study aimed to determine whether the SEFL procedure can be refined to reduce potential animal pain and suffering while retaining the same behavioral and neurobiological changes. Here we showed that PTSD-relevant changes could be produced in both behavior and the brain of rats that were group- rather than single-housed and that received lower-magnitude electric shocks in the 'trauma analog' session. We also varied the number of shock exposures in the trauma analog session, finding SEFL-susceptible and SEFL-resilient populations at all levels of shock exposure, but with greater levels of shock increasing the proportion of rats showing the SEFL-susceptible phenotype. These data demonstrate that the SEFL procedure can be used as an animal analog of PTSD with reduced potential pain and suffering to the animals and that variations in the procedure could be used to generate specific proportions of SEFL-susceptible and SEFL-resilient animals in future studies.

Post-traumatic stress disorder (PTSD) is a debilitating mental health disorder with a lifetime prevalence of 5–10% (ref. [1]). Although both pharmacological[2] and psychological[3] therapies are available, these are only effective in treating PTSD in approximately 50% of patients[4]. There is a clear need for better understanding of treatment mechanisms, and the development of new treatments for PTSD.

Animal models have great potential for providing mechanistic insights into mental health disorders, and for testing of novel treatment strategies[5,6]. However, in the context of animal analogs of PTSD[7], the difference between adaptive and maladaptive fear learning is critical. It is often claimed that studies of adaptive fear learning—for example, Pavlovian fear conditioning—have relevance to PTSD. However, PTSD is a maladaptive memory disorder that goes beyond adaptive fear learning. Patients with PTSD show neuroendocrine, neurobiological and behavioral changes[8] including enhanced fear memory generalization[9] and impaired fear memory extinction[10,11] relative to trauma-exposed individuals without PTSD. Thus, several trauma analog procedures have been developed for use in rodents to model a PTSD-like phenotype, including exposure to ethologically relevant stressors[12] and the single prolonged stress procedure[13].

One prominent rodent analog of trauma is the stress-enhanced fear learning (SEFL) procedure[14–17]. SEFL involves exposing rats (usually males, but see refs. [18,19] for studies of both sexes) to a trauma-like experience of 15 massed electric footshocks before assessing subsequent fear learning and behavior in a context distinct from the 'trauma' context. SEFL is well validated, producing non-associative increases in fear learning that persist for at least 90 days following the massed shock session, independent of the explicit memory of the massed shock context (for example, it can be observed when the 'trauma' has occurred in the window for infantile amnesia[20,21]) and producing 'susceptible' and 'resilient' subpopulations[17]. Studies of SEFL have also revealed neurobiological changes relevant to PTSD. For example, in the basolateral amygdala (BLA)—a nucleus critical for fear conditioning[22–24]—SEFL-susceptible rats have been found to show an increase in the expression of the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of glutamate receptor[17], suggestive of increased amygdala excitability[25].

A potential advantage of SEFL over other rodent models of PTSD-like behavior is its potential for parametric manipulation. The 'standard' SEFL procedure uses 15 1 mA, 1 s footshocks in the massed shock session; nevertheless, there have been reports of SEFL-susceptible populations with as few as four massed shocks[26]. The four-shock protocol produces a lower proportion of SEFL-susceptible individuals in the population[17], but regardless indicates that there may be scope for refining SEFL to minimize potential animal pain and suffering, while providing a robust model of PTSD in animals that show the susceptible phenotype. This would be consistent with the '3Rs' (reduction, refinement and replacement) of animal research[27].

Manipulation of housing conditions may represent another potential refinement of the SEFL procedure. Although rats undergoing the SEFL procedure are usually housed in social isolation[14–17,21], the SEFL-susceptible phenotype has recently been demonstrated in rats that had been pair-housed throughout the procedure[28]. Thus, despite group-housing reducing the impact of stress in both rats[29,30] and mice[31], the SEFL-susceptible phenotype appears to be sufficiently robust that it can be observed despite the potential for social buffering offered by group housing (for review, see ref. [32]).

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Thus, in this Article, we sought to refine the SEFL procedure with three different modifications. First, male rats were group-housed rather than socially isolated throughout the experiments, with the aim of determining whether the SEFL-susceptible phenotype could be observed in rats that had been group-housed, rather than conducting a comparison of socially isolated and group-housed animals. Second, we reduced the magnitude of shock from 1 mA for 1 s to 0.5 mA for 0.5 s in the massed shock session, and to 0.5 mA for 1 s during subsequent contextual fear conditioning. Third, we systematically investigated the number of shocks required in the massed shock session to find the minimum number of shocks that produced a similar proportion of SEFL-susceptible animals as the 15-shock procedure. We assessed the proportion of SEFL-susceptible and SEFL-resilient individuals for each shock condition, and measured extinction learning and retention in these populations. Finally, we examined the expression of the glutamate receptor subunits GluN2B, GluR1 and GluA2 in the BLA, to determine whether changes in receptor expression could be correlated to the SEFL phenotype or previous shock experience.

Results

The SEFL-susceptible phenotype could be observed in a subset of rats, with the proportion showing the phenotype dependent on the number of massed shocks experienced. All rats conditioned fear to the context, with SEFL-susceptible rats showing greater levels of fear. All rats were pre-exposed to the 'massed shock' context (context A), with the number of footshocks presented in context A varying between 0, 4, 9, 12, 13 and 15. The following day, rats were exposed to context B for a 3 min contextual fear conditioning session in which a 0.5 mA, 0.5 s footshock was delivered (Fig. 1a).

All rats conditioned to context B (Fig. 1b) showed more freezing after shock than before (PrePost: $F_{(1,72)}=144$, $P<0.001$, $\eta^2=0.67$). Rats responded similarly to the shock in context B regardless of context A shock experience (PrePost × Shock: $F_{(5,72)}=1.17$, $P=0.33$), though rats that showed the SEFL-susceptible phenotype were generally more fearful (SEFL: $F_{(1,72)}=7.73$, $P=0.007$, $\eta^2=0.097$), particularly after the shock in context B (PrePost × SEFL: $F_{(1,72)}=6.50$, $P=0.013$, $\eta^2=0.083$). Šidák-corrected pairwise comparisons exploring the PrePost × SEFL interaction showed no differences between SEFL-susceptible and SEFL-resilient rats before the shock ($P=0.30$), but increased fear in SEFL-susceptible rats following shock ($P=0.003$). Therefore, as has been reported previously[14,15], rats subsequently classified with the SEFL-susceptible phenotype showed greater fear conditioning to a novel context, regardless of the number of shocks that had been used to model trauma in context A.

SEFL-susceptible rats showed slower extinction of contextual fear (Fig. 1c). Animals reduced their conditioned freezing across the 30 min extinction session (Bin: $F_{(11.3,813)}=42.6$, $P<0.001$, $\eta^2=0.37$), though the pattern of extinction differed between SEFL-susceptible and SEFL-resilient rats (SEFL: $F_{(1,72)}=137$, $P<0.001$, $\eta^2=0.66$; Bin × SEFL: $F_{(11.3,813)}=16.2$, $P<0.001$, $\eta^2=0.18$). SEFL-susceptible rats began the extinction session with higher levels of freezing than SEFL-resilient rats, and maintained these higher freezing levels throughout extinction (Šidák-corrected pairwise comparisons of the Bin × SEFL interaction showed that the freezing of SEFL-susceptible rats was higher across all time bins with $P<0.041$). Overall, SEFL-resilient rats showed extinction learning that plateaued to a consistently low level of freezing from 5 min into the 30 min extinction session (Šidák-corrected pairwise comparisons of the Bin × SEFL interaction showed that the levels of freezing observed from 5 min into the session did not differ from the final minute of extinction, all $P>0.99$). By contrast, SEFL-susceptible rats did not show stably low levels of freezing until 10 min into the session (Šidák-corrected pairwise comparisons showed that the levels of freezing observed from 10 min into the session did not differ from the final minute of extinction, all $P>0.69$).

The number of shocks received in context A did not affect extinction learning in context B per se (Shocks: $F_{(5,72)}=1.13$, $P=0.35$), but the number of massed shocks did affect the rate of extinction (Bin × Shocks: $F_{(56.5,813)}=2.92$, $P<0.001$, $\eta^2=0.17$), and this differed if rats showed the SEFL-susceptible phenotype (Shocks × SEFL: $F_{(5,72)}=4.25$, $P=0.002$, $\eta^2=0.23$; Bin × Shocks × SEFL: $F_{(56.5,813)}=2.56$, $P<0.001$, $\eta^2=0.15$). The number of shocks received in context A affected the speed of extinction in SEFL-susceptible rats, with Šidák-corrected pairwise comparisons of the Bin × Shocks × SEFL interaction showing that freezing had reduced to end-of-session levels by 12 min for the 4-shock group (all $P>0.12$ until the end of the session), 7 min for the 9-shock group (all $P>0.15$), 10 min for the 12-shock (all $P>0.99$) and 13-shock groups (all $P>0.43$) and 25 minutes for the 15-shock group (all $P>0.53$). Unexpectedly, the single rat in the SEFL-susceptible, 0-shock group showed an increase in conditioned freezing in the final 4 min of the session (Fig. 1c). By contrast, SEFL-resilient rats showed low levels of conditioned freezing throughout the extinction session, such that their fear at the end of extinction did not statistically differ from their fear at any other point in the extinction session (Šidák-corrected pairwise comparisons of the Bin × Shocks × SEFL interaction, all $P>0.24$) regardless of the number of massed shocks received. However, both SEFL-susceptible and SEFL-resilient rats showed numerical reductions in conditioned freezing between the beginning and end of extinction training.

SEFL-susceptible rats showed greater recovery and retention of fear memory following extinction (Fig. 1d). In the 8 min retention test conducted 24 h after extinction training, SEFL-susceptible rats showed greater levels of conditioned fear than SEFL-resilient rats (SEFL: $F_{(1,72)}=54.7$, $P<0.001$, $\eta^2=0.43$), and this varied depending on the number of shocks received in context A (SEFL × Shock: $F_{(5,72)}=5.41$, $P<0.001$, $\eta^2=0.27$). Šidák-corrected pairwise comparisons of the SEFL × Shock interaction revealed that, while there were no differences in fear at test between SEFL-susceptible and SEFL-resilient rats in the 0-shock ($P=0.13$), 9-shock ($P=0.28$) and 13-shock ($P=0.097$) groups, SEFL-susceptible rats showed greater fear at test than SEFL-resilient rats that had received the same number of massed shocks for the 4-shock ($P<0.001$), 12-shock ($P<0.001$) and 15-shock groups ($P<0.001$).

To assess the degree of fear recovery between extinction and test, difference scores were calculated between freezing during the

Fig. 1 | The behavioral phenotype associated with SEFL could be observed in rat populations given different degrees of massed shock exposure. **a**, Experimental schematic. Rats were exposed to context A, where they received either 0, 4, 9, 12, 13 or 15 0.5 mA, 0.5 s footshocks. Twenty-four hours later, they were contextually fear conditioned to a single 0.5 mA, 0.5 s footshock in context B. Twenty-four hours after fear conditioning, rats were re-exposed to context B for a 30 min extinction session. Twenty-four hours later, rats were returned to context B for an 8 min memory retention test. Twenty-four hours after the final test, rats were killed and brains were collected for subsequent molecular analyses. **b**, All rats showed increased freezing following the footshock in context B, with rats subsequently classified as showing the SEFL-susceptible phenotype showing greater freezing post-shock. **c**, Rats subsequently classified as SEFL-susceptible showed higher levels of fear at the beginning of extinction and were slower to extinguish the contextual fear memory than SEFL-resilient rats. **d**, Rats subsequently classified as SEFL-susceptible showed impaired retention of extinction memory compared with SEFL-resilient rats. Inset panels show the difference in freezing between the first minute of the retention test and the final minute of extinction training. Data shown as mean ± standard error of the mean (s.e.m.). Group sizes, SEFL-resilient rats: 0-shock, $n=13$; 4-shock, $n=11$; 9-shock, $n=11$; 12-shock, $n=8$; 13-shock, $n=7$; 15-shock, $n=7$; SEFL-susceptible rats: 0-shock, $n=1$; 4-shock, $n=3$; 9-shock, $n=3$; 12-shock, $n=6$; 13-shock, $n=7$; 15-shock, $n=7$.

first minute of the retention test and the final minute of extinction training (Fig. 1d, inset panels). The recovery of fear varied depending on massed shock experience (Shocks: $F_{(5,72)}=4.63$, $P=0.001$, $\eta^2=0.24$). Rats in the 0-shock group showed better retention of the extinction memory (that is, less recovery of fear) than any rats receiving shocks in context A (all $P<0.022$), though the number of massed shocks in context A did not affect retention when SEFL-susceptible and SEFL-resilient rats were considered together (all $P>0.64$). However, SEFL-susceptible and SEFL-resilient rats showed different levels of fear recovery between extinction and the retention test (Shocks × SEFL: $F_{(5,72)}=5.00$, $P=0.001$, $\eta^2=0.26$). While none of the SEFL-resilient rats differed from the 0-shock group (Šidák-corrected pairwise comparisons of the Shocks × SEFL interaction, all $P>0.99$), all of the SEFL-susceptible rats that

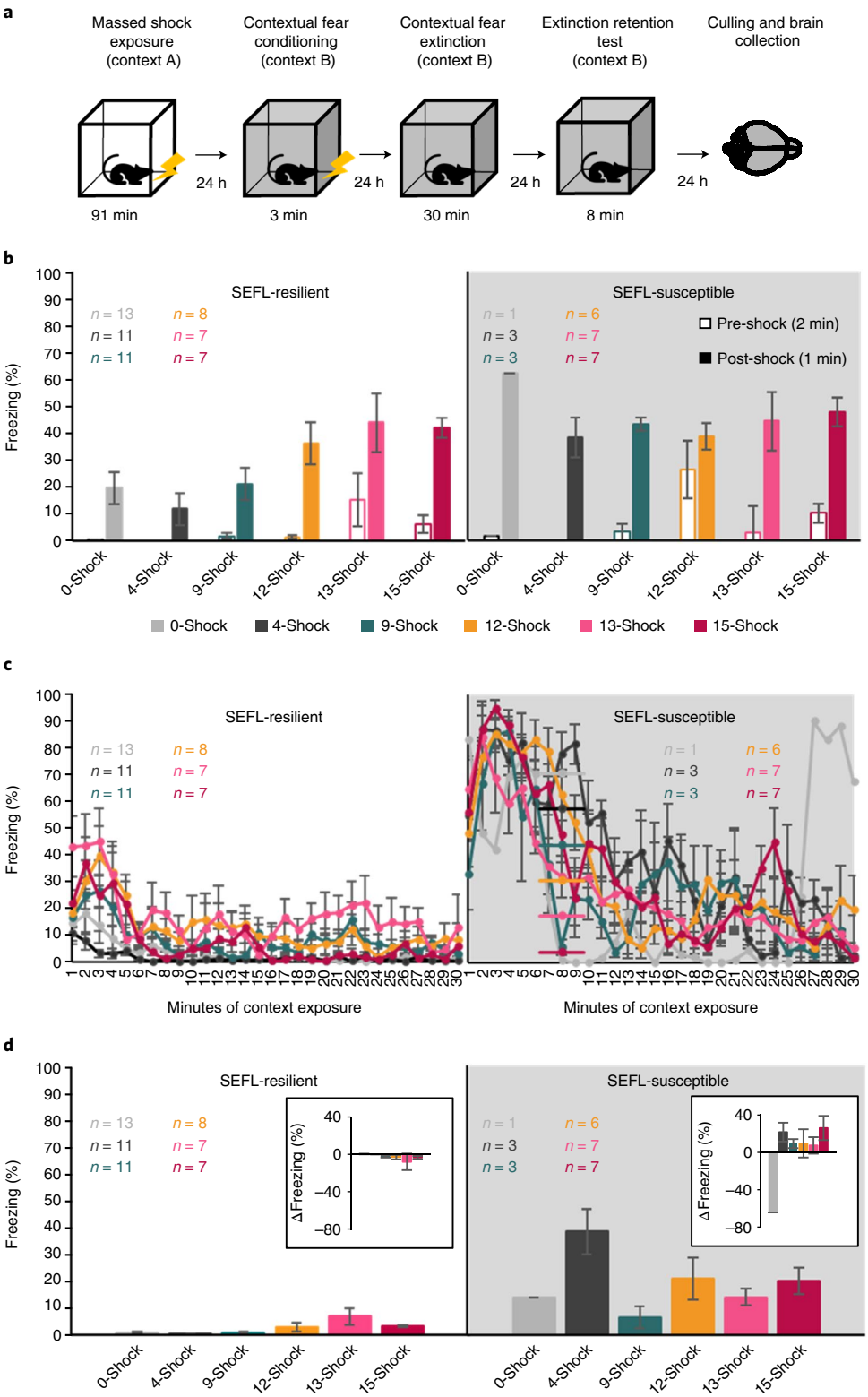

Table 1 | The proportion of SEFL-susceptible animals increased with increasing numbers of shock experienced in the massed shock session in context A

Shocks in context A	Total number of rats receiving massed shock exposure	Number of SEFL-susceptible rats	Proportion of SEFL-susceptible rats
0	14	1	7%
4	14	3	21%
9	14	3	21%
12	14	6	43%
13	14	7	50%
15	14	7	50%

had received shock in context A showed greater recovery of fear than the 0-shock group (Šidák-corrected pairwise comparisons of the Shocks × SEFL interaction, all $P<0.003$), though this did not differ on the basis of the number of shocks received in context A (Šidák-corrected pairwise comparisons of the Shocks × SEFL interaction, all $P>0.69$). Therefore, rats with the SEFL-susceptible phenotype showed enhanced fear recovery, regardless of the number of shocks used to simulate trauma.

The data reported above support the assertion that SEFL-susceptible rats behaved similarly, regardless of the number of massed shocks experienced in context A. However, the proportion of rats showing the SEFL-susceptible phenotype (Table 1) increased in groups that had experienced a greater number of shocks in context A (Fisher's exact test, one-tailed, $P=0.031$) with a moderate effect size (Cramer's V, 0.35).

Rats with the SEFL-susceptible phenotype showed reduced expression of GluN2B and GluA2 glutamate receptor subunits in the BLA as compared with SEFL-resilient rats, while GluR1 expression was related to previous shock experience. Glutamate receptor subunit expression differed across experimental groups, and was differentially affected by massed shock experience and SEFL phenotype. GluN2B expression was reduced in SEFL-susceptible rats (Fig. 2a; SEFL: $F_{(1,67)}=22.9$, $P=0.00001$, $\eta^2=0.26$), independently of the number of massed shocks experienced in context A (Shocks: $F<1$; Shocks × SEFL: $F<1$). GluR1 expression was also affected by SEFL phenotype (Fig. 2b; SEFL: $F_{(1,67)}=8.60$, $P=0.005$, $\eta^2=0.12$) with the massed shock experience affecting expression at the trend level (Shocks: $F_{(5,67)}=2.29$, $P=0.055$, $\eta^2=0.15$) but with no interaction (Shocks × SEFL: $F<1$). GluA2 expression was also reduced in SEFL-susceptible rats (Fig. 2c; SEFL: $F_{(1,67)}=7.78$, $P=0.007$, $\eta^2=0.10$) independently of the number of shocks experienced in context A (Shocks: $F_{(5,67)}=1.25$, $P=0.30$; Shocks × SEFL: $F<1$).

The relative expression of GluR1, GluA2 and GluN2B differed in SEFL-resilient and SEFL-susceptible rats. The expressions of GluN2B, GluR1 and GluA2 proteins within the BLA, 24 h after the final behavioral session, were examined for partial correlations with specific behaviors of interest, controlling for the number of shocks delivered in context A. The behaviors of interest were pre-shock freezing in context B, taken as indicative of fear memory generalization; post-shock freezing in context B, indicative of shock sensitivity; freezing during the first minute of the extinction session, indicative of retention of the context B fear memory; freezing during the final minute of the extinction session, indicative of the strength of extinction learning; freezing during the final retention test, indicative of the retention of the extinction memory, and recovery of fear between the end of extinction training and the beginning of the retention test. The SEFL-susceptible and SEFL-resilient animals were analyzed separately.

For the SEFL-resilient rats, there was no correlation between the expression of the GluN2B or GluR1 proteins and pre-shock freezing in context B (all $P>0.53$), though there was a correlation between GluA2 expression and pre-shock freezing in context B ($r=0.38$, $P=0.005$). There were no other correlations between protein expression and behaviors of interest. This included freezing during the first minute of extinction (all $P>0.46$), freezing at the end of extinction (all $P>0.17$), freezing during the retention test (all $P>0.21$) or recovery of the fear memory (all $P>0.38$).

For the SEFL-susceptible rats, no significant correlations were observed between expression of GluN2B, GluR1 or GluA2 and the behaviors of interest. This included pre-shock freezing in context B (all $P>0.36$), post-shock freezing in context B (all $P>0.11$), freezing at the start of extinction (all $P>0.59$), freezing at the end of extinction (all $P>0.27$), freezing during the retention test (all $P>0.10$) or recovery of the fear memory (all $P>0.42$).

The correlations between the expression of the different glutamate receptor subunits differed between SEFL-resilient and SEFL-susceptible rats. While for SEFL-resilient rats there was a strong correlation between the expression of GluR1 and GluA2 (Fig. 3a; $r=0.74$, $P<0.0001$), GluR1 and GluN2B (Fig. 3b; $r=0.61$, $P<0.0001$) and GluA2 and GluN2B (Fig. 3c; $r=0.67$, $P<0.0001$), for SEFL-susceptible rats there was no correlation of GluR1 and GluA2 subunits (Fig. 3a; $P=0.61$) and no correlation between GluR1 and GluN2B expression (Fig. 3b; $P=0.92$), though GluA2 and GluN2B expression was correlated (Fig. 3c; $r=0.70$, $P<0.0001$). Comparison of the correlation coefficients revealed that the correlation in GluR1–GluA2 expression differed between SEFL-resilient and SEFL-susceptible rats ($z_{diff}=3.51$, $P<0.0005$), as did the correlation between GluR1 and GluN2B expression ($z_{diff}=3.11$, $P<0.005$). The correlation between GluA2 and GluN2B expression was not statistically different between the SEFL-resilient and SEFL-susceptible rats ($z_{diff}=-0.26$, $P=0.79$).

Discussion

This study aimed to determine whether a putative analog of PTSD, the SEFL procedure, could be refined to produce the previously reported[14,15] behavioral phenotype with reduced potential animal pain and suffering, consistent with the '3Rs' of animal research[27]. The original SEFL procedure was modified as follows: (1) group-housing male rats rather than social isolation housing; (2) administering lower-magnitude shocks during the massed shock session; and (3) parametrically manipulating the number of shocks administered in the massed training session. Furthermore, brains were collected following the completion of behavioral procedures to assess changes in receptor expression in the BLA that might correlate with behavior in the same animals.

Behavioral changes associated with SEFL susceptibility. As previously, the SEFL-susceptible phenotype was observed in a subset of male rats and manifested as an increase in fear memory expression when tested 24 h after fear conditioning in a novel context, without generalization between the massed shock and novel contexts[17]. Furthermore, SEFL-susceptible rats showed impaired extinction learning and impaired extinction memory retention, compared with SEFL-resilient rats with the same massed shock experience. Our finding that the SEFL-susceptible phenotype can be observed in group-housed rats extends a previous report showing that pair-housing animals does not protect against the development of the SEFL phenotype[28] and a recent report showing that group-housed animals are susceptible to a restraint-stress-based SEFL procedure[33]. Thus, group housing appears to be a potential refinement to the SEFL procedure. However, as we did not compare group-housed with socially isolated animals, it is not

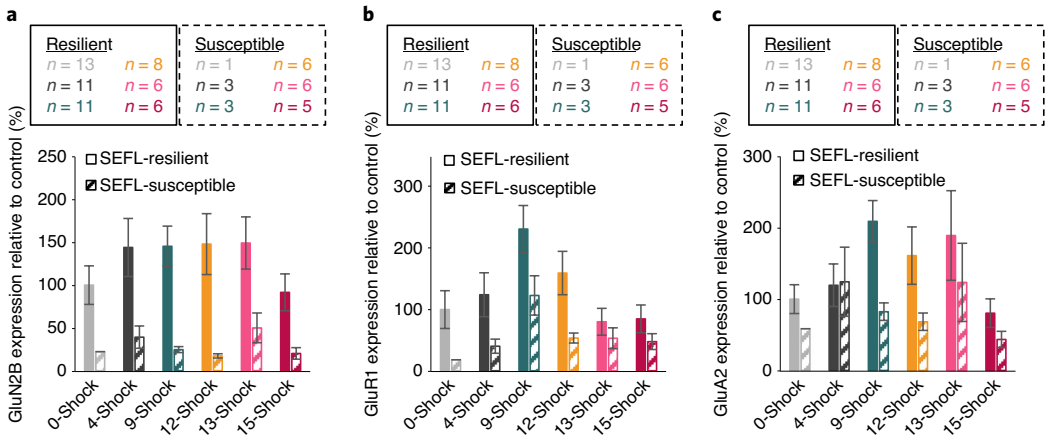

Fig. 2 | Expression of glutamate receptor subunits varied differentially depending on SEFL classification and the number of shocks experienced in context A. a, Expression of GluN2B was reduced in rats subsequently classified as SEFL-susceptible but did not vary depending on the number of shocks experienced in context A. **b**, Expression of GluR1 varied as a function of the number of shocks experienced in context A but did not vary reliably between SEFL-susceptible and SEFL-resilient rats. **c**, As with GluN2B expression, GluA2 expression was reduced in SEFL-susceptible rats but did not vary as a function of the number of shocks experienced in context A. All data are normalized to the expression of the relevant protein in the 0-shock, SEFL-resilient group and are expressed as mean ± s.e.m. Group sizes, SEFL-resilient rats: 0-shock, $n=13$; 4-shock, $n=11$; 9-shock, $n=11$; 12-shock, $n=8$; 13-shock, $n=6$; 15-shock, $n=6$; SEFL-susceptible rats: 0-shock, $n=1$; 4-shock, $n=3$; 9-shock, $n=3$; 12-shock, $n=6$; 13-shock, $n=6$; 15-shock, $n=5$.

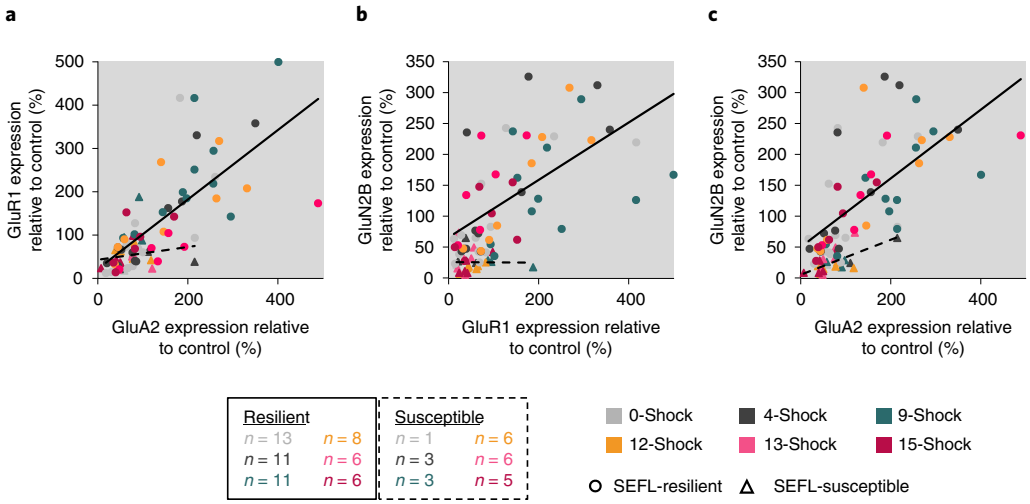

Fig. 3 | SEFL-resilient and SEFL-susceptible populations showed different correlations of glutamate receptor subunits. a, Although GluR1 and GluA2 expression correlated in SEFL-resilient rats, they did not for SEFL-susceptible rats. **b**, Similarly, the correlation between GluR1 and GluN2B expression was observed only in the SEFL-resilient group. **c**, GluA2 and GluN2B expression correlated in both SEFL-resilient and SEFL-susceptible rats. All protein expression data are normalized to expression of the relevant protein in the 0-shock, SEFL-resilient group. Solid lines denote linear trend line for the SEFL-resilient group, dashed lines linear trend line for the SEFL-susceptible group. Group sizes, SEFL-resilient rats: 0-shock, $n=13$; 4-shock, $n=11$; 9-shock, $n=11$; 12-shock, $n=8$; 13-shock, $n=6$; 15-shock, $n=6$; SEFL-susceptible rats: 0-shock, $n=1$; 4-shock, $n=3$; 9-shock, $n=3$; 12-shock, $n=6$; 13-shock, $n=6$; 15-shock, $n=5$.

possible to determine whether the proportion of rats showing the SEFL-susceptible phenotype differs on the basis of housing conditions, although it is of note that, when pair-housed and socially isolated rats are exposed to 15 massed footshocks in the trauma session, their subsequent fear behavior is highly comparable[28]. Thus, we suggest that the lower proportion of SEFL-susceptible individuals that we observed in these experiments is more likely due to our use of a lower footshock magnitude throughout the experiment, although to demonstrate this unequivocally would require explicit comparison with socially isolated animals.

As predicted, the proportion of animals showing the SEFL-susceptible phenotype increased with increasing numbers of shocks delivered in the massed shock training session. This is consistent with findings that, for both males and females, fewer rats show the SEFL-susceptible phenotype following exposure to 4, rather than 15, shocks in the massed shock exposure session[18,26]. The proportion of SEFL-susceptible rats was lower in the present study than the previous literature (for example, in the four-shock group, 21% here versus 43% in ref. [18]), most likely attributable to the difference in shock magnitude in the massed shock session. Importantly, the proportion of SEFL-susceptible rats in the 13-shock and 15-shock conditions was equivalent (50%), suggesting a potential refinement in the procedure to not only reduce the magnitude but also the number of shocks in the massed shock session. To our knowledge, this is

the first study to have conducted a parametric analysis to determine the proportion of SEFL-susceptible rats with different massed shock experiences. Lower shock levels are less stressful for animals[34], suggesting that a reduction in footshock magnitude is another potential refinement of the SEFL procedure. Although we acknowledge the limitations of drawing strong conclusions from a single study, this work suggests that, in future studies, investigators may be able to manipulate the proportion of rats showing the SEFL-susceptible and SEFL-resilient phenotypes to a desired number by altering the parameters of the massed shock session. This may allow a balancing of the refinement and reduction requirements of the '3Rs' animal ethics framework, depending upon the specific research question of interest.

One limitation of the present research is that only male rats were studied. Although a recent study[18] showed no sex differences in susceptibility to the SEFL phenotype when a massed shock session was used as a previous stressor, there were differences in the behavior of males and females when restraint stress was used to induce SEFL[33]. Differences in the expression of fear behavior have been reported between males and females[35], and the estrus cycle may also influence fear behavior[36]. Considering the increased prevalence of anxiety disorders, including PTSD, in females[37] and the importance of sex as a biological variable[38], further research is needed to determine the optimal approaches for refining the procedure to induce SEFL in females.

Table 2 | Summary of the differences in glutamate receptor expression, and procedural differences, between the present and previous studies of SEFL

Study	Expression			Notes
	GluR1	GluA2	GluN2B	
Present study	↓	↓	↓	Rats were group-housed; procedure also included extinction training before brain collection
Perusini et al.[17]; Meyer[46]	↑	NC	↓	Comparison of 0-shock and 15-shock groups. GluN2B reduction was significant at trend level
Conoscenti et al.[51]	↑	↑	NC	Comparison with home cage and non-context-exposed controls

Data from Perusini et al.[17] and Meyer[46] are combined as they refer to the same dataset. NC, no change.

Molecular changes associated with SEFL susceptibility. Behavioral differences between SEFL-susceptible and SEFL-resilient rats were also reflected in the expression of glutamate receptors within the BLA, an area critical for Pavlovian fear conditioning[24,39,40]. We examined the expression of three different glutamate receptor subunits within the BLA: the GluN2B subunit of the *N*-methyl-D-aspartate (NMDA) receptor, which is downregulated after strong fear conditioning[41] and is necessary for updating fear memories[42,43]; the GluR1 subunit of the AMPA receptor (AMPAR), which shows enhanced expression in SEFL-susceptible rats[17]; and the GluA2 subunit of the AMPAR, which controls the calcium permeability of the AMPAR and has been shown to undergo trafficking following fear conditioning[44].

Consistent with the previous literature on adaptive fear conditioning[41,45] and SEFL[46] in male rats, we observed a reduction in BLA GluN2B expression of SEFL-susceptible rats, independently of massed shock experience. This reduction in GluN2B expression is potentially suggestive of a less plastic fear memory and, somewhat speculatively, may be consistent with reports that GluN2B-containing NMDA receptors are required for the acquisition of extinction memories[47,48]. GluN2B-containing NMDA receptors are required for Pavlovian fear memories to destabilize before reconsolidation[42,43,45] in male rats, and genetically modified mice of both sexes expressing fewer GluN2B subunit-containing NMDA receptors show enhanced rigidity of fear memories[49]. Enhanced rigidity of fear memories, and impaired extinction learning, are characteristic of PTSD and thus support the validity of this refined SEFL procedure.

The expression of the AMPAR subunits GluR1 and GluA2 were differentially affected by the SEFL phenotype. AMPARs are heterotetramers consisting of multiple subunits, with the GluA2 subunit determining the permeability of the AMPAR channel pore to calcium. There were reductions in GluR1 and GluA2 expression in SEFL-susceptible rats. A switch between calcium-impermeable (GluA2-containing) and calcium-permeable (GluA2-lacking) AMPARs is associated with memory formation[44] and updating[50]. Thus, a reduction in GluA2 subunit expression in the SEFL-susceptible rats may indicate that the composition of AMPARs differs between the SEFL-susceptible and SEFL-resilient rats. This may also account for the change in correlation between GluR1 and GluA2 expression observed between the SEFL-resilient and SEFL-susceptible rats.

The findings reported here contrast with previous reports showing an increase in GluR1 subunit expression[17,51], and no change in GluA2 subunit expression[17], in SEFL-susceptible male rats (Table 2). However, there are important procedural differences between the previous reports and the present study that probably account for these apparent discrepancies. First, both previous reports found increased GluR1 subunit expression in the brains of SEFL-susceptible rats exposed to 15 massed shocks compared with unstressed (that is, nonshocked) controls. The present study found that GluR1 expression was also affected, at trend level, by the number of shocks experienced in the massed shock session. Thus, one potential explanation for the apparent discrepancy in GluR1 subunit expression between the studies is that the increased GluR1 expression reported previously was attributable to shock experience, rather than susceptibility to the SEFL phenotype itself. Secondly, the rats in the previous report were socially isolated rather than group-housed, and social isolation has been shown to increase both GluR1 and GluA2 expression in the amygdala of mice[52], which is suggestive that the same may be true for rats (with the caveat that there are reported differences in gene expression between the two species[53]). Thirdly, the present study included an assessment of contextual fear memory extinction, which was not the case for the previous report. As extinction training reduces GluR1 expression in the BLA[54], it is also possible that the reduction was attributable to the more recent (relative to brain collection) extinction training, and differences in extinction learning between SEFL-resilient and SEFL-susceptible rats.

Also in apparent contrast to the previous report[17], we observed a reduction in GluA2 subunit expression in SEFL-susceptible rats. In addition to the potential confound of comparing SEFL-susceptible rats with 0-shock controls described above, the timing of brain collection differed from 24 h in the present study, to 1 week (ref. [51]) or 2 weeks (ref. [17]) in the previous reports. The timing of brain collection relative to the retention test in the present study is more consistent with reports indicating a rapid switch in membrane expression between calcium-impermeable (GluA2-containing)

and calcium-permeable (GluA2-lacking) AMPARs[55]. Speculatively taken together, the present study may indicate that shortly after behavioral experience there is a reduction in GluA2 subunit expression that reflects a limited opportunity for memory manipulation related to the Pavlovian contextual fear learning, which abates by 2 weeks post-behavior.

Conclusions

The present study demonstrates that it is possible to refine the SEFL procedure to reduce potential animal pain and suffering. SEFL susceptibility was observed in rats that were group-housed rather than socially isolated and that received lower magnitude electric footshocks. Consistent with previous reports, an SEFL-susceptible population was observed with fewer than the 15 footshocks used in the standard SEFL procedure, though the proportion of animals showing the SEFL-susceptible phenotype increased with an increasing number of shocks. This parametric analysis of SEFL raises the possibility that future studies using this procedure could be designed to achieve a desired proportion of SEFL-susceptible and SEFL-resilient animals, and to minimize potential animal pain and suffering while still providing a robust model of the non-associative learning mechanism that facilitates fear learning and impairs extinction in patients with PTSD.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41684-022-01054-4.

Received: 15 September 2021; Accepted: 15 August 2022;
Published online: 20 October 2022

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Methods

Subjects. Eighty-four behaviorally naïve, adult male outbred Lister Hooded rats (weighing 150–250 g at the start of the experiment) were obtained from Charles River. Rats were housed in groups of four in a humidity- and temperature-controlled (21 °C) environment under a reversed 12 h light/dark cycle (lights off at 7:00). Rats were allowed to acclimatize to the animal facility for at least 7 days upon arrival. Rats had ad libitum access to standard lab chow and water throughout the experiments, except for during behavioral procedures. Humane endpoints were predetermined and included showing of two clinical signs of 'moderate' severity or 15% weight loss; no animals showed any adverse effects throughout the study, and no humane endpoints were reached. This study was regulated under the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations (2012) on Project Licence PA9FBFA9F following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Behavioral procedures. The experimental protocol was not a published preregistered report, but the protocol was registered with the University of Cambridge Biomedical Services division before the start of the experiments.

Conditioning chambers. Two contexts (A and B) were used during the experiment. In both contexts, the ceiling was made of clear Plexiglas allowing a video camera to record the behavior of the rats for behavioral scoring of freezing. The conditioning chambers comprising both contexts A and B were interfaced to the FearCond software written in C++ for the Whisker control system[56].

Context A consisted of Med Associates conditioning chambers (29.5 × 32.5 × 23.5 cm; Med Associates) housed within sound- and light-attenuating cubicles. The grid floors were composed of 19 stainless-steel rods connected to a shock generator and scrambler that delivered the footshocks. Below the grid floor was a removable stainless-steel tray. The rats were transported to and from the context in an opaque plastic box with a lid in groups of six using an elevator.

Context B consisted of Paul Fray conditioning chambers (40 × 30 × 40 cm; Paul Fray Ltd.) housed within sound- and light-attenuating cubicles in a different room to Context A. The grid floors were composed of 17 stainless-steel bars connected to a shock generator and scrambler that delivered the footshock. During each session, a 2.8 W, 24 V white ceiling houselight illuminated the chamber. Below the grid floor was a removable stainless-steel tray lined with white absorbent paper. A ventilation fan was used for background noise. The rats were transported to and from the context in the same opaque plastic box in groups of four using the stairs and a different route through the animal facility.

SEFL 'trauma' session (context A). The procedure used for SEFL was based upon that of Fanselow and colleagues[14], but with the following key refinements: (1) housing in groups of four rather than in social isolation; (2) reduction in the magnitude of footshock from the use of 1 mA, 1 s footshocks to 0.5 mA, 0.5 s footshocks; (3) a parametric analysis of the minimum number of footshocks required to elicit the SEFL phenotype. Sample size was determined by reference to the previous literature, which typically has $n = 5–8$ per group; however, in light of our modifications to the procedure, we assumed a smaller effect size would be observed and consequently increased the group sizes to $n = 14$ per group. Rats were run in squads of 12, beginning with the 0-shock and 15-shock conditions, followed by the 4-shock condition, which has previously been reported[26] to induce the SEFL phenotype. However, as the proportion of rats showing the SEFL phenotype was relatively low (Table 1) in the 4-shock group in light of our other task modifications, additional squads of animals were run with shock numbers intermediate between ineffective and effective conditions ('splitting the difference' in the numbers of shocks, that is, as four shocks produced a lower proportion of SEFL-susceptible rats than 15 shocks, a 9-shock group was next included, followed by the 12-shock group and finally the 13-shock group). This led to final groups receiving 0, 4, 9, 12, 13 or 15 0.5 mA, 0.5 s electric footshocks in context A over a 91 min session, spaced by fixed and equal intervals. All rats were included in the behavioral analyses, as there were no a priori exclusion criteria. Rats were run as seven separate squads over an 8 week period.

Contextual fear conditioning (context B). The day after the context A 'trauma' session, all rats underwent contextual fear conditioning in context B, receiving a single 0.5 mA, 0.5 s footshock after a 2 min pre-shock period. The session continued for a further 60 s post-shock period before the houselight was extinguished. This and all following sessions in context B were video recorded for offline quantification of freezing behavior.

Extinction training (context B). Twenty-four hours after contextual fear conditioning, rats underwent 30 min of extinction training in context B, during which time no footshocks were delivered.

Retention test (context B). Twenty-four hours after extinction training, rats underwent a final memory retention test in context B. During this 8 min session, no footshocks were delivered.

Behavioral analyses. Freezing behavior, defined as the suspension of all bodily movements except those associated with respiration, was used as a measure of the rats' fear response. Freezing was continuously measured manually by an experimenter blinded to experimental condition using a custom-written MATLAB script (courtesy of Dr. G. H. Vousden).

Rats were classified into SEFL-resilient and SEFL-susceptible subgroups using the procedure previously established by Fanselow and colleagues[17]. Briefly, this involved quantifying the conditioned freezing for the first 8 min of the extinction session as a measure of retention of the Pavlovian fear memory to context B. The mean level of conditioned freezing was calculated for the 0-shock control group, and any rat freezing more than two standard deviations beyond the 0-shock control mean was classified as showing the SEFL phenotype (that is, 'SEFL-susceptible'). This method of classification identified 27 rats as SEFL-susceptible (n in each group: 0-shock = 1; 4-shock = 3; 9-shock = 3; 12-shock = 6; 13-shock = 7 and 15-shock = 7) and 57 rats as SEFL-resilient.

Brain collection and sample preparation. The day after the completion of behavioral procedures, rats were culled by exposure to a rising concentration of carbon dioxide and neck dislocation. Brains were extracted and flash frozen on dry ice before subsequent storage at −80 °C. Brains were sectioned at 15 µm at −20 °C within a cryostat (Leica). Samples from the BLA were microdissected using a 0.99 mm diameter biopsy punch (Milltex) over dry ice and placed into 120 µl of cold lysis buffer containing protease inhibitors (0.32 M sucrose, 20 mM Tris, 1 mM EDTA, 1 µg/ml pepstatin A, 10 µg/ml leupeptin, 0.5 mM PMSF and 10 µg/ml aprotinin). Following sonication, samples were centrifuged at 5,000 rpm for 5 min at 4 °C. The supernatant was aliquoted into clean tubes and stored at −80 °C. The protein content of each sample was quantified using a spectrophotometer (Nanodrop), and samples with extremely low protein levels were excluded from all subsequent analysis ($n = 5$ in total: $n = 2$ from the 15-shock SEFL-susceptible group; $n = 1$ from the 15-shock SEFL-resilient group; $n = 1$ from the 13-shock SEFL-susceptible group; $n = 1$ from the 13-shock SEFL-resilient group). Before blotting, all samples were diluted to contain the same amount of protein as the most dilute sample (9.6 µg).

Western blotting. Samples were loaded and separated using a 7.5% SDS–PAGE and electrotransferred (Bio-Rad) onto a nitrocellulose membrane (Thermo Fisher Scientific). Blots were probed with the following antibodies, which had been tested to deliver a linear relationship between the amounts of loaded protein in the blot and signal intensity: rabbit anti-GluA1 (1:200, Merck Millipore, Upstate 08-555), rabbit anti-GluN2B (1:500, AbCam, ab65783), mouse anti-GluR2 (1:400, Merck Millipore, MAB397) and mouse anti-β-actin (1:1000, AbCam, ab8226), diluted in TBS blocking buffer (Odyssey, Li-COR). Signals were visualized using fluorescent anti-mouse (1:20,000, Li-COR IRDye 680RD goat anti-mouse IgG H + L) and anti-rabbit (1:5,000, Li-COR IRDye 800CW goat anti-rabbit IgG H + L) secondary antibodies and imaged using the Li-COR Odyssey system. Optical densities were quantified using Image Studio (Li-COR) by an experimenter blind to experimental condition and normalized using β-actin expression. All samples were run and quantified in duplicate, with the exception of two samples for which there were technical problems with quantification ($n = 1$ where the β-actin band was not fully represented on the membrane; $n = 1$ where high localized background fluorescence interfered with quantification). For these samples, the single valid measurement was used.

Statistical analyses. All statistical analyses were conducted in SPSS Statistics v27 (IBM). For all analyses, the significance level was set at 0.05 and was two-tailed, with the exception of the Fisher's exact test. Where appropriate, Šidák-corrected pairwise comparisons were used to further analyse main effects and interactions[57].

Freezing behavior during conditioning was measured during the 2 min before shock presentation ('pre-shock freezing') and the 1 min after shock presentation ('post-shock freezing'). Repeated measures ANOVAs were used to analyse freezing during the conditioning, extinction and fear memory retention sessions. For conditioning, pre-shock and post-shock freezing were compared within subjects (PrePost), with the number of shocks delivered in context A (Shocks) and classification of SEFL phenotype (SEFL) as between-subjects factors. For the extinction session, freezing was quantified in 30 1 min time bins and analyzed using repeated measures ANOVA with Bin as a within-subjects factor, and Shocks and SEFL as between-subjects factors. For the fear memory retention test, total freezing during the 8 min session was quantified and compared between SEFL and Shock groups using a univariate ANOVA. To compare recovery of freezing between the end of extinction training and the beginning of the retention test, the difference in freezing between the final minute of extinction training and the first minute of the retention test was calculated, and a univariate ANOVA was used to compare these difference scores with SEFL and Shock as between-subjects factors. Where ANOVAs were used, data were tested for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using Levene's test. Although normality and homogeneity of variance assumptions were not always met, ANOVA was used as it is robust to violations in these assumptions, particularly when group sizes are equal[58]. Where repeated measures ANOVAs were used, data were tested for sphericity using Mauchly's test and where sphericity was violated, the Greenhouse–Geisser correction was applied when $\varepsilon < 0.75$ and the Huynh–Feldt correction applied when $\varepsilon > 0.75$, as recommended by Cardinal and Aitken[57].

The proportion of rats showing the SEFL phenotype following different experiences of massed shock in context A was assessed using Fisher's exact test,

as the sample size was too small for Pearson's χ^2 test to be accurate. A one-tailed test was used owing to the directionality of the hypothesis (that is, that greater shock exposure in context A would produce a higher proportion of the population showing the SEFL phenotype).

For analysis of protein expression, individual optical density readings were normalized to the level of β-actin expression for comparison across gels. To facilitate interpretation, optical densities were normalized to the 0-shock, SEFL-resilient control group. The expression of each protein (GluN2B, GluR1 and GluA2) was analyzed separately using two-way (Shock × SEFL phenotype) univariate ANOVAs.

Correlations between conditioned freezing and expression of proteins were analyzed using partial correlations, controlling for the number of shocks delivered in context A. The comparison of correlation coefficients was achieved by using Fisher's method of standardizing the individual *r*'s to *z* scores, then calculating a difference score, z_{diff}, using the equation:

$$z_{\text{diff}} = \frac{z_{r1} - z_{r2}}{\sqrt{\frac{1}{N_1 - 3} + \frac{1}{N_2 - 3}}}$$

z scores were compared with critical values to determine two-tailed probability.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data accompanying this publication are available at the University of Cambridge data repository (https://doi.org/10.17863/CAM.75449).

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Acknowledgements

This research was funded by the Department of Psychology, University of Cambridge. A.L.M. is the Ferreras-Willetts Fellow in Neuroscience at Downing College, University of Cambridge. The authors would like to thank G. Vousden for writing the MATLAB scripts that were used to quantify conditioned freezing.

Author contributions

A.L.M. conceived and designed the experiments and analysis. I.A.V.A. and A.L.M. collected the data and performed the analysis for the behavioral experiments. M.S.P., O.S.R.P.S. and A.L.M. collected the data and performed the analysis for the western blotting experiments. A.L.M. wrote the manuscript, and all authors edited and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41684-022-01054-4.

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Peer review information *Lab Animal* thanks Julia Chester, Jennifer Quinn, Graziano Pinna and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Data were collected using the Fear Conditioning programme, run on the Whisker Server (www.whiskercontrol.com). Freezing behaviour was quantified using a custom-written MATLAB script by G.H. Vousden. Optical density measurements were made using Li-COR's Image Studio for the Odyssey CLx scanner.
Data analysis	Data were statistically analysed using IBM SPSS Statistics (v27).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The data will be freely available on the University of Cambridge data repository on the DOI provided in the manuscript data availability statement. Currently placeholder data have been uploaded to this link, and a finalised dataset will be uploaded if/when the manuscript is accepted for publication.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	As reported in the manuscript, sample size was determined by reference to the previous literature. As we had modified the procedure to reduce the potential suffering of the animals, we conservatively estimated that the effect size would be markedly reduced and thereby increased our sample size from n=5-8 per group (as previously reported) to n=14 per group.
Data exclusions	No data were excluded from the behavioural analyses. For the molecular analyses five samples were excluded due to very low levels of protein expression. All samples were run in duplicate, with the exception of two samples for which there were technical problems with quantification, in which cases the single valid measurement was used. All of these exclusions are stated in the manuscript.
Replication	For the behavioural analyses, multiple squads of rats in different shock conditions were run over an extended period of time. For the molecular analyses, samples were run in duplicate and the measurements averaged for analysis.
Randomization	The first six squads of rats (n=12 per squad) were assigned to shock conditions in different ways. The first two squads included rats randomly assigned to the 0-shock and 15-shock conditions, based on the previous literature. The third squad was assigned to the 4-shock condition, as this level of shock has been reported to induce SEFL in some previous reports. As we observed a lower proportion of animals showing the SEFL-susceptible phenotype in this condition, the fourth, fifth and sixth squads were assigned to the 9-shock, 12-shock and 13-shock conditions by 'splitting the difference' between the 15-shock group (which produced 50% susceptibility) and the last 'non-effective' group that produced a lower proportion of susceptible animals. For the seventh squad, two animals were randomly assigned to each shock condition.
Blinding	All freezing behaviour was quantified before rats were classified as SEFL-susceptible or SEFL-resilient, although it was only possible to blind to shock condition for the first, second and seventh squads of animals. The quantification of optical densities for the molecular analyses was conducted blind to experimental condition.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	As reported in the manuscript, the following antibodies were used for western blotting: - Rabbit anti-GluA1 (Merck Millipore, Upstate 08-555) - Rabbit anti-GluN2B (AbCam, ab65783) - Mouse anti-GluR2 (Merck Millipore, MAB397) - Mouse anti-beta-actin (AbCam, ab8226) - Goat anti-mouse fluorescent secondary (Li-COR IRDye 680RD IgG H+L) - Goat anti-rabbit fluorescent secondary (Li-COR IRDye 800CW IgG H+L)
Validation	As stated in the manuscript, all antibodies were tested on previously collected samples, with known concentrations of proteins, to ensure that the concentrations of antibodies used were delivering optical density signals that were in the linear range.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	As stated in the manuscript, male Lister-Hooded rats, weighing at least 150g at the start of the experiment, were used.
Wild animals	This study did not involve wild animals.
Field-collected samples	This study did not involve samples collected from the field.
Ethics oversight	As stated in the manuscript, this study was regulated under the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations (2012). It was conducted on UK Home Office Project Licence PA9FBFA9F and Personal Licence I574CD98D, following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body.

Note that full information on the approval of the study protocol must also be provided in the manuscript.