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Auditory cortical neurons are recruited to encode fear signals and anxiety by neuroligin-3-mediated synapse formation

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The social stress often induces fear memory and stress-relevant phobias. Molecular and cellular mechanisms of fear memory and anxiety remain to be addressed for the exploration of therapeutic strategies for these deficits. In social defeat mice induced by the resident/intruder paradigm, we have examined how auditory cortical neurons are recruited to encode stress signals that cause fear memory and anxiety by approaches of behavioral tasks, neural tracing, electrophysiology and molecular biology. The social stress in intruder C57 mice by the attack of resident CD1 mouse causes their fear memory and anxiety-like behaviors. In addition to the interconnections between auditory and somatosensory cortices in the mice of fear memory and anxiety, auditory cortical neurons receive new synapses from the somatosensory cortex and the synapses from the medial geniculate body. These auditory cortical neurons are able to encode the stress signals including the pain stimulus to injury areas and the battle sound in a resident/intruder paradigm. *Neuroligin-3* mRNA knockdown in the auditory cortex prevents the recruitment of associative memory neurons that encode fear memory and anxiety-like behaviors. Therefore, neuroligin3-mediated synapse formation is essential for the stress-induced recruitment of associative memory neurons in auditory cortices that encode stress signals, fear memory and anxiety.

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INTRODUCTION

Physical and psychological stressors often evoke fear memory and anxiety, such as posttraumatic stress disorder and phobia to the stress-related events or objects [1–11]. These affective disorders in turn suppress the immune system and the cardiovascular system to induce their secondary diseases [12]. It is important to reveal cellular and molecular mechanisms underlying stress-induced fear memory and affective disorders, which is a primary goal of the therapy of these pathological moods [13]. Endeavor to achieve this goal appears unsatisfied [13–17]. To the less success, the different types of stressors, such as acute severe stress versus chronic mild stress, psychological stress versus physical stress and natural hazard stress versus social stress, may lead to their featured cellular and molecular alterations in the brain specifically correlated to anxiety, depression and their mixtures [4, 5, 13, 18–21]. The comprehensive maps about molecular cascades and neural circuits between different stressors and their associated affective disorders remain to be revealed to explore their specific therapeutic strategies.

The acute severe stress evokes the fear memory and anxiety [1, 3–5, 7, 22–26]. Many brain areas have been presumably involved in the fear memory and anxiety, including the amygdala, the nucleus accumbens and the prefrontal cortex [27–37]. Neuronal circuits in the amygdala and the nucleus accumbens have been thought of as relevance to the balance between fear memory and reward memory [38–43]. The attenuation of the nucleus accumbens facilitates the amygdala to memorize negative events, leading to affective disorders [44–56]. In addition, the interconnection between the amygdala and the auditory cortex appears formed and strengthened in the fear memory induced by

the associations of the bell ring and the electrical foot shock [23, 57–61]. It is possible that stressful fear signals are programmed by the interactions between the amygdala and the auditory cortex. How auditory cortical neurons are recruited to encode the stress signals for the fear memory and anxiety is hypothetically taken into our study.

The auditory cortex has been indicated to encode the joint storage of auditory signals and other signals in associative learning and memory [7, 62–72]. The primary auditory cortex discriminates those stimulations from threat and nonthreat signals and then to regulate the specificity of threat memory, implying its effectiveness on subsequent fear memory [73]. The cellular mechanisms for the information storage in the auditory cortex are largely unknown [74–78]. The associative memory neurons have been identified in sensory cortices including the somatosensory cortices, the piriform cortex, and the gustatory cortex, which are featured by synapse interconnections among these cross-modal sensory cortices as well as the encoding of multiple associated signals [79–84]. Whether the associative memory neurons are recruited in the auditory cortex to encode those fear signals in the social stress and to cause fear memory and anxiety will be examined in our study.

Taken these questions above, we intend to examine the roles of the formation of new synapses and the recruitment of associative memory neurons in the auditory cortex in encoding stress signals, fear memory and anxiety by multiple approaches of behavioral tasks, electrophysiology and neural tracing and molecular biology. In terms of the strategies, C57 mice as intruders were subjected to the social stress by the resident/intruder paradigm, in which they

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were attacked by a resident CD1 mouse [4, 85–90]. The C57 mice in the groups of social stress and control were placed in a social interaction cage to measure the formation of fear memory and on an elevated-plus maze to assess anxious state. Both intruder mice with the stress-induced fear memory/anxiety and control mice were investigated by the neural tracing to track the new synapse formation and axon interconnections and by electrophysiological recordings to analyze the encoding of auditory cortical neurons to the stressful signals, such as the battle sound and the pain signal from somatic injury regions. The interconnections of the associative memory neurons among cross-modal cortices were examined by microinjecting anterograde and retrograde adeno-associated viruses that carried genes of encoding fluorescent proteins in one of brain areas and by detecting their expression in its interconnected cortical areas, or the other way around. The recruitment of associative memory neurons was ensured when new synapse contacts along with those presented synapse contacts were detected on the dendritic spines of auditory cortical neurons in the convergent manner [79, 81, 91] and when the auditory cortical neurons showed a raised spike-encodings in response to stress signals [79, 84, 92]. The essential role of associative memory neurons recruited by neuroligin-3, a synapse linkage protein, in stress-induced fear memory and anxiety was examined by using short-hairpin RNAs (shRNA) specific to silence *neuroligin-3* mRNA, which were carried by adeno-associated virus (AAV).

MATERIALS AND METHODS

Studies approved in mice and animals feeding

Experiments were conducted in accordance with guidelines and regulations by the Administration Office of Laboratory Animals in Beijing China. All of the experiment protocols were approved by the Institutional Animal Care and Use Committee in the Administration Office of Laboratory Animals in Beijing China (B10831).

In our study, two species of mice were applied. C57BL/6J Thy1-YFP mice (Jackson Lab, USA) were used and divided into intruder and control groups as well as neuroligin-3 knockdown and its scramble control. The glutamatergic neurons in the cerebral brain of these mice were genetically labeled by the yellow fluorescent protein (YFP), hereafter referred as C57 mice in our paper. CD-1 (ICR) mice were used as resident mice. These mice were accommodated in specific pathogen-free facilities (SPF) with the circadian about twelve hours for night and day plus the self-help feeding. Well-developed C57 mice in postnatal 21 days were selected to experience the resident/intruder paradigm (also named as intruder) or control. Male CD-1 mice with the stronger aggressive above three months old were chosen as residents.

Behavioral study

The C57 mice were taken into the laboratory to accommodate experiment operators and the training apparatus for two days and were randomly assigned into control group and intruder group. In the resident/intruder paradigm [85–89], an intruder C57 mouse was placed in a cage of male CD-1 mouse, where this male CD-1 mouse has been housed with a female CD-1 mouse above three days. When placing an intruder C57 mouse into this cage and taking female CD-1 mouse out, this male resident CD-1 mouse attacked and bit intruder C57 mouse at least 10 times or 5 min per day for 12 days (Fig. 1A, lower left). Subsequently, C57 mice were examined by the social interaction (SI) test to identify their fear memory to CD-1 mouse and by elevated-plus maze (EPM) test to evaluate the anxious state. The social interaction test was performed in an open field box (50*50 cm) that included a transparent and perforated box (10*10 cm). One CD1 mouse or a tiny audio recorder that broadcasted the battle sound were placed in this small box about five minutes to examine the avoidance behavior of C57 mice as the indicator of fear memory to CD-1 mouse or the battle sound. The area within 5 cm around the small box was defined as an interaction zone (Fig. 1A, lower median). The stay duration of C57 mice in the interaction zone was named as the interaction time in this test. The EPM consisted of two open arms (30 × 5 cm) opposite to two closed arms (30 × 5 × 15.25 cm). The arms extended from a central platform (5 × 5 cm). The EPM was located 40 cm above the floor (Fig. 1A, lower right). The data of behavior test about the time on open arms and interaction time of C57 mice

were analyzed by Matlab. The detailed method was described in our previous studies [4, 11, 93, 94].

Retrograde and anterograde neural tracing

To trace the formation and change of neuronal circuits in intruder C57 mice induced by the social stress with the resident/intruder paradigm, we used adeno-associated viruses (AAV purchased from OBIO Inc, Shanghai, China) for neural tracing. The AAV2/retro-CMV-mCherry was applied for a retrograde tracing. The AAV2/8-CMV-tdTomato and AAV2/8-CMV-EBFP were used as an anterograde tracing. The locations for the microinjection of AAVs and the confocal view of neural images were based on the brain map for mouse [95].

In tracing the interconnections between the auditory cortex and the primary somatosensory cortex for a trunk area (S1-Tr cortex), 0.2 μ L AAV2/retro-CMV-mCherry was microinjected into the auditory cortex (2.30 mm posterior to the bregma, 4.00 mm lateral to the middle line and 0.65 mm depth away from the cortical surface) or the S1-Tr cortex (1.50 mm posterior to the bregma, 1.50 mm lateral to the middle line and 0.50 mm depth away from the cortical surface) in the mice from groups of intruders before a resident/intruder paradigm and control three weeks before the neural tracing. The injection of AAVs into cerebral cortices was conducted by using a glass pipette. The microinjection quantity and duration were controlled by a microsyringe system held with the three-dimensional stereotaxic apparatus (RWD Life science, Shenzhen, China). The glass pipettes were reserved no less than 15 min before withdrawing from the mouse brain. In principle, AAV2/retro-CMV-mCherry injected into the auditory cortex or the S1-Tr cortex was taken up by axon terminals and boutons of these cortical neurons, and was subsequently transported toward their somata to express red fluorescent protein, so that the somata of neurons as the source area and their projected axons as the target areas (or AAV injection area) were traced in a retrograde manner. It is noteworthy that the transfection efficiency of AAVs in the S1-Tr cortex is presented in Figure S4. The averaged percentage of the AAV transfected neurons is similar to the averaged percentage of cFos-labelled neurons (Fig. 2K), indicating AAV transfection to active neurons.

In the study of the convergent synapse innervations on auditory cortical neurons from the S1-Tr cortex and the medial geniculate body (MG), 0.2 μ L AAV2/8-CMV-tdTomato was injected into the S1-Tr cortex (1.5 mm posterior to the bregma, 1.5 mm lateral to the middle line and 0.5 mm depth below the cortical surface), and 0.2 μ L AAV2/8-CMV-EBFP was injected into the MG (3.2 mm posterior to the bregma, 1.9 mm lateral to the middle line and 2.7 mm depth away from the cortical surface). CMV-coded AAVs transfected nerve cells in the injected areas and expressed their carried-genes and fluorescent proteins. Subsequently, these fluorescent proteins produced in neuronal somata were transported over entire axons in an anterograde manner, such that their axonal boutons and terminals labelled by these fluorescent proteins were detected in the target areas. The contacts by these axon boutons and terminals on dendritic spines of auditory cortical neurons was deemed as synapse contacts. The raised contacts in the experiments included newly formed synapse contacts. The auditory cortical neurons with convergent synapses newly from the S1-Tr cortex and innately from the medial geniculate body were presumably associative memory neurons [79, 81, 84, 91].

The resident/intruder paradigm was conducted three days after the surgical operation and microinjections in order to allow C57 mice recovery from the operation. In the next two weeks, fluorescent proteins were transported to entire axon boutons and terminals along with the stress-induced axon prolongation. At last, the mice were anesthetized by the intraperitoneal injections of urethane (1.5 g/kg) and perfused through the left ventricle with 25 ml 0.01 M phosphate buffer solution (PBS) followed by 25 ml of 4% paraformaldehyde until their bodies were rigid. The brains were quickly isolated and soaked in 4% paraformaldehyde for the fixation no less in 24 h. The cerebral brains were sliced by a vibratome in a series of coronal sections with a thickness of 100 μ m in PBS. These slices were air-dried and cover-slipped with 50% glycerin in PBS. The images of neurons, dendrites, spines and axonal boutons were taken and collected at a 60X lens for high magnification in a confocal microscope (Nikon A1R plus). The anatomic images of the cerebral brain were taken by a 4X lens for a low magnification under this confocal microscope.

In C57BL/6JThy1-YFP mice, postsynaptic neuron dendrites and spines were labelled by the YFP. The presynaptic axon boutons whose somata were infected AAV were labelled by either RFP (mCherry or tdTomato) or BFP. The wavelength of an excitation laser-beam 561 nm was used to activate RFP. The wavelength of an excitation laser-beam 405 nm was used to activate the BFP. The wavelengths of the emission spectra of the BFP, YFP and RFP are 412–482 nm, 522–552 nm and 572–652 nm, respectively. The contacts between yellow dendritic spines and

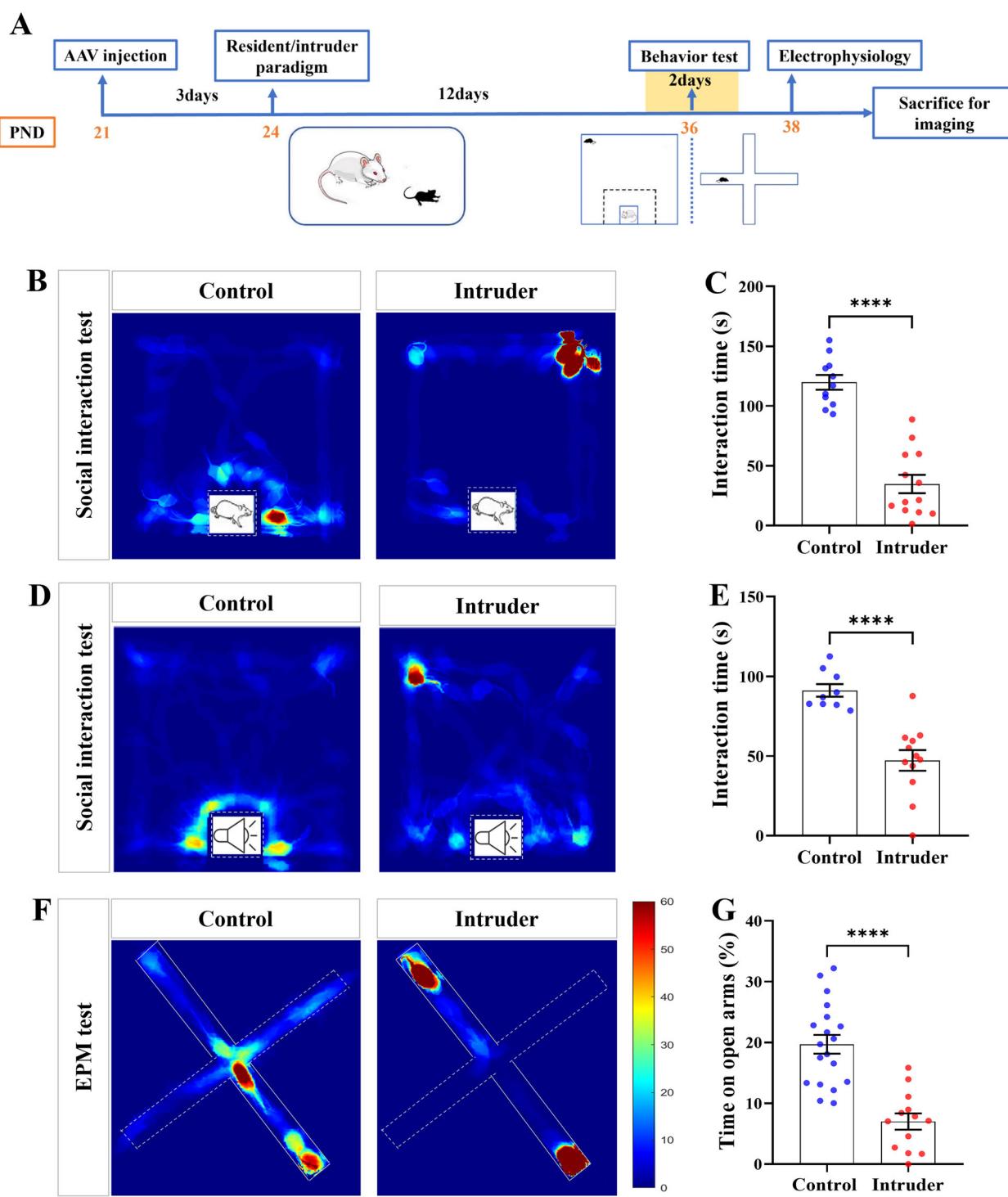


Fig. 1 The social stress induces fear memory and anxiety-like behaviors. A Schematic diagram of experiment timeline. A resident/intruder paradigm (lower left under the timeline) as the social stress protocol was applied to C57 mice aged 24 days. The social interaction test (lower median) was used to evaluate fear memory retrievals by CD-1 mice and battle sound, respectively. The elevated plus maze test (lower right) was used to evaluate the anxiety-like behaviors of C57 mice. **B** The hot-spot maps of C57 mice in response to CD-1 resident mouse in an interaction cage. **C** The interaction time of C57 mice in intruder group is 34.80 ± 7.69 s, and the interaction time of C57 mice in control group is 119.7 ± 6.14 s. C57 mice in intruder group show less interaction time with this CD-1 mouse in open field (control, $n = 11$; intruder, $n = 13$; ****, $P < 0.0001$; one-way ANOVA). **D** The hot-spot maps of C57 mice in response to the battle sound in an interaction cage. **E** The interaction time of C57 mice in intruder group is 47.22 ± 6.51 s, and the interaction time of C57 mice in control group is 91.23 ± 3.95 s. C57 mice in intruder group show less interaction time with the battle sound in an interaction cage (control, $n = 9$; intruder, $n = 12$; ****, $P < 0.0001$; one-way ANOVA). **F** The hot-spot maps of C57 mice on an elevated plus maze. **G** The time on open arms of C57 mice in control group is $19.7 \pm 1.55\%$. C57 mice in intruder group show less duration of staying open arms (control, $n = 19$; intruder, $n = 13$; ****, $P < 0.0001$; one-way ANOVA). Error bars indicate SEM.

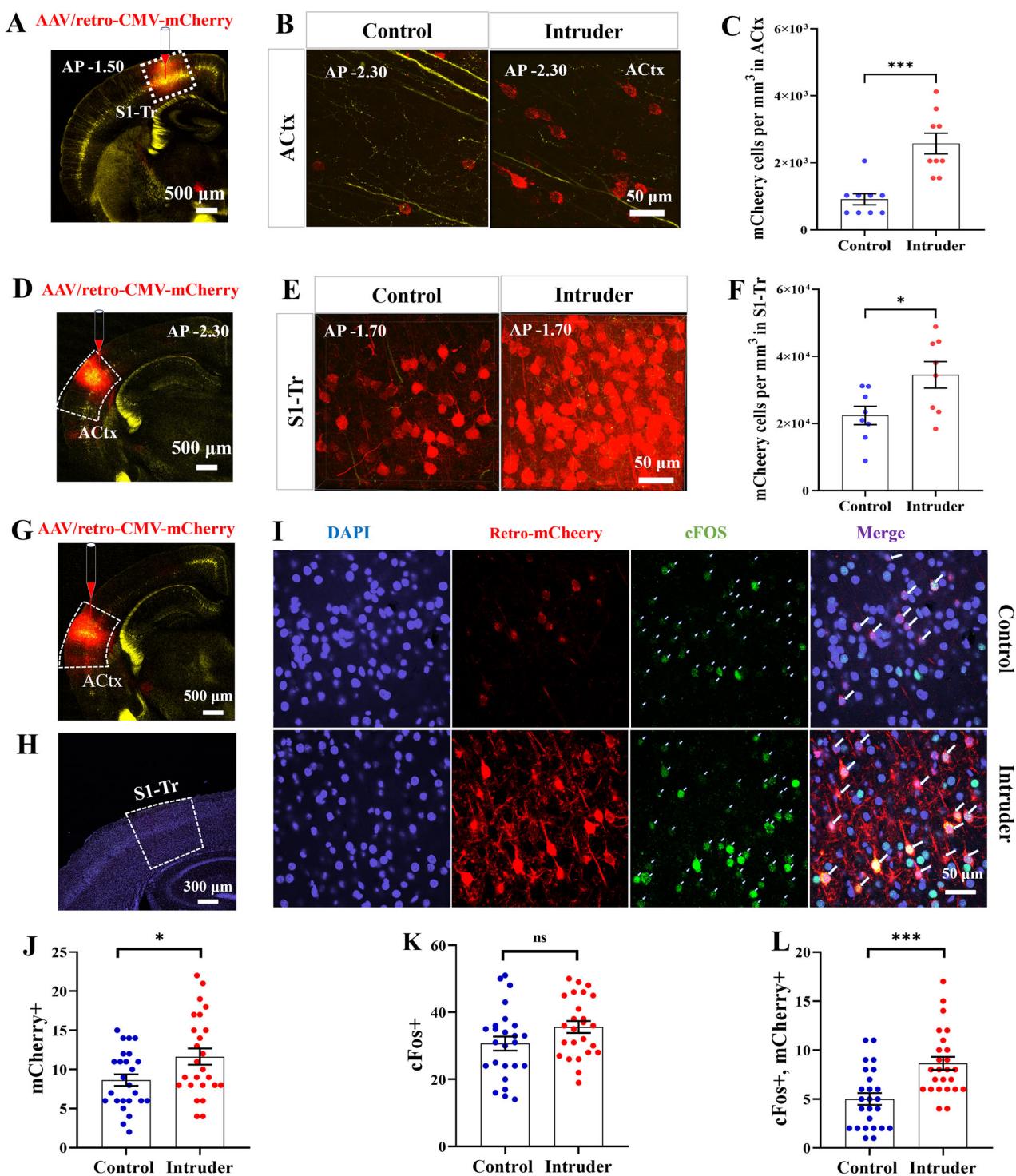


Fig. 2 The social stress induces the mutual innervation between auditory cortex (ACtx) and trunk area of somatosensory cortex (S1-Tr). **A** Retro-AAVs-mCherry are microinjected in the S1-Tr cortex. **B** The retrogradely traced neurons in the auditory cortex (post bregma 2.30 mm, scale bar, 50 μm) in samples of control mouse (left panel) and intruder mouse (right panel). **C** Statistical analyses show mCherry-labeled cells per mm³ in the auditory cortices from control mice (blue symbols) and intruder mice (red symbols; ***P < 0.001, one-way ANOVA). **D** Retro-AAVs-mCherry are microinjected in the auditory cortex. **E** The retrogradely traced neurons in the S1-Tr cortex (post bregma 1.70 mm, scale bar, 50 μm) in samples of control mouse (left panel) and intruder mouse (right panel). **F** Statistical analyses show mCherry-labeled cells per mm³ in the S1-Tr cortices from control mice (blue symbols) and intruder mice (red symbols; *P < 0.05, one-way ANOVA). **G** An image shows the injection site of retro-AAVs-mCherry in the auditory cortex. **H** An immunofluorescent staining image with low power, in which the white frame shows the S1-Tr cortex. **I** Images with high power in the S1-Tr cortices from control mice (top panels) and intruder mice (bottom panels). The images show DAPI-labeled neurons, mCherry-labeled neurons, cFos-labeled neurons and their merges, respectively, in left-to-right panels. **J** mCherry-labeled neurons in the S1-Tr cortex are significantly higher in intruder mice (red symbols) than in control mice (blue symbols; *P < 0.05, one-way ANOVA). **K** cFos-labeled neurons in the S1-Tr cortex in control mice (blue symbols) and intruder mice (red symbols). **L** The neurons labeled by both mCherry and cFos in the S1-Tr cortex are higher in intruder mice (red symbols) than control mice (blue symbols; ***P < 0.001, one-way ANOVA). Error bars indicate SEM.

red or blue axon boutons with less than 0.1 μm space cleft were presumed as chemical synapses. The images of dendritic spines, axon boutons and synapse contacts were analyzed quantitatively by ImageJ and Imaris [79, 81, 84, 91]. Associative memory neurons were accepted by detecting at least two sources of boutons onto the dendritic spines of YFP-labelled auditory cortical neurons.

Immunofluorescence

Mice were anesthetized by the intraperitoneal injections of urethane (1.5 g/kg) and perfused through the left ventricle with 25 ml 0.1 M phosphate buffer solution (PBS) followed by 25 ml of 4% paraformaldehyde until their bodies were rigid. Brains were postfixed with 4% paraformaldehyde for 20 min and then kept in PBS. The cerebral brains were sliced by a vibratome in a series of coronal slices with the thickness of 30 μm in PBS. These slices were washed in PBS, blocked by a buffer solution containing 5% goat serum for 1 h, incubated with the buffer solution that contained the primary antibody in 1% bull serum albumin (free for cFos staining) and 0.3% Triton X-100 at 4 °C for overnight. After washed three times in PBS, these slices were incubated with the secondary antibody for two hours and then, if necessary, incubated with 4',6-diamidino-2-phenylindole for 20 min at room temperature. The secondary antibody for the staining was Alexa Fluor 647 goat anti-rabbit immunoglobulin G (4414S, CST). The slices were lastly washed three times, mounted on microscope slides, air-dried and cover-slipped with 50% glycerin in PBS. Images were captured under a confocal laser-scanning microscope (Nikon A1R plus) and colocalization analysis and merged images were processed according to our previous work [79, 81, 84, 91].

Electrophysiology

Before the electrophysiological recording of auditory cortical neurons *in vivo*, the mice in control or intruder were anesthetized by intraperitoneal injections of urethane (1.5 g/kg) for surgical operations after training paradigms had been done. The body temperature was kept at 37 °C by a computer-controlled heating blanket. The craniotomy (1 mm in diameter) was done on the mouse skull above the left side of the auditory cortex (~2.40 mm posterior to the bregma and 4.00 mm lateral to the midline). The location for electrophysiological recordings was based on the brain mapping for mouse [95]. Electrophysiological recordings to auditory cortical neurons *in vivo* were conducted in the mice under a light anesthetic condition with a withdrawal reflex by pinching, the eyelid blinking reflex by the air-puffing and the muscle relax. The electrical discharges of unitary cortical neurons were recorded in layers IV-V of the auditory cortex by using glass pipettes filled with the standard solution (150 mM NaCl, 3.5 mM KCl and 5 mM HEPES). The resistance of those recording pipettes was 40–50 MΩ. The electrical signals of auditory cortical neurons in their spontaneous spikes and evoked-spikes by the battle sounds or the stimulus to injury areas were recorded and acquired by AxoClamp-2B amplifier and Digidata 1322 A, and were analyzed by pClamp 10 system (Axon Instrument Inc. CA, USA). Spike signals were digitized at 20 kHz and filtered by low-pass at 5 kHz. A 100–3000 Hz band-pass filter and a second-order Savitzky-Golay filter were used to isolate the spike signal. Spiking frequencies were quantitatively analyzed [79, 81, 84, 91].

Normalized spike frequency in response to either one of stimuli was the number of the spike frequency in response to the stimulus in 20 s divided by spontaneous discharge frequency in 20 s before the stimulation. When the ratio reached 1.5 or above, the auditory cortical neurons were deemed to be response to this stimulus. Associative memory neurons (AMN) were accepted by detecting a situation that auditory cortical neurons responded to both stimulations. The identification of associative memory neurons versus those neurons in response to a stimulus is presented in Figure S5. The spectra of spike frequencies in these neurons are presented as the Z-score in Figure S5.

Neuroligin-3 knockdown by shRNA carried by AAV

In the study of the role of neuroligin-3 in the formation of new synapse innervations, one of the proteins for the synapse linkage [84, 96–101], the approach of its mRNA knockdown was used by the short-hairpin RNA (shRNA) specific for neuroligin-3 mRNA which was carried by AAV (AAV2/8-CMV-EGFP-shNlg3), in which the AAV was injected into the auditory cortex. The piece of scramble sequence carried by AAV (AAV2/8-CMV-EGFP-scramble) as a control was injected in the auditory cortex too. The microinjections were operated three days before the resident/intruder paradigm. Theoretically, this approach suppresses the expression level of neuroligin-3 in auditory cortical neurons, the formation of new synapse innervations from S1-Tr neurons and the recruitment of associative memory neurons in the auditory cortex.

Similarly, the behavior tests, AAV-mediated neural tracing and electrophysiological recording were done to evaluate the effectiveness of

neuroligin-3 knockdown on the fear memory, synapse formation and associative memory neuron recruitment in the auditory cortex. Specifically, the encoding capability of neurons in response to the battle sound and the somatic pain signals were analyzed and compared in two subgroups. The effectiveness of shRNA specific for a neuroligin-3 inhibition on new synapse formation and associative memory cell recruitment was confirmed if the number of new synapse contacts and associative memory neurons in the mice of neuroligin-3 knockdown was lowered significantly in comparison with the subgroup of scramble control mice.

Statistics

All data are presented as arithmetic mean \pm SEM. The statistical analyses of all our data were conducted by using GraphPad Prism 9. One-way ANOVA was used for the statistical comparisons of the changes in behavioral and morphology study between the groups of control and intruder as well as between the neuroligin-3 knockdown subgroup and scramble subgroup in the intruder group. A Chi-test was used for the statistic comparison of changes in the percentage of recruited associative memory neurons in the electrophysiological study among these groups. *P* values equally and above 0.05 in the comparisons among the groups were set to be no statistical differences, or vice versa. The one asterisk, two asterisks, three asterisks and four asterisks were presented to be *P* < 0.05, 0.01, 0.001 and 0.0001 respectively.

RESULTS

In this section, we present our studies about the essential role of associative memory cells at the auditory cortex in stress-induced fear memory and anxiety. In the resident/intruder paradigm for the social stress, the fear memory and anxiety-like behaviors in intruder C57 mice was induced by attacks from a resident CD-1 mouse. In this resident/intruder paradigm, the stressful signals included the pain signal due to their trunk injury bitten by resident CD-1 mouse and the battle sound. The neural tracing was applied to examine the mutual synapse innervations between the auditory cortex and the S1-Tr cortex induced by this social stress. The electrophysiological recording and neural tracing were jointly utilized to identify the recruitment of associative memory neurons in the auditory cortex. Associative memory neurons were surely recruited when the auditory cortical neurons became to encode these stressful signals during the electrophysiological recordings *in vivo* and when the convergent synapse innervations were made onto auditory cortical neurons by the axon boutons of S1-Tr cortical neurons and medial geniculate neurons. The shRNA specific for the neuroligin-3 knockdown was used to test the essential roles of neuroligin-3 in the formation of new synapses, the recruitment of associative memory neurons and the emergence of fear memory and anxiety-like behavior induced by this social stress.

In the resident/intruder paradigm, one of intruder C57 mice was placed into the home cage of a resident CD1 mouse. The resident mouse recognized this intruder mouse as a stranger and attacked it. Intruder mice experienced this stress situation once a day and twelve days in total. Control C57 mice in this period were treated without the exposure to the resident CD1 mouse. Subsequently, the social interaction (SI) test and elevated-plus maze (EPM) test were conducted in such two groups of mice to evaluate the emergence of fear memory specific to this resident mouse and of anxiety-like behaviors (Fig. 1A).

In the social interaction test, intruder mice appear to stay away from this resident mouse placed in a small box of the interaction cage (right panel in Fig. 1B), compared to control mice (left panel). The stay durations in the interaction zone (interaction time) in response to this resident CD1 mouse are 34.8 ± 7.69 s in intruder mice (red symbols in Fig. 1C, $n = 13$) and 119.7 ± 6.14 s in control mice (blue symbols; $n = 11$, $p < 0.0001$, ANOVA). These intruder mice also appear to stay away from the battle sound broadcasted by an audio recorder in the small box of the interaction cage (right panel in Fig. 1D), compared with control mice (left panel). The interaction time in response to the battle sound is 47.22 ± 6.51 s in intruder mice (red symbols in Fig. 1E, $n = 12$) and 91.23 ± 3.95 s in control mice (blue symbols; $n = 9$, $p < 0.0001$, ANOVA). This result

indicates that the social stress to intruder mice induces their fear memory specific to the resident mouse and the battle sound. Furthermore, in the elevated-plus maze test, intruder mice appear to avoid the open fields since they prefer to stay in the closed arms (right panel in Fig. 1F), compared to control mice (left panel). The percentages of the stay time in open arms are $7.01 \pm 1.34\%$ in intruder mice (red symbols in Fig. 1G, $n = 13$) and $19.7 \pm 1.55\%$ in control mice (blue symbols, $n = 19$, $p < 0.0001$, ANOVA). That is, intruder mice become more anxious after experienced the resident/intruder paradigm. The data above indicate that the social stress leads to fear memory and anxiety-like behaviors.

In terms of the cellular mechanism underlying the fear memory to associative stressful signals in a resident/intruder paradigm, we assumed that the battle sound activated the auditory cortex by the auditory system and the pain signal in their injury trunk areas activated the somatosensory cortex by the somatic system simultaneously. Based on the principle of coactivity together and interconnection together that recruits associative memory neurons [13, 83, 84], we further assumed that such stressful signals included in the resident/intruder paradigm to induce fear memory and anxiety instigated the new synapse interconnections between auditory and S1-Tr cortices as well as recruited the auditory cortical neurons to be the associative memory neurons that encode the fear memory to stressful signals and the anxiety-like behaviors.

Mutual innervations between S1-Tr and auditory cortical neurons are associated with fear memory

The formation of interconnections between auditory and S1-Tr cortices was examined by neural tracing, in which adeno-associated viruses that carried the genes encoding fluorescent proteins were used. In the retrograde neural tracing, 0.2 μ l AAV/retro-CMV-mCherry was microinjected in the trunk area of somatosensory cortex (S1-Tr cortex) and detected in the auditory cortex (Fig. 2A), or the other way around (Fig. 2D). Three days after injections, C57 mice experienced a resident/intruder paradigm or control for twelve days. In microinjections to the S1-Tr cortex, auditory cortices from two groups were scanned under a confocal microscope. mCherry-labelled neurons in the auditory cortex appear higher in intruder mice with the fear memory (right panel in Fig. 2B) than control mice (left panel). mCherry-labelled neurons per mm^3 in the auditory cortex are 2577 ± 309.7 in an intruder group (red symbols in Fig. 2C, $n = 9$ cubes from 9 mice) and 916.2 ± 166.9 in the control group (blue symbols, $n = 9$ cubes from 9 mice, $p < 0.001$, ANOVA). Moreover, in microinjections into the auditory cortex, S1-Tr cortices from two groups were scanned under confocal microscope. mCherry-labelled neurons in the S1-Tr cortex appear higher in intruder mice with fear memories (right panel in Fig. 2E) than control mice (left panel). mCherry-labelled neurons per mm^3 in the S1-Tr cortex are $34,524 \pm 3965$ in intruder group (red symbols in Fig. 2F, $n = 8$ cubes from 8 mice) and $22,402 \pm 2431$ in the control group (blue symbols, $n = 8$ cubes from 8 mice, $p < 0.001$, ANOVA). These results indicate that the interconnections are formed and increased between auditory and S1-Tr cortices in intruder mice with stress-induced fear memory and anxiety. These results also imply that auditory cortical neurons receive new synapse innervations from the S1-Tr cortex alongside synapse inputs from the medial geniculate body as well as encode the battle sound and trunk pain signals, or their recruitment to be associative memory cells, which are presented below.

In order to examine these interconnected neurons being functionally active, we have conducted the experiment of immunolabelling them with proteins coded by activity-dependent genes, e.g., cFos. Three days after microinjections of AAV-mCherry into the auditory cortex (Fig. 2G), these C57 mice experienced a resident/intruder paradigm or control for twelve days. The mCherry- and cFos-labelled S1-Tr cortical neurons appear higher in intruder mice with fear memories (bottom panels in Fig. 2I) than control mice (top panels). mCherry-labelled neurons per mm^2 are 11.64 ± 1.04 in intruder group (red symbols in Fig. 2J, $n = 25$ fields from 3 mice) and

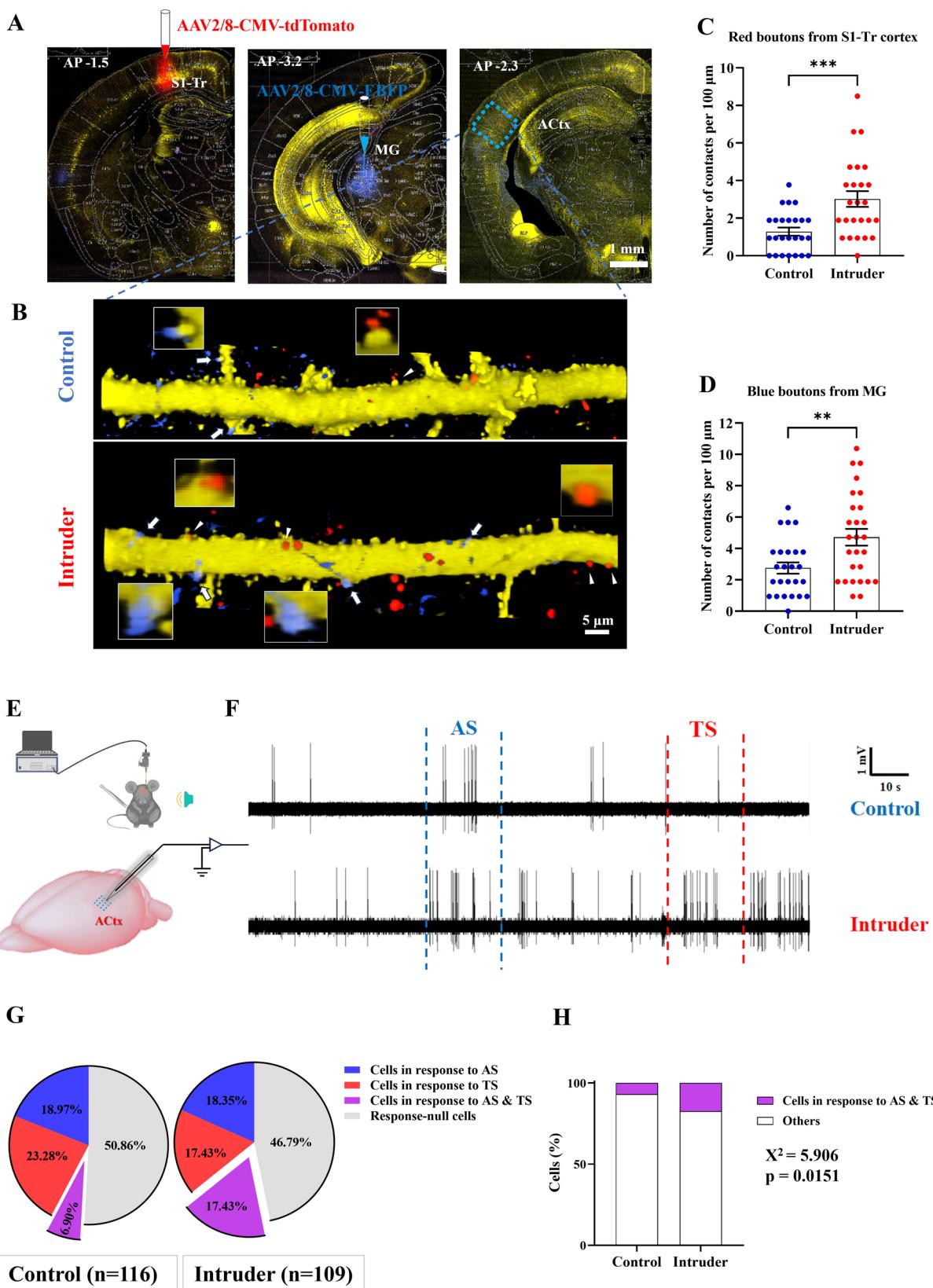
8.64 ± 0.74 in the control group (blue symbols, $n = 25$ fields from 3 mice, $p < 0.05$, ANOVA). mCherry/cFos-labelled neurons per mm^2 are 8.64 ± 0.67 in intruder group (red symbols in Fig. 2L, $n = 25$ fields from 3 mice) and 5.0 ± 0.61 in the control group (blue symbols, $n = 25$ fields from 3 mice, $p < 0.001$, ANOVA). These results imply that the interconnected neurons between S1-Tr and auditory cortices are functionally active, i.e., a functional interconnection. It is noteworthy that those mCherry-labelled neurons and c-Fos labelled neurons are not fully overlap (Figure S1), or they are not identical in nature.

Associative memory neurons are recruited in the auditory cortex to encode stressful signals

The recruitment of associative memory neurons in the auditory cortex has been morphologically examined by the anterograde neural tracing in that AAV-carried fluorescent genes were microinjected in the medial geniculate body and the S1-Tr cortex in intruder and control mice. As showed in Fig. 3A, 0.2 μ l AAV2/8-CMV-tdTomato was injected in the S1-Tr cortex and 0.2 μ l AAV2/8-CMV-EBFP was injected in the medial geniculate body. After the resident/intruder paradigm and control periods went through, auditory cortical neurons were examined in their convergent synapse innervations from the S1-Tr cortex and the medial geniculate body.

Convergent synapse innervations onto the dendritic spines of auditory cortical neurons appear higher in intruder mice (bottom panel in Fig. 3B) than control mice (top panel), where white arrows point synapse contacts made by presynaptic boutons and postsynaptic spines. Synapse contacts per 100 μm dendrites on auditory cortical neurons made by axonal boutons of S1-Tr cortical neurons are 3.02 ± 0.42 in intruder group (red symbols in Fig. 3C, $n = 25$ slices from 3 mice) and 1.28 ± 0.22 in control group (blue symbols in Fig. 3C, $n = 25$ slices from 3 mice, $p < 0.001$, ANOVA). Synapse contacts per 100 μm dendrites on auditory cortical neurons made by axonal boutons of neurons in the medial geniculate body are 4.94 ± 0.56 in intruder group (Fig. 3D red symbols, $n = 25$ slices from 3 mice) and 2.76 ± 0.36 in control group (blue symbols in Fig. 3D, $n = 25$ slices from 3 mice, $p < 0.01$, ANOVA). The results indicate that auditory cortical neurons in intruder mice with fear memories specific to resident mice receive more convergent synapse innervations from the S1-Tr cortex and the medial geniculate body. It is noteworthy that the rise of axonal buttons in the auditory cortex projected from the S1-Tr cortex in intruder mice with the fear memory and anxiety (Figure S2) supports this result. In addition, these axons are colocalized with type-I of glutamate transporter (Figure S3), indicating that the axons are glutamatergic. Therefore, associative memory neurons are substantially recruited in the auditory cortex during the social stress.

Whether these auditory cortical neurons are able to encode stressful signals including the battle sound and the painful signal from injury trunk regions was examined by electrophysiology *in vivo*. The experiment was conducted by recording the responses of auditory cortical neurons to the pain signal that was presumably inputted through the newly established pathway from the S1-Tr cortex to the auditory cortex. The spike frequency was used as an index of the strength of neuronal activity. When the auditory cortical neurons responded to both somatosensory signal and auditory signal, they were presumably associative memory neurons, similar to previous studies [13, 79, 83, 84, 92]. The unitary discharges of auditory cortical neurons were electrophysiologically recorded *in vivo* (Fig. 3E). The battle sound and the somatic pain stimulus (tweezers to injury trunk areas that mimicked the bite of resident mouse) were sequentially given to intruder mice with fear memories and/or control mice. The evoked spikes on the background of spontaneous spikes were recorded and analyzed. The normalized spike frequencies in response to one of stimuli were calculated by the ratio of the frequency of stimulus-induced spikes to the frequency of spontaneous spikes in twenty seconds before the



stimulation. If the ratio reached 1.5 or above, the auditory cortical neurons was deemed as the responses to this stimulation.

Auditory cortical neurons appear to respond to both painful and battle sound signals in intruder mice with fear memory (bottom

trace in Fig. 3F), but not control mice (top panel). The percentages of associative memory neurons in total recorded neurons were 17.43% in intruder group (right panel in Fig. 3G, $n = 18/109$ from 9 mice) and 6.90% in control group (left panel, $n = 7/116$ from

Fig. 3 Auditory cortical neurons in intruder mice with fear memory become to encode the battle sound and the pain signals. **A** Low power images show the injection of AAVs-tdTomato into the S1-Tr cortex (left panel), the injection of AAVs-eGFP in medial geniculate body (middle panel) and the neural tracing at the auditory cortex (white frame). **B** Images show synaptic connections made on dendritic spines of auditory cortical glutamatergic neurons by axonal boutons from the medial geniculate body (white arrow to blue boutons) and from the S1-Tr cortex (white triangle to red boutons) in samples of control mouse (top panel) and intruder mouse (bottom panel). Boxes show enlarged synapse contacts pointed by arrows. **C** Synapse contacts made by red axon boutons from the S1-Tr cortex are higher in intruder mice (red symbols) than in control mice (blue symbols, $***, P < 0.001$, one-way ANOVA). **D** Synapse contacts made by blue axon boutons from the medial geniculate body are higher in intruder mice (red symbols) than in control mice (blue symbols, $**, P < 0.01$, one-way ANOVA). **E** Schematic diagram shows *in vivo* electrophysiological recordings. The auditory stimulus (AS) by the battle sound and the tactile stimulation (TS) to trunk injury-areas were given to mice. The auditory cortices (ACtx) were recorded by the electrode. **F** Illustrates the examples about the responses of single neurons to the battle sound and the pain stimulus recorded from a control mouse (top panel) and intruder mouse (bottom panel), respectively. The auditory cortical neuron in an intruder mouse responds to the battle sound and the tactile stimulus, i.e., associative memory neuron. **G** Pie charts show the distributions of neurons with different response patterns recorded in the auditory cortices of control mice (left panel, $n = 116$) and intruder mice (right panel, $n = 109$), respectively. **H** The portions of associative memory neurons in response to both stimuli in the intruder group and control group are 17.43% ($n = 18/109$) and 6.9% ($n = 8/116$), respectively. A chi-square test yields $\chi^2 = 5.91$ ($P = 0.0151$).

11 mice). The statistical analysis with Chi-test shows $\chi^2 = 5.91$ ($p < 0.05$, Fig. 3H). The result indicates that some auditory cortical neurons are recruited to encode the battle sound and somatic pain signals for their integrative storage in stress-induced fear memory. Both morphological and electrophysiological data verify the stress-induced recruitment of associative memory neurons in the auditory cortex.

Neuroligin-3 is required for fear memory, anxiety and associative memory cell recruitment

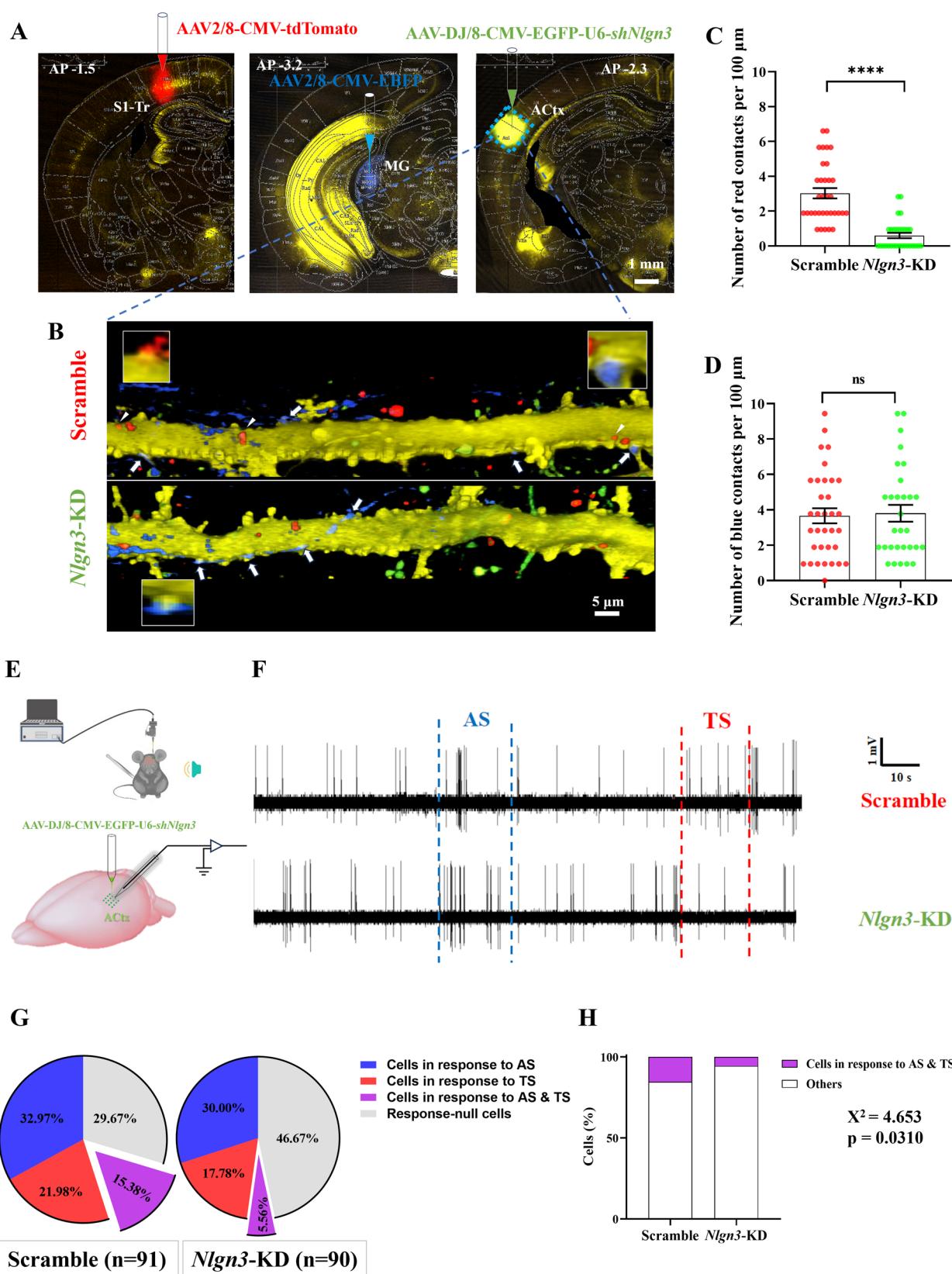
To the emergence of fear memory and the recruitment of associative memory neurons in social stress, new synapses arise in the auditory cortex. The formation of new synapses requires the linkage proteins between presynaptic and postsynaptic membranes, such as neuroligin-3 and neurexin [96, 98–101]. We hypothesized that this neuroligin-3 played the essential role in the new synapse formation for the stress-induced emergence of fear memory/anxiety and recruitment of associative memory neurons in the auditory cortex. This hypothesis was examined by a *neuroligin-3* knockdown in the auditory cortex to observe whether the *neuroligin-3* downregulation precludes the emergence of fear memory and anxiety, the formation of new synapses and the recruitment of associative memory cells in response to the social stress. AAV-carried shRNA specific to *neuroligin-3* mRNA [102–106] was injected into the auditory cortex of intruder mice. The shRNA-scramble control was injected in another group of intruder mice. In terms of time line for experiments, pAAV[shRNA]-GFP-U6-mNlgn3 was injected into the auditory cortex (the right panel in Fig. 4A) three days before resident/intruder paradigms. This shRNA ensured to lower neuroligin-3 expression expectedly prevented the stress-induced formation of synapse connection from the S1-Tr cortex to the auditory cortex and the recruitment of associative memory neurons in the auditory cortex. The effectiveness of this pAAV[shRNA]-GFP-U6-mNlgn3 on neuroligin-3 knockdown has been validated and presented in the supporting datum of our previous publication [107]. The experiments in *neuroligin-3* knockdown were conducted with the behavior tasks, AAV-mediated neural tracing and electrophysiology *in vivo* applied in Figs. 1–3. The essential roles of *neuroligin-3* in the emergence of fear memory/anxiety, the formation of new synapse innervations and the recruitment of associative memory neurons would be ensured if these processes in the group of intruder plus *neuroligin-3* knockdown mice were downregulated.

In the meantime of pAAV[shRNA]-GFP-U6-mNlgn3 injections in the auditory cortex, AAV-EBFP and AAV-tdTomato were injected into the medial geniculate body and the S1-Tr cortex, respectively (Fig. 4A). The synapse contacts between the dendritic spines on auditory cortical neurons (yellow) and the axonal boutons of S1-Tr cortical neurons (red) appear lower in intruder plus neuroligin-3 knockdown mice (bottom panel in Fig. 4B) than in intruder plus scramble control mice (top panel).

Synapse contacts per 100 μm dendrites are 0.60 ± 0.15 in intruder plus neuroligin-3 knockdown group (green symbols in Fig. 4C, $n = 30$ dendrites from 3 mice) and 3.02 ± 0.30 in intruder plus scramble control group (red symbols, $n = 34$ dendrites from 3 mice, $p < 0.0001$, ANOVA). Moreover, the synapse contacts formed by the dendrite spines of auditory cortical neurons (yellow) and the axon boutons of neurons in the medial geniculate body (blue) appear no difference in these two subgroups. Synapse contacts per 100 μm dendrites are 3.81 ± 0.48 in intruder plus neuroligin-3 knockdown group (green symbols in Fig. 4D, $n = 30$ dendrites from 3 mice) and 3.66 ± 0.42 in intruder plus scramble control group (red symbols, $n = 34$ dendrites from 3 mice). This result indicates that neuroligin-3 knockdown in the auditory cortex downregulates the stress-induced formation of new synapse innervation from the S1-Tr cortex, but not synaptic inputs from the medial geniculate body. That is, neuroligin-3 is required for the formation of new synapse innervations and the recruitment of associative memory neurons in the auditory cortex induced by the social stress.

The electrophysiological recording *in vivo* was conducted in the auditory cortices of the intruder plus neuroligin-3 knockdown and intruder plus scrambler control mice (Fig. 4E). The examples in Fig. 4F illustrate the recording of auditory cortical neurons in response to the battle sound and the somatic stimuli in the injury trunk area from an intruder plus scramble control mouse (top trace) and an intruder plus neuroligin-3 knockdown mouse (bottom trace). The percentages of auditory cortical neurons in response to both battle sound and somatic stimulus, i.e., associative memory cells, were 15.38% in intruder plus scramble control subgroup ($n = 15/91$ from 8 mice) and 5.56% in intruder plus neuroligin-3 knockdown subgroup ($n = 5/90$ from 8 mice, Fig. 4G). The statistical analysis by Chi-test shows $\chi^2 = 4.65$ ($p < 0.05$, Fig. 4H). The neuroligin-3 knockdown prevents the responses of auditory cortical neurons to the stressful signals including the battle sound and the somatic stimulus to injury trunk area. That is, neuroligin-3 is required for the stress-induced recruitment of associative memory neurons in the auditory cortex.

In the experiment of behavioral tasks, the interaction time in response to the resident mouse is 43.64 ± 8.29 s in those intruder plus scramble control mice (red symbols in Fig. 5C, $n = 17$) and 35.36 ± 7.39 s in intruder plus neuroligin-3 knockdown mice (green symbols, $n = 18$). In addition, the interaction time in response to the battle sound appears lower in intruder plus scramble control mice (left panel in Fig. 5D) than in the intruder plus neuroligin-3 knockdown mice (right panel). The interaction time in response to the battle sound is 110.1 ± 16.99 s in intruder plus neuroligin-3 knockdown mice (green symbols in Fig. 5E, $n = 10$) and 62.81 ± 6.56 s in intruder plus scramble control mice (blue symbols, $n = 10$, $p < 0.05$, ANOVA). This result indicates that neuroligin-3 knockdown in the auditory cortices of intruder mice prevents the retrievals of fear memories to the battle sound, but not the appearance of resident CD1 mouse. In other words, the fear memory to stressful signals in the resident/intruder paradigm is mainly caused by the storage and



retrieval of the stressful signals in the auditory cortex, which supports our focus on studying the auditory cortex.

The influence of *neuroigin-3* knockdown on anxiety-like behavior in intruder mice was examined by using the elevated-plus maze

(EPM). The stay time on the plate of open arms in the EPM appears longer in those intruder plus *neuroigin-3* knockdown mice (right panel in Fig. 5F) than intruder plus scramble control mice (left panel). The ratios of the stay time on the plate of open arms to the total time

Fig. 4 **Neuroligin-3 knockdown in auditory cortical neurons precludes the formation of synapse innervation from S1-Tr cortex and medial geniculate body as well as the recruitment of associative memory neurons.** **A** Lower power images show the injections of AAV-CMV-tdTomato into the S1-Tr cortex (left panel), AAV-CMV- EGBP into the medial geniculate body (middle panel), and AAV-DJ/8-CMV-EGFP-U6-sh*Nlgn3* or AAV-DJ/8-CMV-EGFP-U6-scramble into the auditory cortex (right panel) in intruder mice, respectively. The neural tracing images are taken at the auditory cortex (blue frame). **B** Images show synaptic connections on dendritic spines of auditory cortical neurons made by axonal boutons from the medial geniculate body (white arrow to blue boutons) and from the S1-Tr cortex (white triangle to red boutons) in samples of scramble group mouse (top panel) and *Nlgn3*-KD group mouse (bottom panel). Boxes show enlarged synapse contacts pointed by arrows. **C** The number of red synaptic connections decreases in *Nlgn3*-KD group, in comparison with scramble group (****, $P < 0.0001$, one-way ANOVA). **D** The number of blue synaptic connections has no difference between two groups ($P > 0.5$, one-way ANOVA). Error bars denote SEM. **E** The schematic diagram of electrophysiological recordings in vivo. AAV-DJ/8-CMV-EGFP-U6-sh*Nlgn3* or AAV-DJ/8-CMV-EGFP-U6-scramble was injected into the auditory cortex (ACtx) of intruder C57 mice. After the resident-intruder paradigm, auditory cortical neurons in the intruder mice are electrophysiologically in vivo recorded in response to the battle sound stimulus (auditory signal, AS) and the tactile stimulus (TS). **F** Illustrates the examples about the responses of single neurons to the battle sound and the pain stimuli recorded from a mouse in scramble group (top panel) and a mouse in *Nlgn3*-KD group (bottom panel). Auditory cortical neuron in a mouse in the intruder plus scramble group responds to the battle sound and the tactile stimulus, i.e., associative memory neuron. **G** Pie charts on the left and right panels show the proportions of response types of auditory cortical neurons recorded in mice of scramble and *Nlgn3*-KD groups, respectively. **H** The proportions of associative memory neurons are 15.38% ($n = 15/91$) in the scramble mice and 5.56% ($n = 5/90$) in *Nlgn3*-KD mice. A chi-square test yielded $\chi^2 = 4.65$ ($P < 0.05$), indicating a significant decrease in the proportion of associative memory neurons in the *Nlgn3*-KD group compared to the scramble group.

in the EPM are $9.2 \pm 1.31\%$ in intruder plus neuroligin-3 knockdown mice (green symbols in Fig. 5G, $n = 14$) and $4.15 \pm 1.04\%$ in intruder plus scramble control mice (red symbols, $n = 17$, $p < 0.01$, ANOVA). Neuroligin-3 knockdown in the auditory cortex of intruder mice prevents their anxiety-like behaviors induced by the social stress based on resident/intruder paradigm. The data indicate that neuroligin-3 in the auditory cortex plays essential role in stress-induced fear memory and anxiety-like behaviors.

DISCUSSION

The social stress by the resident/intruder paradigm induces the fear memory and anxiety in intruder mice, in which the stressful signals include the pain from trunk injury area and the battle sound (Fig. 1). In the mice of expressing stress-induced fear memory and anxiety, the synapse interconnections between auditory and Sr-1Tr cortices are newly formed and functionally active (Fig. 2). The auditory cortical neurons receive new synapse innervations from the S1-Tr cortex alongside those synapse innervations from the medial geniculate body (Fig. 3). Some auditory cortical neurons in these mice become to encode the pain signal from trunk injury areas and the sound signal during their battles (Fig. 3). Therefore, the social stress recruits auditory cortical neurons to be associative memory neurons that encode stress signals, fear memories and anxiety. Moreover, the recruitment of associative memory neurons and the emergence of fear memory and anxiety are precluded by knocking down neuroligin-3-mediated synapse linkage (Figs. 4–5). In summary, the social stress makes auditory cortical neurons recruited to be associative memory neurons that encode fear memory and anxiety by neuroligin-3-mediated new synapse formation.

Our data reveal that the social stress induces the fear memory in those intruder mice specific to the stressful signals including the battle sound generated during the attack of a CD-1 resident mouse and the pain signal from trunk injury areas bitten by this CD-1 resident mouse. As a resident/intruder paradigm was commonly used to study the suffering of depression and anxiety [4, 85–90], the anxiety and depression in the intruder mice may result secondarily from the fear memory to stressful signals. It is known that the physical and psychological stressors lead to anxiety and depression [1–3, 5–11, 13, 18, 20]. Such affective disorders may suppress the immune system and the cardiovascular system to induce the secondary diseases [12]. Based on our present study, the downregulation of stress-induced memory to negative outcome may be the primary step to reduce these pathological moods and to prevent their relevant secondary diseases.

The auditory cortex encoded the joint storage of auditory signals with other signals in associative learning and memory [7, 62, 64–70, 72].

The primary auditory cortex has been thought to work for the discrimination of stimulations from threat versus nonthreat signals and to regulate the specificity of threat memory [73]. However, the cellular mechanism underlying the information storage in the auditory cortex is largely unknown [74–78]. Associative memory cells have been identified in the sensory cortices including the barrel cortex, piriform cortex, somatosensory cortex and gustatory cortex, which are characterized to be the coactivity-dependent interconnections among cross-modal cortices and the encoding of relevant associated signals [79–84, 108]. By using the resident/intruder paradigm, we discover that the social stress induces the recruitment of associative memory neurons in the auditory cortex that are featured by the formation of synapse interconnections with S1-Tr cortical neurons and the encoding of the fear signals including the battle sound and the painful signal essential for fear memory and anxiety. These associative memory cells can be specified to the fear memory cells and anxiety cells. Whether these associative memory cells also encode the emotional negative valence led by the negative memory of aversive tones in the auditory cortex remains to be examined once the test and the approach for the emotional reaction is well developed. In other words, the auditory cortical neurons may be recruited to have multiple functions, such as the fear memory and anxiety as well as their correlated negative emotional valence. In addition, all types of the associative memories to the signals from the external environment and endogenous brain activities are presumably based on the recruitment of associative memory cells in the brain. This logical prediction encourages researchers to test whether associative memory neurons are widely present in the memory formation as basic units of memory trace [7, 13].

Acute severe stress often induces fear memory and anxiety [1, 3–5, 7, 22, 25, 26, 107]. Many brain regions, such as the amygdala, the nucleus accumbens and the prefrontal cortex, have been presumably correlated to the fear memory and anxiety [27–37]. The neural circuits in the amygdala and the nucleus accumbens have been thought of the relevance to the balance between the fear memory and the reward memory [38–43]. The imbalance of these structures causes dominant memories to negative events, leading to affective disorders [44–49, 52–56]. In addition to the interaction of amygdala neurons with other brain areas, the connections between the amygdala and the auditory cortex appear to be strengthened during the fear memory induced by the association of bell ring and foot shock [23, 57–61]. These data indicate that the stress-related fear signals may be encoded by the interaction between the amygdala and the auditory cortex. Our data here indicate the recruitment of associative memory neurons in the auditory cortex for the stress-induced fear memory and anxiety. Those secondary associative memory neurons [7, 13] may be recruited in the amygdala for stress-induced fear memory and anxiety, because we have observed the convergent synapse innervations onto the neurons in the lateral area of the amygdala projected from the S1-Tr

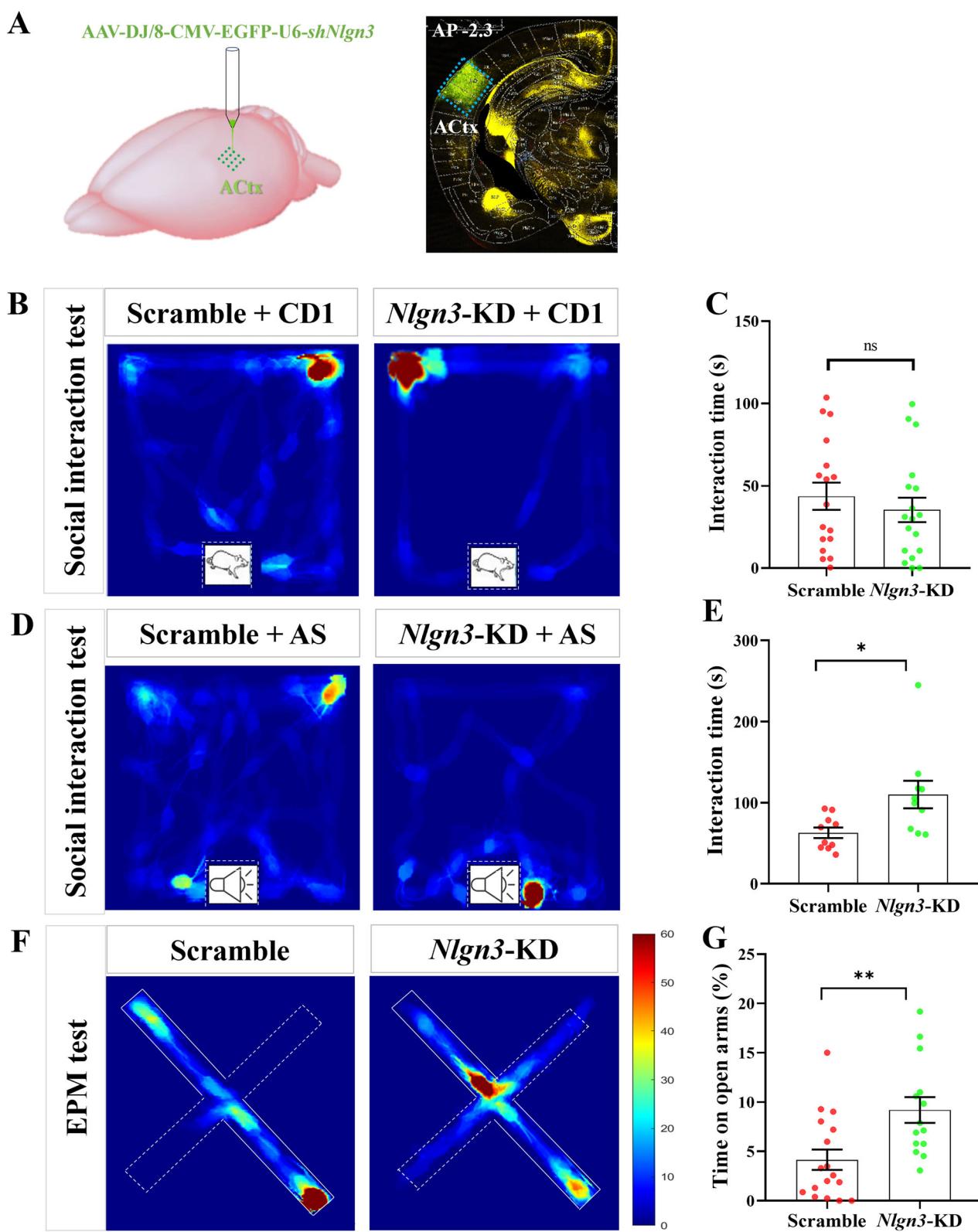


Fig. 5 **Neuroligin-3 knockdown in the auditory cortex attenuates the stress-induced fear memory and anxiety-like behaviors.** **A** AAV-DJ/8-CMV-EGFP-U6-scramble or AAV-DJ/8-CMV-EGFP-U6-sh*Nlgn3* are injected into the auditory cortex to suppress the expression of *neuroligin-3* in intruder mice. **B** The hot-spot maps of an intruder C57 mouse in response to a CD-1 resident mouse in an interaction cage. **C** The interaction time of intruder C57 mice with this CD-1 mouse is not significantly difference between a scramble subgroup (red symbols, $n = 17$) and a *Nlgn3*-KD subgroup (green symbols, $n = 18$, $P > 0.05$, one-way ANOVA). **D** The hot-spot maps of an intruder C57 mice in response to the battle sound in an interaction cage. **E** C57 mice in intruder plus *Nlgn3*-KD subgroup ($n = 10$) express more interaction time with the battle sound, in comparison with intruder plus scramble subgroup ($n = 10$; $^*P < 0.05$, one-way ANOVA). **F** The hot-spot maps of an intruder C57 mouse on an elevated plus maze. **G** C57 mice in intruder plus *Nlgn3*-KD subgroup ($n = 14$) show more duration of staying open arms, compared with scramble subgroup ($n = 17$; $^{**}P < 0.01$, one-way ANOVA). Error bars indicate SEM.

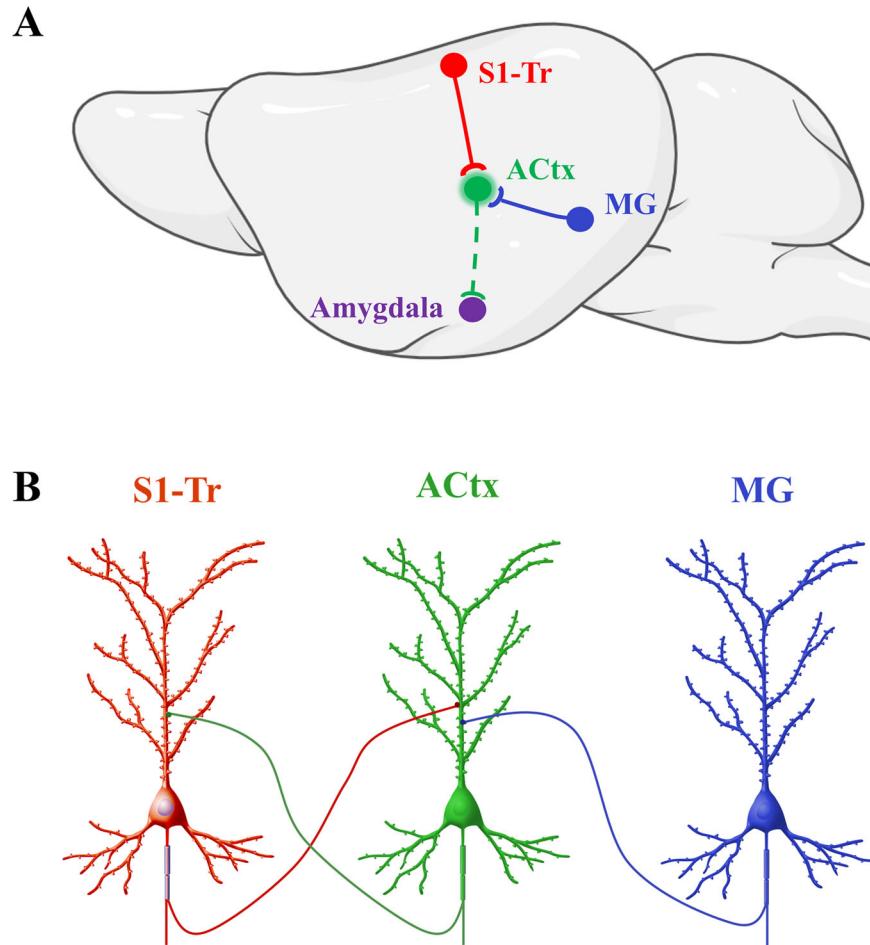


Fig. 6 Auditory cortical neurons are recruited to encode both auditory and somatosensory stress signals. **A** Auditory neurons turn into encoding stressful auditory and somatosensory signals. These stressful signals are transmitted to amygdala, leading to the onset of fear memory and anxiety-like behaviors. **B** After the social stress, the auditory neurons form synapse interconnection with the somatosensory neurons, alongside receiving synapse innervations from the medial geniculate body.

cortex and the auditory cortex in the mice with stress-induced fear memory and anxiety.

In terms of the molecular mechanism underlying the recruitment of associative memory neurons in the auditory cortex for stress-induced fear memory and anxiety, our studies show that neuroligin-3 knockdown in the auditory cortex by its shRNA prevents the emergence of fear memory and anxiety, the formation of new synapse interconnections and the recruitment of associative memory neurons (Figs. 4–5). The co-disappearance of associative memory cells and fear memory/anxiety by knocking down neuroligin-3 suggests the essential role of synapse linkage protein neuroligin-3 in stress-induced fear memory and anxiety by recruiting associative memory neurons. There are two strategies to test the causal relation of the stress-induced interconnections between the auditory cortex and the S1-Tr cortex to the formations of associative memory cells, fear memory and anxiety. In the present study, the prevention of the formation of this interconnection by downregulating neuroligin-3 is conducted to test whether the recruitment of associative memory neurons as well as the emergences of the fear memory and anxiety are precluded. Another strategy worthy to be applied is to examine whether the inhibition of the function of this stress-induced interconnection by an optogenetic approach can block the activity of associative memory neurons as well as the expression of the fear memory and anxiety. It is noteworthy that the neuroligin-3 knockdown in the auditory cortex is unable to prevent the fear memory by the visual system seeing the resident CD-1 mouse, indicating that the associative

memory neurons may also be recruited in the visual cortex, which has been observed in other studies from our group.

To the question whether the interconnection between auditory and S1-Tr cortices is functional, we have experimentally examined the colocalization of those interconnection neurons labeled by AAV-fluorescents and the neurons labelled by the antibody of *cFos*, one of immediate early genes that are used to show active neurons [109–111]. As shown in Fig. 2, both interconnection neurons and *cFos*-labelled neurons are raised in the auditory cortices from the mice with stress-induced fear memory and anxiety, indicating those interconnected neurons as associative memory neurons more active. It is noteworthy that the partial colocalization of the interconnected neurons and the *cFos*-labelled neurons in the auditory cortex (Figure S1) indicates that they are not the identical population of auditory cortical neurons, or not mutually represented.

The formation of new synapse interconnections among cross-modal cortices and the recruitment of associative memory neurons to encode stress signals for fear memory and anxiety are functionally and morphologically identified in the mouse model of resident/intruder paradigm. The stress-induced psychological behaviors and cellular changes are based on neuroligin3-mediated new synapse linkage. A diagram in Fig. 6 illustrates that the social stress induces auditory cortical neurons to receive new synapse innervations from the S1-Tr cortex alongside synapses from the geniculate body as well as to interconnect S1-Tr cortex, so that auditory cortical neurons become able to encode all of these stress

signals inputted from auditory and somatosensory systems. The associative memory neurons in the auditory cortex may play a central role between the input of fear signals from the sensory system and the processing of fear memory and pathological mood in the amygdala and the prefrontal cortex. The stress-induced recruitment of associative memory neurons is strengthening the concept of associative memory neurons being recruited in all types of associative learning [7]. Our studies also reveal that the cellular working principle for fear memory and anxiety is based on the associative memory neurons in the auditory cortex to encode stress signals and psychological deficits, which has not been indicated in previous studies.

DATA AVAILABILITY

The datasets used and analyzed in the current study are available from the corresponding author based on the request without commercial purpose.

CODE AVAILABILITY

The datasets used and analyzed in the current study are available from the corresponding author based on the request without commercial purpose.

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AUTHOR CONTRIBUTIONS

Conceptualization: JHW. Methodology: YZ, BC, LW, JL, YX, JHW. Validation: YZ, BC, LW, JL, YX, JHW. Formal analysis: YZ, BC. Investigation: YZ, BC, LW, JL, YX, JHW. Resources: JHW. Data curation: YZ, BC. Writing-Original draft: JHW. Writing-Review & Editing: JHW. Visualization: YZ, BC, JHW. Supervision: JHW. Project administration: JHW. Funding acquisition: JHW.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Experiments were done in accordance with guidelines and regulations by the Administration Office of Laboratory Animal in Beijing China. All experiment protocols were approved by Institutional Animal Care and Use Committee in Administration Office of Laboratory Animal at Beijing China (B10831).

CONSENT FOR PUBLICATION

In the publication of this manuscript, no individual' data, such as personal details, images, videos and voice are included. All necessary consent for publication has been secured.

ADDITIONAL INFORMATION

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