Neuregulin-1-dependent control of amygdala microcircuits is critical for fear extinction

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Research Article

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Abstract

Anxiety disorders, especially posttraumatic stress disorder, are marked by an impaired ability to understand that a conditioned stimulus previously paired with a noxious stimulus is no longer noxious. Although previous studies suggest that fear extinction depends on the function of the amygdala, the underlying mechanisms are unclear. We found that NRG1 receptors (ErbB4) were abundantly expressed in the intercalated amygdala (ITC). The NRG1-ErbB4 pathway in the ITC promotes fear extinction. The NRG1-ErbB4 pathway in the ITC did not affect excitatory input to ITC neurons from BLA neurons but increased feed-forward inhibition of CeM neurons through increased GABAergic neurotransmission of ITC neurons. We also found that the NRG1-ErbB4 signaling pathway in ITC might regulate fear extinction through P/Q-type voltage-activated Ca^{2+} channels (VACCs) but not through L- or N-type VACCs. Overall, our results suggest that the NRG1-ErbB4 signaling pathway in the ITC might represent a potential target for the treatment of anxiety disorders.

Introduction

Healthy individuals can process fearful and other emotional stimuli and extinguish fear in situations that are no longer threatening. However, patients with posttraumatic stress disorder (PTSD) or other anxiety disorders have difficulty extinguishing fearful memories. Pavlovian fear conditioning is a classical model for research on anxiety disorders in the laboratory [1][2, 3]. In some respects, fear conditioning resembles PTSD [4, 5]. To extinguish learned fear, an animal is given the conditioned stimulus (CS) repeatedly without the unconditioned stimulus (US) [6]. Therefore, understanding the mechanisms underlying fear memory extinction is important for understanding the mechanism of fear inhibition and may help to improve the treatment of anxiety disorders.

The amygdala is a key brain region for fear learning [7-12]. As the main entry for sensory inputs into the amygdala, the lateral amygdala (LA) delivers CS information to the amygdala [13, 14]. The central medial nucleus of the amygdala (CeM) is the primary output for fear, which projects to brainstem fear effector structures and controls fear behaviors [8, 15]. The LA can influence the activity of the CeM indirectly by regulating the basolateral nucleus of the amygdala (BLA) [16-18]. Meanwhile, glutamatergic neurons of the BLA can project to GABAergic neurons of the intercalated amygdala (ITC), leading to increased feed-forward inhibition of CeM neurons [16-20]. Previous studies also demonstrated that the fear extinction process is associated with increased inhibition of CeM neurons [21]. Despite these advances, we still know very little about the mechanisms underlying how BLA inputs regulate ITC neurons and increase the inhibition of CeM neurons.

The NRG1-ErbB4 signaling pathway has an important function in fear regulation, synaptic plasticity, neuronal survival, etc. [22, 23]. NRG1 contains an epidermal growth factor (EGF)-like domain that stimulates its receptors. As NRG1 receptors, ErbB receptors are transmembrane receptor tyrosine kinases and act in a dimerization paradigm [23]. However, ErbB4 is the only ErbB receptor autonomously activated by NRG1[23]. In contrast, ErbB2 must function with other ErbBs as heterodimers [23]. The

kinase function of ErbB3 is impaired, although it can bind to NRG1 [23]. We found that ErbB4 is primarily expressed in GABAergic neurons, and NRG1 binds to ErbB4 and promotes GABA release in BLA slices [24]. Thus, we focused our research on NRG1-ErbB4 signaling. In past research, there was some evidence showing that NRG1 and ErbB4 are important for innate fear and conditioned fear memory expression [23, 25]. However, the role of NRG1-ErbB4 signaling in fear extinction and the underlying mechanism have not yet been investigated.

Previous studies have found that inhibitory synaptic transmission occurs through Ca^{2+} -dependent modulation [26, 27]. Previous studies also indicate that the spontaneous release of GABA requires the function of multiple coupled voltage-activated Ca^{2+} channels (VACCs) of variable subtypes [28]. Pharmacological studies have found that at least six different subtypes of Ca^{2+} channels, L, N, T, P, Q, and R, exist [29, 30]. At cortical synapses, P/Q channels have the strongest function in GABA release compared to N and R channels [31]. However, to date, the mechanism underlying Ca^{2+} -dependent modulation of GABAergic neurotransmission is not well understood. Since NRG1-ErbB4 is important for the regulation of GABAergic neurotransmission, whether VACC-dependent GABAergic neurotransmission could be regulated by NRG1-ErbB4 has not yet been determined; therefore, we attempted to use specific channel-type blockers to test this hypothesis.

In this study, we examined the function of NRG1-ErbB4 signaling in the ITC and explored the function of NRG1-ErbB4 signaling in fear extinction and the underlying mechanism. Our observations revealed an important function for NRG1-ErbB4 signaling in controlling GABAergic neurotransmission of the amygdala and suggested that the NRG1-ErbB4 signaling pathway in the ITC constitutes a potential target for the treatment of anxiety disorders.

Methods And Materials

Animals

Mice were given free access to water and food. Mice were housed under a 12 h light/dark cycle. Behavioral testing was performed in the daytime around 10:00 A.M. All procedures followed the Chinese Council on Animal Care Guidelines. All experimental protocols were also performed according to guidelines set by Wuhan University and were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC). Efforts were made to reduce the number of sacrificed mice and to minimize the suffering of mice.

Reagents

NRG1 (rHRG β 177–244) is a β -type NRG1 that contains an EGF domain. BMI was purchased from Tocris Bioscience. AG1478 was obtained from Calbiochem. Other chemicals were purchased from Sigma–Aldrich.

Intracerebral infusions

Mice (5-7 weeks old, male) were anesthetized and placed onto the stereotaxic instrument (Stoelting, USA). One stainless-steel guide cannula was placed into the random and unilateral ITC areas according to the following coordinates: anterior, ± 1.4 mm; lateral, ± 3.2 mm; ventral, ± 4.2 mm. Seven days after surgery, drug infusion and behavioral tests were performed. Mice were injected at a flow rate of 0.05 $\mu\text{l}/\text{min}$ with 0.1 $\mu\text{l}/\text{side}$. Detailed information is provided in the Supplemental Experimental Procedures.

Viral injection

As previously described [24, 32], mice were anesthetized and placed onto the stereotaxic frame. Virus (0.5 μl) was injected into the bilateral BLA (anterior, -1.5 mm; lateral, ± 3.2 mm; and ventral, -4.7 mm), and virus (0.1 μl) was infused into the bilateral ITC areas (anterior, -1.4 mm; lateral, ± 3.2 mm; and ventral, -4.2 mm). Detailed information is provided in the Supplemental Experimental Procedures.

Histological identification

Detection of viral expression, the accuracy of fiber implantation and fluorescent protein expression were performed as previously described [24].

Behavioral tests

As previously described [33], mice underwent 20 min habituation in the training chamber (context A) for 3 consecutive days before the experiments. On day 4, mice were placed in fear conditioning chamber A. Mice were given a 3-min undisturbed period, followed by four CS (70 dB; 30 s duration; 80 s intershock interval); each CS session was terminated with a US (0.6 mA; 1 s duration). Mice were left in the training chamber for another 2 min and immediately returned to their home cages. On day 5, the no-extinction mice were exposed to context B but were not given the CS or US. However, mice in the extinction group were presented with 20 unpaired CSs in context B on day 5. The intervals between CSs were pseudorandomly chosen from intervals ranging between 80–180 s (10 s increments). The percentage of freezing time in each period was quantified by DigBehv animal behavior software. Detailed information is provided in the Supplemental Experimental Data.

Electrophysiological Recordings

Mice was anesthetized. Brains were quickly removed and chilled in ice-cold modified artificial cerebrospinal fluid (ACSF). The amygdala slices were cut using a VT-1000S vibratome and transferred to a chamber filled with ACSF. Whole-cell patch-clamp recordings of neurons were obtained using IR-DIC visualization (Zeiss, Axioskop 2). Detailed information is provided in the Supplemental Experimental Procedures.

Statistical analyses

The number of animals is indicated by “n”. Data met the assumption of normality. Data were analyzed by one-way ANOVA, two-way ANOVA or Student’s t-test. For multiple comparisons, an SNK test was used

when equal variances were assumed, and Dunnett's test was used when equal variances were not assumed. Throughout the study, statistical analyses were performed using SPSS software (SPSS, Inc.). All data are expressed as the mean $\pm$ SEM. Values of $p < 0.05$ were considered significant.

Results

The NRG1-ErbB4 signaling pathway in the ITC area is required for fear extinction and inhibition in CeM neurons.

GABAergic ITC neurons are critical for the modulation of fear memory extinction. Although we observed that ErbB4 was found primarily in GABAergic interneurons in the BLA [24], there is no morphological or behavioral evidence to show whether ErbB4 is expressed in GABAergic ITC neurons or is involved in the regulation of fear extinction. To address this issue, we first explored whether ErbB4 is expressed primarily in the interneurons of the ITC area using transgenic mice to identify the colocalization of GAD67-positive interneurons and ErbB4-positive neurons. Figure 1A shows the coimmunofluorescence image of tdTomato (red) reporting ErbB4 expression and GAD67 expression (green) in the ITC. Most ErbB4-expressing neurons in the ITC area coexpressed GAD67 ($98.99 \pm 0.007\%$; Figure 1A). These results provide histological evidence that ErbB4 is abundantly expressed in GABAergic ITC neurons.

Based on previous studies suggesting important roles for the ITC area in fear extinction and the NRG1-ErbB4 pathway in GABAergic neurotransmission, we hypothesized that the ITC area was the amygdalar subregion requiring the NRG1-ErbB4 pathway in fear extinction. To test this hypothesis, the drug was infused into the ITC area. One hour later, mice underwent fear extinction training. We observed significantly reduced freezing levels during fear extinction in the NRG1 group than in the control group ($F_{3, 684} = 71.943$, $P < 0.001$; Figure 1C). Mice treated with ErbB4's antagonist (AG1478) exhibited impaired fear extinction compared with the control mice ($F_{3, 684} = 71.943$, $P < 0.001$; Figure 1C). There were no significant differences between the AG1478 group and NRG1+AG1478 group ($F_{3, 684} = 71.943$, $P = 0.56$; Figure 1C). Taken together, these findings suggest that NRG1 promotes fear extinction through ErbB4 receptors in the ITC area.

To test whether extinction depends on NRG1-mediated increased feed-forward inhibition in the CeM, we first compared responses of CeM neurons to BLA inputs (Figure 1D) in amygdala slices obtained from mice that were previously treated with AAV-ectoErbB4 and subjected to fear extinction versus control mice that were treated with AAV-GFP and trained on extinction. AAV-ectoErbB4 was designed to express a polypeptide that includes the entire extracellular domain of ErbB4 and binds to NRG1, preventing NRG1 from activating endogenous receptors [34]. We confirmed that expression of the virus was limited only in the ITC three weeks after virus injection (Supplementary Figure 1). We also identified ITC neurons projected to the CeM (Supplementary Figure 2).

We examined the behavior of these mice (Figure 1F) and then analyzed the responsiveness of CeM neurons to BLA inputs in vitro (Figure 1G, 1H). Analysis of percent time spent freezing (Figure 1F)

demonstrated that the Ecto-E group exhibited higher levels of conditioned freezing during fear extinction (day 5) than the control group ($F_{2,513} = 392.994$, $P < 0.001$; Figure 1F). These observations thus confirm the conclusion that NRG1 in the ITC area is necessary for the regulation of fear extinction.

We anesthetized the mice 24 h after exposure to the extinction training context and prepared coronal sections from their amygdala tissues. We obtained patch recordings from samples of 10 CeM neurons per group and compared their responsiveness to electrical stimuli delivered at a standard position in the BLA (Figure 1D). The eIPSC amplitudes of CeM neurons from the Ecto-E mice and the Control-No E mice were significantly lower than those from the Control-E mice ($F_{2,27} = 5.113$, $P = 0.007$ and 0.016 , respectively; Figure 1G and 1H). However, eIPSCs from Ecto-E mice were not significantly different from those of the Control-No E group ($F_{2,27} = 5.113$, $P = 0.719$; Figure 1G and 1H). Blocking ITC NRG1 did not affect the increased excitatory input to CeM neurons by stimulating BLA neurons (Supplementary Figure 3). Next, we investigated the presynaptic or postsynaptic mechanism involved in the regulation of GABAergic transmission by NRG1. The mIPSCs were measured at CeM neurons. The mIPSC frequencies, but not the mIPSC amplitudes, of the Control-E group were higher than those of the Control-No E group ($F_{2,27} = 6.203$ and 0.197 , $P = 0.005$ and 0.619 , respectively; Figure 1I-1K). Inhibiting NRG1 produced no effect on the mIPSC amplitudes but significantly decreased the frequencies compared to the Control-E group ($F_{2,27} = 0.197$ and 6.203 , $P = 0.941$ and 0.005 , respectively; Figure 1I-1K). These results indicate that fear extinction is associated with enhanced BLA-evoked IPSCs in the CeM that could be reversed by inhibiting ITC NRG1. ITC NRG1 is important for the regulation of BLA-evoked presynaptic inhibition of the CeM, which is involved in fear extinction.

Blocking ITC NRG1 does not affect the increased excitatory input to ITC neurons from BLA neurons but leads to decreased inhibition of CeM neurons.

To explore how extinction depends on increased inhibition of the CeM mediated by ITC NRG1, we first compared the responses of ITC neurons to BLA inputs (Figure 2A) in amygdala slices obtained from mice treated with AAV-ectoErbB4 and subjected to fear extinction versus control mice treated with AAV-GFP and trained on extinction (Figure 1E).

We anesthetized the mice 24 h after extinction training and prepared amygdala slices. We first obtained patch recordings of the ITC neurons and compared their responsiveness to the electrical stimuli delivered at the BLA (Figure 2A). Thereafter, we measured evoked excitatory postsynaptic currents (eEPSCs) in ITC neurons. AP5 (100 mM) and CNQX (20 mM) were included in ACSF when we recorded AMPAR-EPSCs and NMDAR-EPSCs, respectively. The AMPAR-EPSC amplitudes of ITC neurons from the Ecto-E mice and the Control-E mice were significantly higher than those from the Control-no E mice ($F_{2,27} = 6.356$, $P = 0.019$ and 0.009 , respectively; Figure 2B). However, AMPAR-EPSCs from Ecto-E mice were not significantly different from those of the Control-E group ($F_{2,27} = 6.356$, $P = 0.993$; Figure 2B). However, the NMDAR-EPSCs showed no differences between these groups ($F_{2,27} = 0.097$, $P = 0.908$; Figure 2D).

Next, we investigated the responses of CeM neurons to ITC inputs (Figure 2F) in amygdala slices obtained from mice treated with AAV-ectoErbB4 and subjected to fear extinction versus control mice that were treated with AAV-GFP and trained on extinction. We then obtained patch recordings of the CeM neurons and compared their responsiveness to the electrical stimuli delivered at the ITC (Figure 2A). The eIPSC amplitudes of CeM neurons from the Control-E mice were significantly larger than those from the Control-no E mice ($F_{2,27} = 5.147$, $P = 0.028$; Figure 2H). However, the eIPSCs from Ecto-E mice were significantly smaller than those of the Control-E group ($F_{2,27} = 5.147$, $P = 0.031$; Figure 2H). These results indicate that fear extinction is associated with enhanced BLA-evoked EPSCs in the ITC, which elicits feed-forward inhibition of CeM neurons. The feed-forward inhibition of CeM elicited by ITC neurons is regulated by the NRG1 signaling pathway.

ITC NRG1 might regulate GABAergic transmission through P/Q-type VACCs but not through L- or N-type VACCs.

The decreased IPSCs observed after blocking NRG1 were mediated by different neurotransmission mechanisms. One possibility is that they decrease Ca^{2+} -dependent synaptic transmission. To examine this, we studied the function of an ErbB4 antagonist (AG1478) and inhibitors of VACC subtypes on eIPSC amplitudes of CeM neurons. We measured the eIPSCs of CeM neurons and compared their responsiveness to electrical stimuli delivered at the ITC (Figure 3A). A series of experiments were performed by additively applying AG1478 (5 μ M) and one of the following blockers: L-type VACC blocker nifedipine (NFD, 4 μ M), N-type VACC blocker ω -Ctx-GVIA (CTx, 1 μ M), or P/Q-type VACC blocker ω -Aga-IVA (Aga, 0.5 μ M).

As shown in Figure 3B-3D, NFDP exerted weak blocking effects on eIPSCs on CeM neurons (<6%), whereas CTx and Aga had significant blocking effects on CeM neurons. Interestingly, by applying AG1478 after VACC antagonist treatments, we obtained significantly increased inhibition of the eIPSCs on NFDP- and CTx-treated neurons but not on Aga-treated pyramidal neurons [$t(10) = -4.162$, -3.925 , and -0.665 ; $P = 0.002$, 0.003 and 0.521 , respectively; Figure 3B-3D], suggesting that the NRG1-ErbB4 signaling pathway may act through P/Q-type channels but not through L- or N-type channels. To further identify the possible effects of Ca^{2+} channel antagonists on the reaction of AG1478 in Figure 3, we analyzed the contribution of AG1478 in the absence or presence of Ca^{2+} channel antagonists. The decreased percentage of eIPSC amplitudes mediated by AG1478 did not change in the presence of L- or N-type channel antagonists ($F_{3,20} = 6.581$, $P = 0.818$, 0.851 , respectively; Figure 3E). However, AG1478 barely worked in the presence of the P/Q-type antagonist Aga compared to the control group ($F_{3,20} = 6.581$, $P < 0.001$, Figure 3E). These data indicate that the NRG1-ErbB4 signaling pathway may regulate GABAergic transmission through P/Q-type channels but not through L- or N-type channels.

Since the above results suggest that the NRG1-ErbB4 signaling pathway might affect IPSCs via presynaptic mechanisms, we next directly measured the effects of NRG1-ErbB4 on VACCs of ITC interneurons (Figure 3F). Then, we additively applied the AG1478, Aga, CTx, and NFDP to measure VACC

current changes. We found that AG1478 significantly decreased the amplitude of VACC currents ($F_{4,25} = 203.744$, $P = 0.007$, Figure 3G). We did not observe a significant reduction in VACC currents in response to Aga compared to the AG1478 treatment ($F_{4,25} = 203.744$, $P = 0.895$, Figure 3G). However, we found that VACC currents were significantly reduced by CTx in the presence of AG1478 and Aga ($F_{4,25} = 203.744$, $P = 0.002$, Figure 3G). NFDP further reduced VACC currents in the presence of AG1478, Aga and CTx ($F_{4,25} = 203.744$, $P = 0.001$, Figure 3G). To exclude the possible effect of AG1478 on Aga, we added Aga first, and AG1478 still did not cause a significant difference between the Aga group and the AG1478 group ($F_{4,25} = 98.538$, $P = 0.295$, Figure 3H). These data were quite coincident with the above conjecture that the NRG1-ErbB4 signaling pathway regulates GABAergic transmission through P/Q-type VACCs but not through L- or N-type VACCs.

Blocking ITC NRG1 has no effect on the excitability of BLA synapses projecting to the ITC but leads to decreased feed-forward inhibition of CeM neurons mediated by reduced P/Q-type VACC currents of ITC neurons.

To further support the function of ITC NRG1 in fear extinction and the underlying mechanism, we first compared the responses of ITC neurons to BLA inputs (Figure 4A) and then compared the responses of CeM neurons to BLA inputs (Figure 4D) in amygdala slices obtained from mice that were treated with AAV-ectoErbB4 and subjected to fear extinction versus control mice that were treated with AAV-GFP and trained on extinction. BLA glutamatergic projection neurons were transfected with adeno-associated virus serotype 5 (AAV5) carrying codon-optimized channel rhodopsin (ChR2)–egfp under the control of the CaMKIIa promoter. For all groups, the virus was injected into the ITC areas three weeks before the behavioral experiments. We anesthetized the mice 24 h after extinction training and prepared amygdala sections. We also confirmed that expression of the virus was limited only in the BLA and identified that BLA neurons projected to the ITC three weeks after virus injection (Supplementary Figure 4).

We first obtained patch recordings from ITC neurons and compared their responsiveness to photostimulation of BLA terminals in the ITC (Figure 4A). We performed these tests at a membrane potential of -70 mV. The eEPSC amplitudes of ITC neurons from the Control-E mice and the Ecto-E mice were significantly higher than those from the Control-No E mice ($F_{2,27} = 5.091$, $P = 0.042$ and 0.023 , respectively; Figure 4B and C). However, eEPSCs from Ecto-E mice were not significantly different from those of the Control-E group ($F_{2,27} = 5.091$, $P = 1$; Figure 4B and C). Next, we investigated the responses of CeM neurons to photostimulation of BLA terminals in the ITC (Figure 4D). The eIPSC amplitudes of CeM neurons from the Control-E mice were significantly higher than those from the Control-No E and Ecto-E mice ($F_{2,27} = 10.097$, $P = 0.009$ and 0.017 , respectively; Figure 4E and 4F). However, eIPSCs from Ecto-E mice were not significantly different from those of the Control-No E group ($F_{2,27} = 10.097$, $P = 1$; Figure 4E and 4F). The latency of eIPSCs recorded on CeM neurons was longer than that of eEPSCs recorded on ITC neurons [$t(58) = -8.766$; $P < 0.001$, Figure 4G]. Altogether, the above results again demonstrated that BLA glutamatergic neurons projected to ITC GABAergic neurons and elicited feed-forward inhibition of CeM neurons.

We next directly measured the effects of NRG1-ErbB4 on P/Q-type VACC currents of ITC interneurons and compared the amplitudes of P/Q-type VACC currents in coronal slices of the amygdala obtained from mice that were previously treated with AAV-ectoErbB4 and subjected to fear extinction versus control mice that were treated with AAV-GFP and trained on extinction (Figure 4I and 4J). We applied the P/Q-type VACC blocker Aga to measure the P/Q-type VACC current changes using Ba^{2+} as the charge carrier. We found that the amplitudes of P/Q-type VACC currents in the Control-E group were higher than those in the Control-No E group and in the Ecto-E group ($F_{2,15} = 7.619$, $P = 0.005$ and 0.011 , respectively; Figure 4I and 4J). No differences were observed between the Control-No E group and the Ecto-E group ($F_{2,15} = 7.619$, $P = 1$; Figure 4I and 4J). These data again supported the above conjecture that the ITC NRG1-ErbB4 signaling pathway regulates GABAergic transmission through P/Q-type channels involved in fear extinction.

The ITC NRG1 signaling pathway might regulate fear extinction through P/Q-type VACCs.

These results indicate that the ITC NRG1-ErbB4 signaling pathway regulates BLA-evoked presynaptic inhibition of the CeM through P/Q-type VACC channels, which are involved in extinction training. To further test this, drug (control ACSF, NRG1, NRG1+Aga, Aga) was infused into the ITC area through guide cannulas, and 1 h later, mice underwent fear extinction training (Figure 5B). We observed significantly lower freezing levels during fear extinction in the NRG1 group than in the control group ($F_{3,513} = 1.706$, $P < 0.001$; Figure 5C). Mice treated with NRG1+Aga or Aga exhibited impaired fear extinction compared to control mice ($F_{3,513} = 1.706$, both $P < 0.001$; Figure 5C). No differences were observed between the NRG1+Aga and Aga groups ($F_{3,513} = 1.706$, $P = 0.988$; Figure 5C). These observations suggest that NRG1 promotes fear extinction through P/Q-type VACC channels in the ITC area.

To further elucidate the function of ITC NRG1 in fear extinction and the underlying mechanism, we compared the responses of CeM neurons to BLA inputs in amygdala slices (Figure 2; Figure 5A). We anesthetized mice 24 h after extinction training and prepared amygdala slices. We then measured the responses of CeM neurons to photostimulation of BLA terminals in the ITC (Figure 2; Figure 5D). The eIPSC amplitudes of CeM neurons from the NRG1-treated mice were significantly higher than those from the Control mice ($F_{3,37} = 17.726$, $P = 0.044$, Figure 5D and 5E). However, the amplitudes of eIPSCs from the NRG1+Aga group or the Aga group were significantly lower than those of the Control group ($F_{3,37} = 17.726$, $P = 0.019$ and 0.006 , respectively; Figure 5D and 5E). No differences were observed between the NRG1+Aga group and the Aga group ($F_{3,37} = 17.726$, $P = 0.728$; Figure 5D and 5E). Altogether, the above results again support that BLA glutamatergic neurons elicited ITC feed-forward inhibition of CeM neurons during the fear extinction process, which could be regulated by ITC NRG1-ErbB4 signaling. The ITC NRG1-ErbB4 signaling pathway might regulate GABAergic neurotransmission required for fear extinction through P/Q-type channels.

Discussion

Since some human anxiety disorders are related to an extinction deficit, understanding the mechanisms of fear extinction might help to improve treatment of anxiety disorders [35]. Previous studies have shown that fear extinction depends on an increased inhibition of CeM neurons mediated by enhanced activity of ITC neurons [36]. How does fear extinction promote the activity of ITC neurons? Herein, we found that increased CeM inhibition elicited by fear extinction was associated with increased BLA inputs to ITC neurons, which activated the NRG1-ErbB4 signaling pathway in the ITC. The NRG1-ErbB4 pathway then enhanced inhibition of CeM neurons by increasing the GABAergic transmitter release probability through promoting P/Q-type VACC currents of ITC neurons. Our results suggest that enhancing the excitability of ITC neurons by regulating NRG1-ErbB4 signaling might represent a potential therapeutic target for anxiety disorders.

Our past research demonstrated that NRG1 and ErbB4 in the BLA are important for the regulation of GABAergic neurotransmission, which is required for the regulation of innate fear and conditioned fear memory expression [24]. However, the role of NRG1-ErbB4 signaling in fear extinction and the underlying mechanism have not yet been investigated. We next explored how ITC NRG1 is involved in this process. We found that during fear extinction, excitatory input from BLA neurons to ITC neurons was increased. Blocking ITC NRG1 did not affect the increased excitatory input to ITC neurons from BLA neurons but led to decreased inhibition of CeM neurons from ITC neurons. The increased P/Q-type channel currents mediated by the ITC NRG1-ErbB4 signaling pathway promoted GABAergic transmission, which underlies the mechanisms of fear extinction. Although our previous study found that the NRG1-ErbB4 pathway in the BLA was critical for the modulation of fear conditioning, we did not identify a fear extinction role for BLA NRG1-ErbB4. Fear extinction is thought to be a new learning process. We also provide additional evidence that fear expression and fear extinction are different in synaptic microcircuits and molecular mechanisms. Our findings also identify a novel pathophysiological mechanism for PTSD.

Previous studies have found that N-, P/Q- and R-type channels are distributed at synaptic terminals and could promote neurotransmitter release from neurons. The distribution patterns and functions of these channels are variable. For example, N- and P/Q-channels are heterogeneously distributed across the presynaptic terminals of excitatory synapses, even if the terminals originate from the same axon [37]. In inhibitory synapses, some presynaptic interneurons may release GABA by P/Q-channels, while others may release GABA by N-channels [38]. At cortical synapses, P/Q channels have the strongest function in GABA release compared to N channels and R channels [39]. To test whether and which VACCs mediate the function of ITC NRG1-ErbB4 in GABAergic neurotransmission, we used specific channel-type blockers: the L-type VDCC blocker nifedipine, the N-type VDCC blocker ω -Ctx-GVIA, and the P/Q-type VDCC blocker ω -Aga-IVA. A series of experiments were performed by sequentially applying AG1478 and one type of VACC antagonist. AG1478 treatment after L- and N-type channel antagonists were administered to neurons further decreased the eIPSCs but not after treatment with the P/Q-type channel antagonist. In addition, our further analysis in Figure 4E certified that L- or N-type channel antagonists, but not the P/Q-type channel antagonist, did affect the function of Ecto-ErbB4 in eIPSCs. All results suggest that ITC NRG1-ErbB4 regulates GABAergic transmission through P/Q-type channels but not through L- or N-type

channels. The precise pharmacological dissection of the voltage-gated Ca^{2+} channels involved in evoked GABAergic transmission also required detailed studies on single ITC synaptic boutons [40]. We next directly measured the effects of NRG1-ErbB4 on P/Q-type VACC currents of ITC interneurons and compared the amplitudes of P/Q-type VACC currents in coronal slices of the amygdala. Our findings indicated that the ITC NRG1-ErbB4 signaling pathway regulates GABAergic transmission through P/Q-type channels involved in fear extinction.

We found for the first time that NRG1-ErbB4 signaling enhances ITC GABAergic outputs in the amygdala and thus promotes fear extinction. Our findings reveal that there is a novel function and mechanism of NRG1–ErbB4 signaling in the regulation of fear memory, which could provide a new method for curing anxiety disorders. We also provide additional evidence that fear expression and fear extinction were different in synaptic microcircuits and molecular mechanisms. Because the susceptibility genes of schizophrenia are shown to include NRG1 and ErbB4, our findings might also help to reveal the mechanism of emotional disorder in schizophrenia.

Declarations

Ethics approval

All procedures were following the Chinese Council on Animal Care Guidelines. All experimental protocols were also conducted under the guidelines set by the Wuhan University and approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC).

Consent to participate

Not applicable

Data availability

All data reported in this study are available from the corresponding authors upon request.

Conflicts of interest

All authors claim that there are no conflicts of interest

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Author contributions

All authors claim that there are no conflicts of interest. Haibo Xu and Linlin Bi are responsible for the conception or design of the work, drafting the work or revising it. Ming Chen has contributions to the the acquisition, analysis, or interpretation of data for the work. Ying Liu has contributions to the collection of supplementary figures, and revising the paper. Ying Li has contributions to the collection of supplementary figures, and revising the paper.

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Consent for publication

Not applicable

Ethical Standards

Disclosure of potential conflicts of interest

There are no conflicts of interest.

Research involving Human Participants and/or Animals

Our research involved adult male C57 BL/6 mice. No human participants were involved.

Informed consent

Not applicable

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Figures

Figure 1

The NRG1-ErbB4 signaling pathway in the ITC area is required for fear extinction and inhibition in CeM neurons. (A) Representative micrograph images of tdTomato (red), reporting ErbB4 expression and GAD67-GFP (green) expression in the ITC. The boxed ITC area is magnified in the lower panel. Scale bar:

500 μ m. (B) The experimental protocol for C. (C) The proportion of freezing time during the various phases (x-axis). During the conditioning phase, all groups were given four CS-US pairings (indicated by the arrows). Data represent the percentage of freezing time during six 30-s periods before conditioning and the four CSs for all groups. During the extinction phase, the percentage of freezing time during CS periods is shown. (D) Experimental setup for 1E-1K. Rec, recording pipette; Stim, stimulus electrode. (E) Control and experimental groups for 1F-1K. Control-No E, control mice without fear extinction; Control-E, control mice with fear extinction; Ecto-E, ITC AAV-ectoErbB4-treated mice with fear extinction. (F) Percentage of freezing time during the various phases of the fear conditioning and extinction protocol. The freezing time of the Control-No E group during corresponding 30-s periods is also shown. (G) Representative traces of BLA-evoked IPSC responses in CeM cells with 10 mM QX-314 in the pipette solution. (H) Statistical analysis of BLA-evoked IPSCs in CeM neurons. (I) Representative traces of mIPSCs in CeM cells are shown. (J) Summarized data of mIPSC frequencies. (K) Summarized data of mIPSC amplitudes. Data are expressed as the mean $\pm$ SEM. n=10/group; *P<0.05, **P<0.01, ***P<0.001; two-way ANOVA test for 1C and 1F, one-way ANOVA test for 1H, 1J and 1K.

Figure 2

Blocking ITC NRG1 did not affect the increased excitatory input to ITC neurons from BLA neurons but led to decreased inhibition of CeM neurons from ITC neurons in fear extinction. (A) Experimental setup. (B) Representative traces of BLA-evoked AMPA-EPSC responses in ITC neurons. (C) Statistical analysis of BLA-evoked AMPA-EPSCs in ITC neurons. (D) Representative traces of BLA-evoked NMDA-EPSC responses in ITC neurons. (E) Statistical analysis of BLA-evoked NMDA-EPSCs in ITC neurons. (F) Experimental setup. (G) Representative traces of ITC-evoked IPSCs in CeM cells are shown. (H)

Summarized data of ITC-evoked IPSC amplitudes. Data are expressed as the mean $\pm$ SEM. n=10/group; *P<0.05, **P<0.01, ns, not significant; one-way ANOVA test.

Figure 3

ITC NRG1 might regulate GABAergic transmission through P/Q-type channels but not through L- or N-type channels. (A, F) Experimental setup. (B, C, D) Inset left, representative traces of ITC-evoked IPSC responses in CeM neurons. Inset right, mean inhibition percentage of eIPSCs mediated by three VACC antagonists, with or without AG1478. VACC antagonists were as follows: 4 μ M nifedipine (NFDP) (B), 1 μ M ω -Ctx-GVIA (CTx) (C), and 0.5 μ M ω -Aga-IVA (Aga) (D). (E) Data analysis of the contribution of AG1478 to eIPSCs in the absence of toxins or the presence of 4 μ M NFDP, 1 μ M CTx, or 0.5 μ M Aga. (G, H) Averaged amplitudes of Ca²⁺ currents recorded from ITC interneurons in amygdala slices were measured from 10 ms at the end of the voltage step (90-100 ms). Left insets are specimen records of Ca²⁺ currents. Data are expressed as the mean $\pm$ SEM. n=6/group; *P<0.05, **P<0.01, ***P < 0.001; t-test for 3B, 3C, 3D; one-way ANOVA test for 3E, 3G, 3H.

Figure 4

Blocking ITC NRG1 had no effect on the excitability of BLA synapses projecting to the ITC but led to decreased feed-forward inhibition of CeM neurons mediated by reduced P/Q-type VACC currents of ITC neurons. (A, D, H) Experimental setup. (B) Representative traces from ITC neurons in response to the illumination of BLA terminals expressed with CaMKII-ChR2. Control-No E, control mice without fear extinction; Control-E, control mice with fear extinction; Ecto-E, ITC AAV-ectoErbB4-treated mice with fear

extinction. (C) Statistical analysis of optogenetically evoked EPSCs in ITC neurons. $n=10/\text{group}$. (E) Representative traces from CeM neurons in response to the illumination of BLA terminals in the ITC expressed with CaMKII-ChR2. (F) Statistical analysis of optogenetically evoked IPSCs in CeM neurons. $n=10/\text{group}$. (G) Summarized data of eEPSC latencies in the ITC and eIPSC latencies in the CeM. (I) Specimen records of Ca^{2+} currents. (J) Averaged amplitudes of P/Q-type Ca^{2+} currents recorded from ITC interneurons in amygdala slices were measured from 10 ms at the end of the voltage step (90-100 ms; $n=6/\text{group}$). Data are expressed as the mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; t-test for 4G; one-way ANOVA test for 4C, 4F, and 4G.

Figure 5

The ITC NRG1 signaling pathway might regulate fear extinction through P/Q-type channels. (A) Experimental setup. (B) Experimental protocol. (C) The proportion of freezing time during the various phases. In the conditioning phase, all groups were given four CS-US pairings (indicated by the arrows). Data represent the percentage of freezing time during six 30-s periods before conditioning and the four CSs during conditioning for all groups. In the extinction phase, the percentage of freezing time during CS periods is shown. All extinguished groups were presented with 20 CSs. (D) BLA glutamatergic projection neurons were transfected with AAV5-CaMKII-ChR2. Representative traces from CeM neurons in response to the illumination of BLA terminals projecting to the ITC terminals. (F) Statistical analysis of optogenetically evoked IPSCs in CeM neurons ($n=10/\text{group}$). Data are expressed as the mean $\pm$ SEM. * $P<0.05$, *** $P<0.001$; two-way ANOVA test for 5C; one-way ANOVA test for 5E.

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