IgSF9b regulates anxiety behaviors through effects on centromedial amygdala inhibitory synapses

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Abnormalities in synaptic inhibition play a critical role in psychiatric disorders, and accordingly, it is essential to understand the molecular mechanisms linking components of the inhibitory postsynapse to psychiatrically relevant neural circuits and behaviors. Here we study the role of IgSF9b, an adhesion protein that has been associated with affective disorders, in the amygdala anxiety circuitry. We show that deletion of IgSF9b normalizes anxiety-related behaviors and neural processing in mice lacking the synapse organizer Neuroligin-2 (Nlgn2), which was proposed to complex with IgSF9b. This normalization occurs through differential effects of Nlgn2 and IgSF9b at inhibitory synapses in the basal and centromedial amygdala (CeM), respectively. Moreover, deletion of IgSF9b in the CeM of adult Nlgn2 knockout mice has a prominent anxiolytic effect. Our data place IgSF9b as a key regulator of inhibition in the amygdala and indicate that IgSF9b-expressing synapses in the CeM may represent a target for anxiolytic therapies.
Understanding the molecular basis of psychiatric disorders is one of the foremost medical challenges of our times, and substantial interest has arisen in the role of synaptic dysfunction in psychiatric pathophysiology. The vast majority of corresponding studies have focused on glutamatergic, excitatory synapses, but it is becoming increasingly clear that an equally important contribution stems from abnormalities in inhibitory synaptic transmission. This is particularly true for anxiety disorders, which have been linked to decreased inhibition in multiple brain regions. To date however, astonishingly little is known about the molecular mechanisms by which abnormalities at inhibitory synapses contribute to the pathophysiology of anxiety disorders.

A primary reason for this dearth of information is that the molecular composition of inhibitory postsynapses is only just being uncovered. In the past 5 years, the list of known inhibitory synapse organizers has expanded dramatically due to major technological advances. A key question now is whether these molecules function uniformly across inhibitory synapses, or whether they display the same heterogeneity of function that is observed at the cellular level in the inhibitory network. Understanding this organization will not only be essential for identifying disease mechanisms in pathological anxiety and other disorders, but may also provide a unique opportunity for uncovering selective targets for circuit-specific therapeutic interventions.

A particularly interesting candidate molecule is IgSF9b, a member of the immunoglobulin superfamily of cell adhesion proteins that was recently shown to localize to inhibitory synapses in dissociated neuron cultures. Variants in IgSF9b are associated with major depression and the negative symptoms of schizophrenia, indicating that IgSF9b may regulate affect in human patients. IgSF9b was recently reported to act in a complex with Neurelin-2, a member of the Neurelin family of adhesion proteins specifically found at inhibitory synapses. Loss-of-function mutations in Nlgn2 have been identified in patients with schizophrenia or pathological anxiety and autism, and in mice, deletion of Nlgn2 robustly enhances anxiety-related behaviors. Together, these findings raise the intriguing possibility that IgSF9b, like Nlgn2, may contribute to psychiatric pathophysiology by regulating synaptic inhibition in behaviorally relevant circuitry. To date, however, the molecular, cellular and circuitry functions of IgSF9b and its potential interactions with Nlgn2 in vivo remain completely unexplored.

In the present study, we investigated the role of synaptic inhibition in anxiety by addressing two key questions: First, does IgSF9b regulate anxiety-related behavior and neural processing in the anxiety circuitry of WT and/or pathologically anxious Nlgn2 KO mice? Second, do IgSF9b and/or Nlgn2 act at specific inhibitory synapses within the anxiety circuitry and thus offer synapse-specific targets for interventions? To this end, we investigated the consequences of IgSF9b deletion and IgSF9b x Nlgn2 double deletion on anxiety-related behavior and circuits, as well as on inhibitory synapses in the amygdala, a brain region that plays a central role in the processing of anxiety information. We show that IgSF9b regulates anxiety-like behaviors in Nlgn2 KO mice and that IgSF9b and Nlgn2 exert differential effects on distinct components of the amygdala inhibitory circuitry. Our data provide the first characterization of IgSF9b function in vivo and uncover a prominent anxiolytic consequence of IgSF9b deletion in Nlgn2 knockout (KO) mice, implicating IgSF9b-expressing neurons in the centromedial (CeM) amygdala as potential targets for anxiolytic therapies.
related consequences. Analysis of CeM activation during OF exposure revealed an overactivation of the CeM in Nlgn2 KO mice (Fig. 2a, lower right panel, red bar), with no effect in IgSF9b KO mice (Fig. 2a, lower right panel, blue bar). Strikingly, the overactivation of the CeM was completely normalized in double KO mice (Fig. 2a, lower right panel, purple bar), which is highly consistent with their WT-like anxiety-related behavior. Similar analysis of the CeL revealed no effect in either Nlgn2 KO or IgSF9b KO mice (Fig. 2a, upper right panel).

To confirm that suppression of BA → CeM projection neurons is indeed the mechanism behind suppression of anxiety-related activity in the CeM, we stereotaxically injected a fluorescent Retrobead retrograde tracer into the CeM to label neuronal populations that project directly to the CeM (Fig. 2c and Supplementary Fig. 3i-m). We measured activation of Retrobead-positive neurons in the BA under anxiogenic conditions using cFos immunohistochemistry (Fig. 2d). Importantly, the stereotaxic surgery did not alter anxiety in any of the KO mice (Supplementary Fig. 3l–m). Analysis of Retrobead- and cFos-double positive neurons revealed that BA → CeM direct projections were strongly overactivated in Nlgn2 KO mice (Fig. 2d, red bar), and surprisingly, overactivation persisted in double KO mice.

Fig. 1 Deletion of IgSF9b normalizes anxiety-related behavior in Nlgn2 KO mice. a, b WT, Nlgn2 KO, IgSF9b KO, and double KO mice were assessed for anxiety-related behavior in an OF test. c Representative tracks of OF exploration. d Time spent in the anxiogenic center of the OF. e Distance traveled in the center of the OF, expressed as percentage of total distance traveled. f Number of entries into the center of the OF. g Total distance traveled in the OF. h Mice were assessed for anxiety-related behavior in EPM. i Representative tracks of EPM exploration. j Time spent in the anxiogenic open arms of the EPM. k Distance traveled in the open arms of the EPM, expressed as percentage of total distance traveled. l Number of entries into the open arms of the EPM. m Total distance traveled in the EPM. Statistically significant ANOVA comparisons are marked in gray at the top of the panels and are listed in Table 1. For all other ANOVA comparisons, F < 1. Post-hoc analysis: *p < 0.05 relative to WT, **p < 0.01 relative to WT, ***p < 0.001 relative to WT, #p < 0.05 relative to double KO, ##p < 0.01 relative to double KO, ###p < 0.001 relative to double KO. Error bars represent SEM, n = 10–12 mice per genotype for OF, n = 9–14 mice per genotype for EPM.
modification prominent in Nlgn2 KO mice, indicating that Nlgn2 deletion (Fig. 4b–c). The increase in the beta frequency band was most during exploration (decrease) in Nlgn2 KO mice (Fig. 3c, d). Beta power increased in Nlgn2 KO mice during exploration of the OF center, and this increase was mainly due to the OF (Fig. 3c, d). Spectral analysis of CeM LFPs revealed that exposure to the OF increased oscillatory activity in all genotypes (Fig. 3a, b and Supplementary Fig. 4a–b). These results indicate that deletion of Nlgn2 increases beta oscillatory activity in the CeM under anxiogenic conditions, while deletion of IgSF9b normalizes anxiety-related beta activity in double KO mice (Fig. 3d, e), a mechanism that may underlie the normalization of anxiety-like behavior.

IgSF9b and Nlgn2 bidirectionally regulate CeM activity. To confirm that changes in the neural activity of the CeM accompany anxiety-related behavior, and to investigate whether IgSF9b and/or Nlgn2 alter specific neural substrates in the CeM in vivo, we recorded local field potential (LFP) oscillations in the CeM in freely moving mice during exploration of the OF (Fig. 3a, b and Supplementary Fig. 4a–b). Spectral analysis of CeM LFPs revealed that exposure to the OF increased oscillatory activity in all genotypes compared to home cage CeM activity, particularly in the theta (4–12 Hz) and beta (18–30 Hz) ranges (Supplementary Fig. 4b–c). The increase in the beta frequency band was most prominent in Nlgn2 KO mice, indicating that Nlgn2 deletion modifies anxiety-related CeM activity in the beta frequency range. To further explore whether this increase in beta power was modulated by anxiogenic conditions, we compared CeM activity during exploration (defined as speed > 1 cm/s) of the relative safety of the OF with the potentially anxiogenic center of the OF (Fig. 3c, d). Beta power increased in Nlgn2 KO mice during exploration of the OF center, and this increase was completely abolished in double KO mice (Fig. 3e, Supplementary Fig. 4d). Furthermore, we found a significant correlation between the magnitude in beta power and the distance from the center of the OF specifically in Nlgn2 KO mice (Fig. 3f–i). The increase in beta power was not induced by changes in locomotion, since beta activity was not modulated by speed in any genotype (Supplementary Fig. 4e). These results indicate that deletion of Nlgn2 increases beta oscillatory activity in the CeM under anxiogenic conditions, while deletion of IgSF9b normalizes anxiety-related beta activity in double KO mice (Fig. 3d, e), a mechanism that may underlie the normalization of anxiety-like behavior.

Table 1 Two-way ANOVA comparisons

| Figure | Main effect Nlgn2 KO | Main effect IgSF9b KO | Nlgn2 KO x IgSF9b KO interaction | Nlgn2 KO x IgSF9b KO interaction |
|--------|---------------------|----------------------|-------------------------------|-------------------------------|
| 1d     | $F_{1,40} = 10.02$  | 0.003                | $F_{1,40} = 18.58$            | $< 0.001$                      |
| 1e     | $F_{1,40} = 12.00$  | 0.001                | $F_{1,40} = 17.60$            | $< 0.001$                      |
| 1f     | $F_{1,40} = 20.80$  | $< 0.001$            | $F_{1,40} = 23.14$            | $< 0.001$                      |
| 1g     | $F_{1,40} = 35.93$  | $< 0.001$            | $F_{1,40} = 25.73$            | $< 0.001$                      |
| 1j     | $F_{1,40} = 3.22$   | 0.080                | $F_{1,40} = 17.77$            | $< 0.001$                      |
| 1k     | $F_{1,40} = 6.32$   | 0.016                | $F_{1,40} = 22.31$            | 0.017                         |
| 1l     | $F_{1,40} = 7.29$   | 0.010                | $F_{1,40} = 21.70$            | $< 0.001$                      |
| 1m     | $F_{1,40} = 1.33$   | 0.255                | $F_{1,40} = 11.04$            | $< 0.001$                      |
| 2a (BA) | $F_{1,40} = 2.78$  | 0.010                | $F_{1,40} = 4.58$             | 0.041                         |
| 2a (CeM) | $F_{1,40} = 7.69$ | 0.012                | $F_{1,40} = 3.33$             | 0.084                         |
| 2d     | $F_{1,40} = 9.68$   | 0.005                | $F_{1,40} = 2.10$             | 0.160                         |
| 2e     | $F_{1,40} = 2.19$   | 0.154                | $F_{1,40} = 1.01$             | n.s.                          |
| 3e     | $F_{1,40} = 18.73$  | $< 0.001$            | $F_{1,40} = 4.90$             | $< 0.05$                       |
| 3f     | $F_{1,40} = 18.73$  | $< 0.001$            | $F_{1,40} = 33.75$            | $< 0.001$                      |
| 4g     | $F_{1,40} = 6.20$   | 0.020                | $F_{1,40} = 5.64$             | 0.026                         |
| 4h     | $F_{1,40} = 1.27$   | 0.243                | $F_{1,40} = 1.22$             | n.s.                          |
| 5j     | $F_{1,40} = 6.83$   | 0.011                | $F_{1,40} = 1.01$             | n.s.                          |
| 6c     | $F_{1,40} = 1.28$   | n.s.                 | $F_{1,40} = 5.07$             | 0.032                         |
| 6d     | $F_{1,40} = 1.20$   | n.s.                 | $F_{1,40} = 1.35$             | 0.259                         |
| 6f     | $F_{1,40} = 1.26$   | n.s.                 | $F_{1,40} = 1.12$             | 0.300                         |
| 6g     | $F_{1,40} = 1.73$   | 0.011                | $F_{1,40} = 6.38$             | 0.02                          |
| 6i     | $F_{1,40} = 6.60$   | 0.0003               | $F_{1,40} = 11.41$            | 0.012                         |
| 6j     | $F_{1,40} = 11.26$  | 0.001                | $F_{1,40} = 6.60$             | 0.001                         |
| 7j     | $F_{1,40} = 14.09$  | 0.0004               | $F_{1,40} = 6.60$             | 0.001                         |
| 7j     | $F_{1,40} = 7.19$   | 0.013                | $F_{1,40} = 6.60$             | 0.001                         |

n.s., not significant

*Refers to IgSF9b shRNA rather than IgSF9b KO

(Fig. 2d, purple bar). In contrast, activation of inhibitory CeL cells projecting to CeM projections did not differ among any of the genotypes (Fig. 2e). Together, these data indicate that Nlgn2 and IgSF9b deletion affect distinct targets within the amygdala: While Nlgn2 regulates anxiety-induced activation of projection neurons in BA, IgSF9b may normalize the CeM anxiogenic output by local mechanisms within the CeM.
valence of the environment (“safe” vs. “anxiogenic”), (2) beta oscillatory activity in the CeM represents a novel neural signature of pathological anxiety induced by deletion of Nlgn2, and (3) Nlgn2 and IgSF9b bidirectionally modulate anxiety-related neural activity in the CeM particularly during risk-assessment behavior.

IgSF9b knockdown in CeM normalizes anxiety in Nlgn2 KO mice. To further confirm that IgSF9b acts in the CeM to modulate anxiety, and to investigate whether targeting IgSF9b-containing synapses in the adult amygdala may recapitulate the anxiolytic effects in the constitutive global KO, we locally reduced IgSF9b levels by adeno-associated virus (AAV)-mediated expression of IgSF9b shRNA (Fig. 4a–b). AAV particles encoding IgSF9b shRNA or control shRNA (a mutant construct that lacks knockdown activity, as reported previously) were injected into the CeM of 8–12-week-old WT and Nlgn2 KO mice using stereotaxic surgery (Fig. 4c–d and Supplementary Fig. 5a), generating four experimental groups (Fig. 4e).

One day before and 6 weeks after surgery, anxiety-related behavior was assessed in an OF (Fig. 4c). IgSF9b shRNA had no significant effect on either the time or the distance traveled in the center of the OF in WT mice (Fig. 4f–g, white vs. blue and white shaded bars). In contrast, Nlgn2 KO mice injected with IgSF9b shRNA showed a pronounced reduction of anxiety-related behaviors compared to Nlgn2 KO mice injected with control shRNA, as evidenced by a significant increase in both the time and the distance traveled in the center (Fig. 4f–g, red vs. red and blue shaded bars, and Fig. 4h, representative traces). Interestingly, anxiety levels appeared to be exacerbated in Nlgn2 KO but not in WT mice over the observed 6-week time period, even in mice that had not undergone surgery (Supplementary Fig. 5b–d), and this effect was completely reversed by local reduction of IgSF9b (Fig. 4f–h, Supplementary Fig. 5e–f). Given that IgSF9b is highly expressed in the CeM (Supplementary Fig. 6a–d), these data confirm that IgSF9b modulates anxiety-related behaviors through a CeM-specific mechanism, and indicates that targeting inhibitory transmission in the CeM can ameliorate anxiety-related behaviors.

IgSF9b deletion alters CeM inhibitory synapse function. To elucidate the synaptic mechanisms that may be responsible for the anxiolytic effect of IgSF9b deletion in the CeM, we recorded miniature inhibitory postsynaptic currents (mIPSCs) from acute brain slices obtained from adult mice (Fig. 5). To assist in the identification of the relevant brain structures and to distinguish between excitatory and inhibitory neurons, we used mice of all four genotypes that additionally expressed the Venus under a vesicular inhibitory amino acid transporter (VIAAT) promoter. Given that IgSF9b is highly expressed in the CeM (Supplementary Fig. 6a–d), these data confirm that IgSF9b modulates anxiety-related behaviors through a CeM-specific mechanism, and indicates that targeting inhibitory transmission in the CeM can ameliorate anxiety-related behaviors.
Neurons in the CeM showed typical firing patterns as previously reported (Fig. 5a, b), and all parameters that reflect membrane excitability were similar between groups (Fig. 5c–e and Table 2). Surprisingly, however, IgSF9b deletion significantly increased mean mIPSC frequency while leaving mean mIPSC amplitudes unaffected (Fig. 5f–k, blue bars). This increase was due to a subset of IgSF9b KO neurons with substantially larger mean mIPSC frequencies, as revealed by a significant shift in the distribution of mean mIPSC frequencies among the neurons tested (Fig. 5i, blue vs. black line). Double KO mice showed a similar albeit less pronounced phenotype, with a trend towards an increase in mean mIPSC frequency (Fig. 5h, purple bar) and a significant shift in the distribution of mean mIPSC frequencies in the neuronal population (Fig. 5i, purple vs. black line). Consistent with our previous findings, Nlgn2 deletion did not affect mean mIPSC frequency, and only modestly reduced mean mIPSC amplitude (Fig. 5h–k, red bars).

Fig. 3  Neuronal activity in the beta frequency range is normalized in the CeM of double KO mice. a Representative image of the location of electrode in the CeM and experimental design. Scale bar, 500 µm. After electrode implantation mice were exposed to the OF for 15 min. b Representative traces of CeM LFPs from all four genotypes during SAP. c, d LFP power spectrum for all four genotypes during movement in the periphery (c) and center (d) of the OF. e Average power increase in the center relative to the periphery of the OF for the beta frequency range (18-30 Hz). f Increase in normalized beta power as a function of distance from the center in WT (f), Nlgn2 KO (g), IgSF9b KO (h), and double KO (i) mice. Statistical analysis of correlations is indicated in the figure. j Schematic of the stretch-attend posture (SAP) scored during the OF test. k Representative wavelet transforms of CeM LFP of the four genotypes during exploration of the OF. White horizontal lines indicate the duration of a representative SAP. l Average beta power during SAP. Statistically significant ANOVA comparisons are marked in gray at the top of the panels and are listed in Table 1. For all other ANOVA comparisons, F < 1. Post-hoc analysis: *p < 0.05 relative to WT, **p < 0.001 relative to WT, ***p < 0.05 relative to double KO, ###p < 0.01 relative to double KO, ####p < 0.001 relative to double KO. SAPs and mice: WT n = 77/6 mice; Nlgn2 KO n = 62/5 mice; IgSF9b KO n = 84/6 mice; double KO n = 71/5 mice. Error bars represent SEM. WT, white bars; Nlgn2 KO, red bars; IgSF9b KO, blue bars; double KO, purple bars.

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IgSF9b deletion increases VIAAT puncta in the CeM. We next investigated the molecular mechanisms by which IgSF9b deletion increases synaptic inhibition in the CeM. Immunohistochemistry for IgSF9b revealed punctate staining in the BA and CeM of adult WT mice, which was absent in IgSF9b KO mice (Supplementary Fig. 6a–d). Co-staining of IgSF9b with Nlgn2 revealed that in the CeM, but not in the BA, these proteins are localized in adjacent overlapping domains (Supplementary Fig. 6e–i), consistent with previous findings in hippocampal cultures. These data indicate that IgSF9b and Nlgn2 likely function in separate subdomains of the inhibitory synapse as previously proposed.

To assess the molecular basis of the increase in inhibitory transmission in IgSF9b KO and double KO mice, we performed immunohistochemistry for several markers of inhibitory synapses, including VIAAT, which labels inhibitory presynaptic terminals; gephyrin, the core scaffolding protein at inhibitory postsynapses; S-SCAM, a scaffolding protein that is only known synaptic interaction partner of IgSF9b; and the GABAAR subunits γ2 and α1 (Fig. 6a–g, Supplementary Fig. 6j–k). Strikingly, IgSF9b KO and double KO mice showed a significant increase in the number of VIAAT puncta (Fig. 6c, blue and purple bars,
respectively), consistent with the increase in mIPSC frequency, indicating that IgSF9b deletion increases the number and/or VIAAT content of inhibitory presynaptic terminals in the CeM. Gephyrin and S-SCAM remained unchanged (Fig. 6d, e, blue bars). IgSF9b deletion also resulted in a trend towards an increase in GABAAR γ2 subunit puncta, as well as a significant reduction in GABAAR α1 subunit puncta (Fig. 6f, g, blue bars). Nlgn2 KO mice showed a slight reduction in the number of GABAAR α1 subunit puncta, with no effects on any other synaptic marker (Fig. 6c–g, red bars), confirming the observation from the mIPSC recordings that Nlgn2 deletion has only very subtle effects in the CeM.

Fig. 4 Local knockdown of IgSF9b in the adult CeM ameliorates anxiety-related behaviors in Nlgn2 KO mice. a Immunoblot for IgSF9b in HEK cell lysates transfected with a Myc-IgSF9b construct and IgSF9b shRNA or control shRNA14. Numbers next to the protein ladder represent molecular weight in kDa. b Representative images and quantification of perisomatic IgSF9b puncta in the CeM following injection of control shRNA or IgSF9b shRNA. n = 3 mice. Scale bar, 20 μm. c Schematic diagram showing experimental design of IgSF9b shRNA experiment. Mice were tested in the OF 24 h before (pre) and 6 weeks after (post) injection. d Representative image of GFP-positive neurons in viral injection site. e Four experimental groups were generated: WT + control shRNA (white), WT + IgSF9b shRNA (white and blue striped), Nlgn2 KO + control shRNA (red), and Nlgn2 KO + IgSF9b shRNA (red and blue striped). f Time in center post-injection expressed as % pre-injection. g Normalized distance in center post-injection expressed as % pre-injection. h Representative tracks of OF exploration of Nlgn2 KO mice pre- and post-injection. Statistically significant ANOVA comparisons are marked in gray at the top of the panels and are listed in Table 1. For all other ANOVA comparisons F < 1. Post-hoc analysis: *p < 0.05 relative to WT, **p < 0.01 relative to WT. Error bars represent SEM, n = 6–9 mice per group.
To test whether the increase in VIAAT puncta in the CeM reflects an acute function of IgSF9b on inhibitory synapses or whether this is a developmental consequence, we quantified VIAAT puncta following injection of IgSF9b shRNA into the CeM (Fig. 6h–j). Deletion of IgSF9b increased the number of VIAAT puncta in Nlgn2 KO mice (Fig. 6i, red and blue striped bars), an effect that may underlie the rescue of anxiety-related behavior in Nlgn2 KO mice following local deletion of IgSF9b in the CeM (Fig. 4). Moreover, acute deletion of IgSF9b increased the size of VIAAT puncta in WT mice (Fig. 6j, white and blue and white bars), consistent with the upregulation of VIAAT puncta in single IgSF9b KO mice (Fig. 6c, blue bar). Together, our data indicate that deletion of IgSF9b in the CeM results in an increase in inhibitory synapse function, which may underlie its behavioral consequences in the anxiety circuitry.

IgSF9b deletion does not affect BA inhibitory synapses. Finally, we investigated the consequences of IgSF9b deletion on synaptic inhibition in the BA, in order to determine whether the differential effects of IgSF9b deletion on anxiety-related neuronal activation in the BA and the CeM are also reflected at the synaptic level. We first recorded mIPSCs in excitatory (in our case Venus-negative) neurons in the BA (Fig. 7a–f). Consistent with previous reports, Nlgn2 deletion resulted in a pronounced reduction in
mean frequency and mean amplitude of mIPSCs in the BA (Fig. 7c, e, red bars)\(^8,20,34,35\). In striking contrast, IgSF9b deletion affected neither mean mIPSC frequency nor mean IPSC amplitude (Fig. 7c, e, blue bars). Double KO mice showed a trend towards a reduction in mean frequency and mean amplitude of IPSCs that was similar to that of Nlgn2 KO mice (Fig. 7c, e, purple bars). Comparison of the probability distribution of mean frequencies or mean amplitudes among all cells to the corresponding WT distribution (Fig. 7d, f) revealed a significant shift towards lower mean frequencies in double KO cells (Fig. 7d, black vs. purple line), indicating that individual subpopulations of cells may be differentially affected in double KO mice. Consistent with the decrease in mIPSC frequency and amplitude, Nlgn2 deletion significantly reduced the number and size of perisomatic gephyrin and GABA\(_{A}\)R \(\alpha 1\) puncta, while no changes were observed for VIAAT or, interestingly, the GABA\(_{A}\)R \(\gamma 2\) subunit (Fig. 7g–j, red bars). IgSF9b deletion did not affect any inhibitory synapse markers in the BA, while double KO mice showed reductions in gephyrin and GABA\(_{A}\)R \(\alpha 1\) staining that were identical to those observed in Nlgn2 KO mice (Fig. 7g–j, blue and purple bars, respectively), consistent with the notion that IgSF9b does not normalize anxiety through a synaptic mechanism in the BA. Thus, combined with the functional changes observed in whole-cell recordings in amygdala slices, these morphological data indicate that IgSF9b and Nlgn2 differentially affect inhibitory synapses in the amygdala, with Nlgn2 deletion primarily impairing synaptic inhibition in the BA, and IgSF9b deletion primarily enhancing synaptic inhibition in the CeM. Altogether, our findings are consistent with a model in which IgSF9b deletion normalizes anxiety-related behaviors and neuronal activity by increasing inhibition onto CeM output neurons and hence counteracting the anxiety-related overactivation of the CeM (Fig. 8).

**Discussion**

In the present study we sought to elucidate the role of the cell adhesion molecule IgSF9b and its interactions with Nlgn2 in amygdala circuits, synapses, and behaviors related to anxiety processing. We show that deletion of IgSF9b has anxiolytic consequences and normalizes the prominent anxiety phenotype observed in Nlgn2 KO mice. This normalization does not occur through a mechanistic interaction of Nlgn2 and IgSF9b at the same synapses, but likely through differential effects on different inhibitory synapses in the BA and CeM, respectively. Specifically, our data support a model in which reduced inhibition in BA of Nlgn2 KO mice results in overactivation of BA → CeM projection neurons under anxiogenic conditions, which is counteracted in the CeM by the increased inhibition resulting from additional deletion of IgSF9b (Fig. 8). Together, our data provide the first description of IgSF9b function in vivo and uncover a novel role for IgSF9b in anxiety-related behavior and amygdala inhibitory synapses. Moreover, our findings highlight that IgSF9b-expressing synapses and neurons in the CeM may represent an important common target for anxiolytic treatments that is independent of individual upstream mutations.

Major efforts have recently been invested in determining the full complement of proteins that governs the formation and function of inhibitory synapses\(^12,15,36–40\). The cell adhesion molecule IgSF9b was identified as one of these candidate molecules in cell cultures\(^14,15\), but the mechanism by which it regulates inhibitory synapse function in vivo in intact circuits remains completely unknown. Here we show that in the CeM, deletion of IgSF9b results in an enhancement of inhibitory synaptic transmission, likely through a presynaptic mechanism. In particular, the increases in mIPSC frequency and VIAAT staining without a concomitant increase in gephyrin indicate that IgSF9b deletion may result in an increase in the number of VIAAT-positive vesicles per synaptic terminal, analogous to effects recently observed in IgSF21 KO mice (albeit with opposite polarity)\(^41\). Together with the observation that IgSF9b, like its mammalian paralog IgS9f and its Drosophila ortholog Turtle, forms primarily or exclusively homophilic interactions\(^14,16,42\), our data indicate that IgSF9b provides a transsynaptic signaling complex that regulates the organization of the inhibitory presynaptic terminal. Moreover, the differential effects of IgSF9b deletion on GABA\(_{A}\)R \(\alpha 1\) and \(\gamma 2\) subunits in the CeM indicate that IgSF9b may also regulate the subunit composition of GABA\(_{A}\)Rs at inhibitory post-synapses, although the mechanism or functional significance of this effect remains unknown.

Intriguingly, IgSF9b appears to play distinct roles at different inhibitory synapses, as evidenced by our finding that IgSF9b deletion affects inhibitory synaptic transmission and synaptic markers only in the CeM but not the BA, despite being expressed in both (Supplementary Fig. 6). One possible explanation for this difference lies in the differential composition of the two structures with respect to neuron types\(^8,43\). The BA is a cortex-like structure composed primarily of excitatory projection neurons with a small number of inhibitory interneurons, while the CeA is similar to striatum in that it is composed almost exclusively of inhibitory neurons. Therefore, the vast majority of inhibitory synapses sampled in the BA and CeM are made onto excitatory and inhibitory neurons, respectively, raising the possibility that IgSF9b differentially affects these synapse subtypes. Consistent

### Table 2 Kinetics of PSCs and passive membrane properties of inhibitory neurons in CeM

|                          | WT          | Nlgn2 KO    | IgSF9b KO   | Double KO   | Interaction  | F-value | p-value |
|--------------------------|-------------|-------------|-------------|-------------|--------------|---------|---------|
| mIPSCs: rise time (µs)   | 830 ± 80(n=22) | 916 ± 86(n=16) | 855 ± 79(n=18) | 781 ± 51(n=18) | Interaction: | F<1,70 = 1.10 | 0.30    |
| mIPSCs: decay (ms)       | 14.97 ± 0.71(n=22) | 17.17 ± 0.89(n=17) | 15.76 ± 1.16(n=18) | 15.25 ± 0.89(n=19) | Interaction: | F<1,70 = 2.19 | 0.14    |
| sEPSCs: rise time (µs)   | 457 ± 39(n=13) | 400 ± 35(n=15) | 515 ± 63(n=13) | 426 ± 44(n=15) | Nlgn2 KO: F<1,52 = 2.36 | 0.13    |
| sEPSCs: decay (ms)       | 3.13 ± 0.17(n=13) | 2.90 ± 0.15(n=15) | 3.07 ± 0.19(n=15) | 2.80 ± 0.16(n=13) | Nlgn2 KO: F<1,52 = 2.10 | 0.15    |
| Membrane resistance (MOhm) | 477 ± 40(n=32) | 501 ± 44(n=30) | 489 ± 38(n=32) | 397 ± 31(n=28) | IgSF9b KO: F<1,105 = 1.62 | 0.21,0.11 |
| Membrane capacitance (pF) | 28.86 ± 1.42(n=33) | 31.75 ± 1.92(n=31) | 28.75 ± 1.76(n=33) | 32.71 ± 2.07(n=28) | Interaction: F<1,103 = 2.56 | 0.05    |

*For all other ANOVA comparisons, F<1*
with this notion, IgSF9b was previously proposed to function primarily at inhibitory synapses onto interneurons in hippocampal cultures. In contrast, there is growing evidence that other known synapse organizers such as Nlgn2, GARLH/LHFPL4, and MDGA1 may function exclusively at inhibitory synapses onto excitatory neurons. Together, these findings give rise to the notion that excitatory and inhibitory neurons may utilize an entirely different complement of organizer proteins at inhibitory postsynapses, and that IgSF9b may be the first example of a synapse organizer with specificity for inhibitory synapses onto inhibitory neurons.

Moreover, the function of IgSF9b at inhibitory synapses may also depend on the identity of the presynaptic neuron. In hippocampal cultures, shRNA-mediated deletion of IgSF9b resulted in a decrease in mIPSC frequency and a reduction in VIAAT-positive gephyrin clusters in contrast to the increase in mIPSC frequency and VIAAT puncta observed here in the CeM (Figs. 5, 6).

In the CeM, the inhibitory neuron subtypes found in the CeM are entirely distinct from those observed in the hippocampus, it is conceivable that these different subtypes may differentially employ IgSF9b at inhibitory postsynapses. Alternatively, IgSF9b may play distinct roles at different stages of synaptic development (i.e., at developing synapses in neuronal cultures vs. mature synapses in adult mice) or in dissociated cultures vs. intact neuronal networks. Cell type– and circuit-specific analysis of IgSF9b function will be essential for fully understanding its role in the brain.

Given that IgSF9b and Nlgn2 function at distinct synapses and do not appear to interact in a cell-autonomous manner, at least in the amygdala, how does deletion of IgSF9b normalize the prominent anxiety phenotype observed in Nlgn2 KO mice? Combined evidence from our cFos analysis (Fig. 2a), retrograde tracing experiments (Fig. 2c–e), and in vivo electrophysiology (Fig. 3) indicates that IgSF9b deletion normalizes anxiety-related output specifically in the CeM of Nlgn2 x IgSF9b double KO mice. Given that the normalization of anxiety-related behaviors can be mimicked by local shRNA-mediated knockdown of IgSF9b in the CeM of Nlgn2 KO mice (Fig. 4), it is likely to occur through a local mechanism within the CeM. In light of our observation that IgSF9b deletion increases inhibitory synaptic transmission in the CeM (Figs. 5, 6), the most parsimonious explanation is that this increased inhibition onto CeM output neurons counteracts the increased excitation originating from Nlgn2 KO BA projection neurons, thus balancing the activity of CeM neurons mediating anxiogenic projections to the brainstem.

An interesting remaining question regards the source of the inhibitory inputs to the CeM that are upregulated by IgSF9b deletion. CeM neurons are known to receive feedforward inhibitory projections from the CeL, the intercalated nucleus and the bed nucleus of the stria terminalis, and synaptic transmission at any of these inhibitory connections or at local interneurons may be upregulated in response to IgSF9b deletion. Importantly, our model does not require the neuronal activity of the upstream inhibitory neurons to be increased, and indeed we show that CeL inputs to the CeM are not differentially activated (Fig. 2e). We cannot rule out that other CeM inputs may show altered activation in IgSF9b or double KO mice. However, the effectiveness of the local deletion of IgSF9b in CeM in mimicking the double KO phenotype indicates that any such upstream alterations cannot be required for the normalization of anxiety-related behaviors by IgSF9b deletion. Our findings, therefore, identify local information processing in the CeM as a key target for the normalization of anxiety-related behaviors by IgSF9b deletion.
mediator of anxiety-related behaviors and highlight the fundamental importance of better understanding the role of the CeM in anxiety processing.

The strong correlation of power of beta oscillations with distance from the center of the OF in Nlgn2 KO mice and its exacerbated increase during risk-assessment behavior implicates beta oscillations in the CeM as a key neural signature of pathological anxiety. To our knowledge this is the first time that oscillatory activity in the CeM is described in anxiety processing in general, and beta oscillations during risk-assessment behavior in particular. At present, both the function of these beta oscillations in anxiety processing and the mechanisms that underlie their modulation by Nlgn2 and IgSF9b remain unknown. Beta oscillations have previously been observed in the basal ganglia and somatosensory cortex during decision-making tasks, highlighting their multifaceted role in information processing. However, their

\[ \text{\textbf{Fig. 7}} \text{ IgSF9b deletion does not affect inhibitory synapses in the BA.} \text{ a, b Schematic diagram illustrating recording sites in the BA, mean mIPSCs and representative mIPSC traces from the BA of WT, Nlgn2 KO, IgSF9b KO, and double KO mice. c Average cumulative distribution of mIPSC inter-event intervals and quantification of mean mIPSC frequency in the BA. d Probability distribution of mean mIPSC frequency of each analyzed cell in the BA. Kolmogorov-Smirnov test: WT vs. Double KO, } p < 0.0001. \text{ e Average cumulative distribution of mIPSC amplitudes and quantification of mean mIPSC amplitude in the BA. f Probability distribution of mean mIPSC amplitude among all analyzed cells in the BA.} n = 13-18 cells/5-6 mice per genotype.} \]

\[ \text{g-j Photomicrographs and quantification of the number of perisomatic puncta of g VIAAT, h gephrin, i GABA}_\text{A},\text{R}72, \text{ and j GABA}_\text{A},\text{R}11 in all four genotypes. Scale bar, 2 } \mu \text{m.} n = 3-8 per genotype. \]
The cellular and synaptic mechanisms leading to the generation of beta rhythms are largely unknown, although changes in inhibitory synaptic activity are known to affect LFPs in general and beta activity in specific. Further experiments will be essential to fully understand how Nlgn2 and IgSF9b regulate beta oscillations in the CeM and how this contributes to the generation and normalization of pathological anxiety processing.

The anxiolytic effects of IgSF9b deletion in Nlgn2 KO mice are intriguing in light of previous studies showing that variants in IgSF9b are associated with major depression and the negative (mainly affective) symptoms of schizophrenia. It is conceivable that the IgSF9b variants in human patients are in fact gain-of-function rather than loss-of-function mutations, which based on our findings, could result in reduced synaptic inhibition in the CeM. Indeed, our data indicate that the function of IgSF9b-containing synapses in the CeM may be key in determining the vulnerability or resilience of individuals towards upstream anxiogenic factors. The precise molecular mechanisms remain to be elucidated both in mice and humans, and it is clear that IgSF9b is emerging as an important regulator of affective behaviors, and that further investigations into its function may substantially contribute to our understanding of a range of psychiatric disorders.

Ultimately, perhaps the most exciting conclusion arising from our study is that IgSF9b-expressing neurons and synapses in CeM may represent a viable common target for anxiolytic therapies, independent of the upstream anxiogenic mutations. Given that both global and local deletion of IgSF9b lead to a remarkably specific anxiolytic effect, targeting IgSF9b pharmacologically may provide a promising strategy for the development of more selective anxiolytic therapies. Moreover, it is tempting to speculate that in the age of circuit psychiatry, targeting IgSF9b-expressing neurons with viral vectors will become feasible, offering entirely new treatment options for patients with anxiety and co-morbid psychiatric disorders.

**Fig. 8** Model of the circuitry-based normalization of the anxiety phenotype in Nlgn2 x IgSF9b double KO mice. Based on our findings, we propose the following model for the normalization of anxiety-related behaviors in Nlgn2 x IgSF9b double KO mice: Nlgn2 deletion enhances activity of excitatory BA → CeM projection neurons, resulting in overactivation of anxiogenic projection neurons in the CeM and hence increased anxiety-related behaviors. IgSF9b deletion enhances synaptic inhibition onto anxiogenic projection neurons in the CeM, which results in reduced activation of CeM projection neurons and hence reduced anxiety-related behaviors. In Nlgn2 x IgSF9b double KO mice, the combination of these two effects results in normalization of the activity of CeM projection neurons and hence a normalization of anxiety-related behaviors.

**Methods**

**Experimental subjects.** Nlgn2 KO mice were generated in our laboratory on an 129/Sv background and were backcrossed onto a C57BL/6J background for at least six generations. IgSF9b KO mice were obtained from Lexicon Pharmaceuticals (The Woodlands, TX, U.S.A.; Omnibank clone 281214, generated through inserter Dr. Atsushi Miyawaki, RIKEN). All mice were 2–3 months old at the beginning of the experiment. For experiments involving OF testing (Figs. 1–4 and Supplementary Fig. 1–4), only male mice were used unless specified otherwise. For the EPM experiment (Fig. 1h–n) and for molecular and slice electrophysiology experiments (Figs. 5–7), both male and female mice were used, based on the observation that the OF phenotype is identical in both sexes. Animals were maintained on a 12 h light/dark cycle (7 am/7 pm), with food and water ad libitum. All experiments were performed during the light phase (with the exception of home cage activity monitoring as described below, Supplementary Fig. 2). The experimenter was blind to genotype during all stages of data acquisition and analysis. All procedures were approved by the State of Niedersachsen (Landesamt für Verbraucherschutz und Lebensmittelsicherheit) and were carried out in agreement with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany and the Max Planck Society.

**Behavioral characterization.** The OF test was conducted in a square arena made of white plastic (50 cm × 50 cm). Mice were placed in the corner of the OF and were permitted to explore the arena for 10 min. The EPM was conducted in an apparatus made of gray plastic, with two open arms and two closed arms (28 cm × 5 cm each, elevated 45 cm from the ground). Mice were placed in the closed arm and were permitted to explore the apparatus for 5 min. Performance was recorded using an overhead camera system and scored automatically using the Viewer software (Biobserve, St. Augustin, Germany). Between each mouse, the arena was cleaned thoroughly with 70% ethanol followed by water to eliminate any odors left by the previous mouse.

In our previous study, the center zone of the OF was defined as a square of 25 × 25 cm. However, closer assessment revealed that a second, intermediate zone (12.5 cm × 25 cm from the walls of the chamber) was also avoided by the Nlgn2 KO mice (Supplementary Fig. 1a–h), and that in fact the behavior in this intermediate zone resembled the behavior in the center, rather than the periphery, in all four genotypes assessed in the present study. For this reason, an extended center zone of 37.5 × 37.5 cm was used throughout this study to more accurately reflect the anxiogenic area.

Recording of home cage activity was performed using the LABORAS system and software (Metris, Hoofddorp, The Netherlands). Mice were habituated to the LABORAS cages for two days. On the third day, activity was recorded for two 6-hour periods, from 9 pm to 3 am (dark cycle) and from 9 am to 3 pm (light cycle). The following parameters were assessed: total duration of locomotor activity, immobility, grooming, and climbing, as well total distance traveled and average velocity during locomotion.

**cFos induction assay.** To assess anxiety-induced cFos activation, mice were first exposed to the OF arena for 10 min. Ninety minutes after exposure, they were anesthetized with Avertin (Triaboromethanol, Sigma) and perfused transcardially first with saline, then with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains were post-fixed in PFA overnight, and cryoprotected in 30% sucrose in 0.1 M PB. Free-floating sections (40 μm thickness) were prepared using a Leica CM3050S cryostat (Leica, Wetzlar, Germany). Sections were incubated in blocking...
solution (3% bovine serum albumin, 10% goat serum, 3% Triton X-100 in 0.1 M phosphate-buffered saline (PBS)) for 1 h, then incubated for 12 h with Rabbit polyclonal antibody (catalog# HPA010802, Sigma Aldrich, Darmstadt, Germany) diluted 1:1000 in blocking solution; and then incubated for 2 h with Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Eugene, OR, USA) diluted 1:600 in blocking solution. The sections were washed with PBS after each incubation, and were finally mounted on glass slides using Aqua-Poly/Mount (Polysciences, Eppelheim, Germany).

For PV/cFos or SOM/cFos immunolabeling, amygdala sections were processed as described above for cFos immunohistochemistry. All secondary antibodies were diluted 1:600 in respective blocking solution and obtained from Invitrogen, Eugene, USA. For PV immunolabeling sections were incubated for 12 h with mouse monoclonal anti-PV antibody (catalog# 235, SWANT, Bellinzona, Switzerland) diluted 1:2000 in blocking solution; and then incubated for two hours with Alexa Fluor 555 goat anti-mouse antibody. For SOM immunolabeling, the blocking solution contained 10% donkey serum and 0.3% Triton X-100 in PBS 0.1 M. Sections were incubated for 12 h in goat polyclonal anti-SOM antibody (catalog# sc-7819, Santa Cruz Biotech, Dallas, Texas, USA) diluted 1:1000 in blocking solution; and then with Alexa Fluor 488 donkey anti-goat antibody. Sections were washed with PBS after each incubation, and were finally mounted on glass slides using Aqua-Poly/Mount (Polysciences, Eppelheim, Germany).

Images of cFos and cellular markers were obtained using a confocal laser scanning microscope (Leica SP2) with a x40 oil immersion objective. For each set, sections were anatomically matched and the settings for laser power, gain and offset were kept constant during imaging. 2 stacks of 5 μm thickness and containing 2 optical sections each were obtained from each amygdala section. In total, 10–12 stacks were imaged from 5–6 amygdala sections per mouse in each group.

For analysis of synaptic markers, immunolabeling for VIAAT was performed on perfusion-fixed brain slices as described for the cFos assay. Briefly, sections were incubated for 24 h with rabbit polyclonal anti-VIAAT antibody (catalog# 131002, Synaptic Systems, Goettingen, Germany) diluted 1:1000 in blocking solution (3% bovine serum albumin, 10% goat serum, 3% Triton X-100 in 0.1 M PBS), washed, and then incubated for 2 h with Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Eugene, OR, USA) diluted 1:600 in blocking buffer. Immunolabeling for IgSf9b, Nlgn2, gephyrin, S-SCAM, GABAaRα1, and GABAaRγ2 was performed on methanol-fixed fresh frozen brain sections using a modified version of a published protocol. Brains were frozen immediately after dissection in an isopentane bath at −35 °C to −40 °C. Coronal sections were prepared using a Leica CM3050 cryostat (Leica, Wetzlar, Germany), mounted on glass slides, and dried at room temperature. Sections were then fixed in methanol at −20 °C for 5 min, and blocked for 1 h. The duration of incubation was 12 h with primary antibody and 2 h with secondary antibody for secondary antibodies. For secondary antibodies were used, diluted in blocking solution: Rabbit polyclonal anti-IgSf9b (catalog# HPA010802, Sigma Aldrich, Darmstadt, Germany) at 1:1000 mouse monoclonal anti-Nlgn2 (catalog# 129511, Synaptic Systems, Goettingen, Germany) at 1:1000 mouse monoclonal anti-Gephyrin (catalog# 115500, Synaptic Systems, Goettingen, Germany) at 1:1000; rabbit polyclonal anti-MAG12 (S–SCAM, catalog# M2441, Sigma Aldrich, Darmstadt, Germany); rabbit polyclonal anti-GABAaRα1 (catalog# 224203, Synaptic Systems, Goettingen, Germany) at 1:1000; guinea pig polyclonal GABAaRγ2 (generously provided by Dr. Jean-Marc Fritschy, University of Zürich) at 1:1000. The following secondary antibodies were obtained from Invitrogen, Eugene, USA, and diluted 1:600 in the blocking buffer: Alexa Fluor 555 goat anti-rabbit antibody, Alexa Fluor 488 goat anti-rabbit antibody, Alexa Fluor 550 goat anti-mouse antibody. Sections were washed with PBS after each incubation. The slides were then dried overnight at 4 °C, and covered with mounting media (Aqua-Poly/Mount; Polysciences, Eppelheim, Germany) and glass coverslips.

In vitro electrophysiology. Adult (8–12-week-old) WT, Nlgn2 KO, IgSf9b KO, and double KO mice additionally expressing a VIAAT-Venus transgene were used. Male mice were housed for 7 days after the surgery and before the recording. For data acquisition, the mice were connected to the electrophysiological equipment and placed in the OF chamber, where they were allowed to explore for 15 min. The electrophysiological signal was amplified and sent to the acquisition board. The raw signal was acquired at 52 kHz sampling rate, band pass filtered (0.1–9000 Hz), and stored for offline analysis. During the experiment, simultaneous electrophysiological and video recordings were made by the Cheetah Data Acquisition System.

All recordings were analyzed using custom-written MATLAB scripts. The signal was filtered between 0.7 and 400 Hz using a zero-phase distortion FIR filter and down sampled to 1 kHz. The multimeter method was used for the power analysis (Chronux Package). The following time windows were used: theta range (4–12 Hz), 1 s with 0.8 s of overlap; beta range (18–30 Hz), 1 s with 0.5 s of overlap; gamma range, 0.15 s with 0.1 s of overlap. To calculate the power spectra during the entire OF session, a 5 tapers spectra were obtained from the time period and the tracks of the movements in the periphery and center were extracted using a modified version of the autototyping toolbox. To evaluate the relative increase in beta power as a function of distance from the center, the distance from the center during the entire OF session was binned for each mouse (3 cm bins). To compute the beta power, the power was first summed across the beta band, and then the summed values were averaged for each location bin. The individual bin values were normalized by the average power in the periphery (>18 cm from center) and the linear correlation coefficients (fitlin function, MATLAB) per genotype were computed. To calculate the correlation between speed and beta power, the beta power was averaged in speed bins of 5 cm/s for each mice and the linear correlation coefficients computed as above. To evaluate power changes during the SAP, the time events at which the mouse showed a clear SAP from the periphery towards the center of the OF were manually extracted. The events were identified by a typical elongation of the body and a very slow forward movement that was followed by a retreat movement. For the beta band, the power across frequencies was summed to produce one value for each time point. Then, the power values at each time point during the SAP were averaged and the mean power values per event were averaged per group. The Morlet wavelet transform was used to visualize the power at different frequencies ranges as shown in Fig. 3k, with 40 wavelets at centered frequencies ranging from 1 to 120 Hz and a length of 10 cycles.

Analysis of synaptic markers. Immunolabeling for VIAAT was performed on perfusion-fixed brains as described for the cFos assay. Briefly, sections were incubated for 24 h with rabbit polyclonal anti-VIAAT antibody (catalog# 131002, Synaptic Systems, Goettingen, Germany) diluted 1:1000 in blocking solution (3% bovine serum albumin, 10% goat serum, 3% Triton X-100 in 0.1 M PBS), washed, and then incubated for 2 h with Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Eugene, OR, USA) diluted 1:600 in blocking buffer. Immunolabeling for IgSf9b, Nlgn2, gephyrin, S-SCAM, GABAaRα1, and GABAaRγ2 was performed on methanol-fixed fresh frozen brain sections using a modified version of a published protocol. Brains were frozen immediately after dissection in an isopentane bath at −35 °C to −40 °C. Coronal sections were prepared using a Leica CM3050S cryostat (Leica, Wetzlar, Germany), mounted on glass slides, and dried at room temperature. Sections were then fixed in methanol at −20 °C for 5 min, and blocked for 1 h. The duration of incubation was 12 h with primary antibody and 2 h with secondary antibody for secondary antibodies. For secondary antibodies were used, diluted in blocking solution: Rabbit polyclonal anti-IgSf9b (catalog# HPA010802, Sigma Aldrich, Darmstadt, Germany) at 1:1000 mouse monoclonal anti-Nlgn2 (catalog# 129511, Synaptic Systems, Goettingen, Germany) at 1:1000 mouse monoclonal anti-Gephyrin (catalog# 115500, Synaptic Systems, Goettingen, Germany) at 1:1000; rabbit polyclonal anti-MAG12 (S–SCAM, catalog# M2441, Sigma Aldrich, Darmstadt, Germany); rabbit polyclonal anti-GABAaRα1 (catalog# 224203, Synaptic Systems, Goettingen, Germany) at 1:1000; guinea pig polyclonal GABAaRγ2 (generously provided by Dr. Jean-Marc Fritschy, University of Zürich) at 1:1000. The following secondary antibodies were obtained from Invitrogen, Eugene, USA, and diluted 1:600 in the blocking buffer: Alexa Fluor 555 goat anti-rabbit antibody, Alexa Fluor 488 goat anti-rabbit antibody, Alexa Fluor 550 goat anti-mouse antibody. Sections were washed with PBS after each incubation. The slides were then dried overnight at 4 °C, and covered with mounting media (Aqua-Poly/Mount; Polysciences, Eppelheim, Germany) and glass coverslips.

Images of synaptic markers were obtained using a confocal laser scanning microscope (Leica SP2) with a ×63 oil immersion objective and ×8 digital zoom. For each set, sections were anatomically matched and the settings for laser power, gain and offset were kept constant during imaging. 12 stacks of 5 μm thickness and containing 2 optical sections each were obtained from each amygdala section (12 stacks from 4 sections for each mouse in total). Images were thresholded in ImageJ, with same threshold applied to all mice in each set. To quantify perisomatic synapses, the perisomatic area was identified by manually tracing the perimeter of the cell body (defined as a circular area large enough to contain the soma). The traced area was then enlarged by 1.4 μm or 2 μm in each direction for quantification of postsynaptic puncta or presynaptic puncta, respectively. The number of particles was quantified in this area using the “count particles” module in ImageJ, and the number of particles per area was divided by the length of the cell body perimeter to obtain the final result.
anesthetized with Avertin and perfused transcardially for 90 s with an ice-cold sucrose-based solution (6 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose, 2.5 mM glucose, and 3 mM potassium acid diluted in artificial cerebrospinal fluid (aCSF), 124 mM NaCl, 2.7 mM KCl, 26 mM NaHCO₃, and 1.25 mM NaH₂PO₄) as described previously for the preparation of amygdala slices from adult mice. The brains were rapidly dissected and placed in the same ice-cold sucrose-based solution. The brainstem was removed and the brains were mounted on a holder and transferred to the recording chamber for a preparation of 300 mOsm solutions. Slices containing the BA and central amygdala were transferred to a chamber filled with aCSF (see above) with additional 2 mM CaCl₂ and 1.3 mM MgCl₂ and equilibrated with 95% O₂/5% CO₂. Slices were allowed to recover for 20 min at 33 °C and maintained at room temperature thereafter. All chemicals were obtained from Merck Millipore (Molsheim, France), Sigma Aldrich (Darmstadt, Germany), or Tocris Bioscience (Bristol, UK).

Whole-cell patch-clamp recordings were obtained at room temperature (−22 °C) with an EPC10 amplifier (HEKA Elektronik, Germany). Slices were kept in a recording chamber and perfused with aCSF with additional 1.3 mM MgCl₂, 2 mM CaCl₂, 18.6 mM glucose, and 2.25 mM ascorbic acid (osmolarity = 320 mOsm) at a rate of 1–2 ml/min. Neurons were visually identified with infrared video microscopy using an upright microscope equipped with a x60 objective. VIAAT-positive neurons were identified by Venus expression. For recordings in BA, VIAAT-Venus-negative neurons were targeted, while for recordings in CeM, VIAAT-Venus-positive neurons were used.

Patch electrodes (3–5 MΩ open tip resistance when filled with internal solution) were pulled from borosilicate glass tubes. For voltage-clamp experiments to record miniature inhibitory postsynaptic currents (mIPSCs) and ERPs, patch electrodes were filled with Cs-based internal solution containing (in mM) 110 CsCl, 30 K-glutamate, 1.1 EGTA, 10 HEPES, 0.3 Na-GTP, and 0.1 CaCl₂, and (125 μM) 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (QX-314; Tocris-Cookson, Ellisville, MO); pH = 7.3 (adjusted with CsOH, 280 mOsM). To block glutamatergic EPSCs, 2 μM NBQX (6-cyano-7-nitroquinoxaline-2,3-dione) and 2 μM CPP ((RS)-3-(2-carboxypropylxyn-4-yl)-propyl-1-phosphonic acid) were added to the bath. Action potential (AP) firing was suppressed by adding 5 mM bicuculline methiodide (TOX) to the aCSF. For current-clamp experiments and recordings for the corresponding spontaneous postsynaptic excitatory currents (iEPSCs), patch electrodes were filled with K-glutamate-based internal solution containing (in mM) 125 K-glutamate, 20 KCl, 0.2 EGTA, 2 Mg-ATP, 10 HEPES, and 0.2 Na-GTP; pH = 7.3 (adjusted with KCl, 280 mOsM). To block GABAergic EPSCs, 25 μM bicuculline methiodide (TOX) was added to the bath. Firing thresholds were estimated from AP phase-plane plots and corrected for a measured liquid junction potential of 7.9 mV. To monitor series resistance on-line and to allow offline estimation of whole-cell membrane resistance and membrane capacitance, a voltage step (10 mV amplitude, 20 msec duration) was delivered at the beginning of each sweep during whole-cell voltage-clamp experiments.

Capacitive current transients were analyzed using a simplified two-compartment equivalent circuit model86. Mean current transients obtained by averaging ≥ 30 consecutive sweeps were fitted using a double-exponential function,

\[ I(t) = A_1 e^{-\tau_1 t} + A_2 e^{-\tau_2 t}, \]

where \( I(t) \) is the amplitude of the current at time \( t \), \( A_1 \) and \( \tau_1 \) denote the amplitude and time constant of the fast component of decay, \( A_2 \) and \( \tau_2 \) represent the amplitude and time constant of the slower component of decay, and \( A \) is the difference between the holding current and the final steady-state current at the end of the depolarizing pulse. The holding potential for whole-cell voltage-clamp recordings was set to −70 mV. Whole-cell voltage-clamp experiments were fitted in the analysis of the success resistance at initial ≥ 13 MΩ and did not change by more than 20% during the recording. Recordings with a leak current > 200 pA were rejected. Data were acquired with Patchmaster software (HEKA Elektronik, Germany), low-pass filtered at cut-off frequency of 5 kHz using a Bessel filter and digitized at 20 kHz. All offline analysis was performed with IgorPro (Wavemetrics, USA). mIPSCs were detected using a sliding template.

Surgical procedures were exactly as described for the injection of Retrobeads, except that 1 ml of virus was injected bilaterally into CeA using a Nanoject II Microinjector (Drummond, Broomall, PA, USA) and a Micro pump controller (WPI). Mice were alternately assigned to receive AAV-control shRNA or AAV-IgSF9b-shRNA injections based on order of birth. The following coordinates relative to Bregma were used: AP (anteroposterior) −0.58, ML (medio-lateral) ±2.48, DV (dorsoventral) and −5.4. After surgery, mice were housed in pairs and were allowed to recover for 6 weeks before assessment of behavior in the OF as described above. Mice were sacrificed following OF exposure for verification of the injection site as defined by GFP expression (Supplementary Fig. 5). Only mice in which both bilateral injection sites were correctly positioned in CeM were included in the study. Moreover, mice with any GFP expression in BLA or CeL were excluded, although minor, low-expression leakage into other border areas was tolerated. In total, 11 mice were excluded due to mistargeting (WT = Ctrl shRNA, 4 animals; WT + IgSF9b shRNA, 1 animal; Nlgn2 KO + Ctrl shRNA, 3 animals; and Nlgn2 KO + IgSF9b shRNA, 3 animals).

**Statistical Analysis**. Sample sizes were estimated based on prior experience with the methods used in this study. All data were analyzed statistically using Prism (GraphPad Software, La Jolla, CA, USA) or Matlab. Outliers were identified using the Grubbs’ test and were removed prior to statistical analysis. Behavioral scores were subjected to two-way ANOVA with post-hoc Tukey’s tests for comparison between groups. Data obtained from histological experiments were analyzed using two-way ANOVA with post-hoc paired t-tests for comparison between groups. Data obtained from in vitro electrophysiological experiments were analyzed using two-way ANOVA with post-hoc Tukey’s test for comparison between groups. Groups. Distributions of mean mIPSC frequencies and amplitudes were analyzed using the Kolmogorov–Smirnov test. Data obtained from in vivo electrophysiological experiments were analyzed using two-way ANOVA with post-hoc Tukey’s test for comparisons between groups.

**Code availability**. Custom MATLAB scripts written for the analysis of LFPs are available from the corresponding author upon request.

**Data availability**. All data produced from this study are available from the corresponding author upon request.

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Author contributions
D.K.-B., O.B. and N.B. conceived the study. D.K.-B. and O.B. designed all experiments (except in vivo electrophysiology experiments, which were designed by H.C.-S. and O.B.). O.B. performed most of the behavioral, immunohistochemical, and in vitro electrophysiology experiments and performed stereotaxic surgeries under supervision of D.K.-B. H.C.-S. performed and analyzed in vivo electrophysiology experiments. C.P.C. assisted with stereotaxic surgeries and behavior experiments. M.H. assisted with immunohistochemical experiments and with establishing the in vitro electrophysiology protocol. S.W. assisted with AAV production and with behavioral experiments. H.A. assisted with cFOS quantification. N.K. and L.D.H. assisted with establishing the in vitro electrophysiology protocol. O.M.S. assisted with establishing a protocol for stereotaxic surgery and AAV virus production. Y.T. provided research reagents. H.E. provided guidance and equipment for behavioral experiments. H.T. assisted with establishing the in vitro electrophysiology experiments and provided software for data analysis. N.B.
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