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Archival Report

Neuroligin-2 Determines Inhibitory Synaptic Transmission in the Lateral Septum to Optimize Stress-Induced Neuronal Activation and Avoidance Behavior

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ABSTRACT

BACKGROUND: Investigations in the neocortex have revealed that the balance of excitatory and inhibitory synaptic transmission (E/I ratio) is important for proper information processing. The disturbance of this balance underlies many neuropsychiatric illnesses, including autism spectrum disorder and schizophrenia. However, little is known about the contribution of E/I balance to the functioning of subcortical brain regions, such as the lateral septum (LS), a structure that plays important roles in regulating anxiety-related behavior.

METHODS: We manipulated E/I balance in the mouse LS by localized conditional deletion of neuroligin-2, a postsynaptic cell adhesion protein located at gamma-aminobutyric acidergic synapses and important for inhibitory synaptic transmission. We then performed analyses of synaptic transmission in the LS, stress-induced expression of immediate early gene *c-fos*, and anxiety-related and depression-related behavior.

RESULTS: The absence of neuroligin-2 in the LS in the mature mouse brain resulted in postsynaptic impairment of inhibitory synaptic transmission. Importantly, the reduced inhibition and resulting E/I imbalance decreased the responsiveness of LS neurons to stress. Furthermore, this E/I imbalance in the LS was associated with impaired stress-induced activation of downstream hypothalamic nuclei and reduced avoidance behavior of the animals in the elevated plus maze.

CONCLUSIONS: Our results described the synaptic function of neuroligin-2 in the LS, uncovered a positive association between c-Fos–expressing neurons in the LS and downstream hypothalamic areas and avoidance behavior, and demonstrated that intact inhibitory synaptic transmission and proper E/I balance are required for the optimal functioning of this subcortical circuit.

Keywords: Anxiety, Avoidance, c-Fos, Excitation-inhibition balance, Hypothalamus, Lateral septum

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The lateral septum (LS) is a subcortical forebrain structure that is important for the regulation of affective behaviors, including anxiety, aggression, social recognition, and food seeking (1–5). The LS is composed of gamma-aminobutyric acidergic (GABAergic) neurons, which receive cognitive and emotional information from multiple brain regions and project to hypothalamus and midbrain to control behavioral responses (6). In particular, the LS receives dense projections from the hippocampus as part of the septo-hippocampal axis, which is considered a major regulator of anxiety-related behavior (7–9).

Classically, the function of LS has been studied using excitotoxic lesions or pharmacological manipulations (8,10,11). More recently, optogenetic tools are being applied to dissect its role in behavior (1,3,5). In contrast to the progress being made in associating LS neuronal subpopulations with specific behavioral functions, the molecules that organize excitatory and inhibitory synapses within the LS have not been examined. This information is crucial because balance of synaptic excitation and inhibition (E/I ratio) is important for optimal functioning of neuronal circuits, as has been elucidated in the neocortex (12,13). However, it is largely unknown which molecules modulate the E/I ratio in the LS and how alteration of this ratio contributes to the ability of this brain region, and more generally a subcortical circuit, to respond to salient experiences and regulate behavior.

To address some of these major deficiencies in our understanding, we focused on the functional analysis of a synaptic protein, neuroligin-2 (NL2), in the LS. NL2 belongs to a family of postsynaptic cell adhesion proteins (NL1–NL4) that play important roles in the developmental maturation and function of synapses (14–16). NL2 is localized to GABAergic synapses (17,18) and is selectively important for inhibitory synaptic transmission, thus affecting E/I balance (19–21). The *Nlgn2* gene is associated with schizophrenia, major depressive disorder, autism spectrum disorder, and anxiety in human patients (22–25). Constitutive NL2 knockout mice as well as

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knockin mice with a loss-of-function R215H mutation show robust anxiety-related behavior (26–29). This makes it crucial to study the function of NL2 in anxiety-mediating regions and circuits of the brain, such as the LS. The investigation of NL2 function in the LS could also potentially elucidate how E/I balance within its circuits determines affective behavior. In this study, we used virus-mediated expression of Cre recombinase to achieve conditional deletion of NL2 in the LS of *Nlgn2*$^{fl/fl}$ mice. We present our results on the effect of the absence of NL2 in the LS on synaptic transmission, local and target neuron responsiveness to stress, and affective animal behavior.

METHODS AND MATERIALS

Detailed methods and materials are described in the Supplement. Briefly, floxed *Nlgn2* (*Nlgn2*$^{fl/fl}$) male and female mice (B6;SJL-NL2$^{tm1.1Sud}$/J, Stock No. 025544, RRID:IMSR_JAX:025544; The Jackson Laboratory, Bar Harbor, ME) were injected with adeno-associated virus (AAV) in the LS, expressing green fluorescent protein (GFP)–tagged Cre recombinase or GFP. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center. Whole-cell patch-clamp recordings were performed on infected neurons to assay excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs). For analyzing the responsiveness of the neurons in the LS and hypothalamic nuclei to stress, mice underwent a single 30-minute session of acute restraint stress (ARS), which was followed by perfusion fixation of their brains and immunohistochemistry on the vibratome sections using an anti–c-Fos antibody. Anxiety-related and depression-related behaviors were tested in mice using the elevated plus maze (EPM), open field test (OFT), and tail suspension test (TST). Statistical analyses were performed using Student unpaired *t* test or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post hoc test (as appropriate).

RESULTS

Conditional Deletion of NL2 in the LS Impairs Inhibitory Synaptic Transmission

To obtain the deletion of *Nlgn2* gene selectively in the LS in a mature mouse brain, we injected AAV-expressing Cre recombinase in the LS of 4- to 5-week-old *Nlgn2*$^{fl/fl}$ mice and allowed the viral expression for more than 6 weeks (Figure 1A, B). Animals injected with AAV expressing GFP were used as control subjects. Accurate and selective targeting of the virus was confirmed by observing the GFP signal, which was mostly confined to the LS (Supplemental Figure S1). Viral expression of Cre recombinase was present in the vast majority of neurons (Supplemental Figure S2), leading to an efficient deletion of NL2, as evidenced in the Western blot derived from LS lysates (Figure 1C) as well as in the immunohistochemical staining of LS sections (Figure 1D; Supplemental Figure S3). The deletion remained confined to the LS, as NL2 expression was unaltered in the adjacent dorsal striatum, nucleus accumbens, and bed nucleus of the stria terminalis (Figure 1D; Supplemental Figure S3).

We examined the effect of NL2 deletion on synaptic transmission in the LS by performing whole-cell patch-clamp recordings in acute slices from adult mice. Excitatory and inhibitory synaptic transmissions were recorded (Figure 1E) from visually identified GFP-tagged Cre recombinase–expressing (Cre+) and GFP-expressing (control) neurons in the intermediate part of the LS (Supplemental Figure S4). We confined our measurements to the intermediate part (also called rostral or rostroventral LS) because previous studies had implicated this LS region in regulating stress-related behavior [reviewed in (30)], and the stress-induced activation of immediate early gene (IEG) *c-fos* is most prominent in this area (31,32). We examined IPSCs at different stimulation intensities and found that the amplitude was markedly decreased in the Cre+ cells compared with control cells (Figure 1F). In contrast, there was no difference in the EPSC amplitude between Cre+ cells and control cells (Figure 1G). We then calculated the ratio of IPSCs to EPSCs and found that this ratio showed a large decrease in Cre+ cells (Figure 1H). These results obtained after postdevelopmental deletion of NL2 in the LS unequivocally indicate that NL2 has a continuing role in maintaining inhibitory synaptic transmission in the LS in the mature brain.

Alteration of Inhibitory Synaptic Transmission in the Absence of NL2 Is Caused by a Postsynaptic Mechanism

To investigate if the effect on inhibitory synaptic transmission observed in the LS after deletion of NL2 had a presynaptic or postsynaptic origin, we recorded and analyzed miniature synaptic transmission in the presence of tetrodotoxin. The amplitude of miniature IPSCs (mIPSCs) was strongly reduced in the Cre+ cells compared with control cells (*t* test, $p = .0002$; n = 10–11 cells, 5 mice per group) (Figure 2A, left graph). The frequency of the mIPSCs did not differ significantly between the two groups (*t* test, $p = .1674$) (Figure 2A, middle graph), although there was a trend toward a decrease. The time constant of mIPSC decay also remained unaltered (*t* test, $p = .3065$) (Figure 2A, right graph). In contrast to a large effect on mIPSC amplitude, no significant difference was observed in the miniature EPSC (mEPSC) amplitude between the two groups (*t* test, $p = .8554$; n = 10–11 cells, 3 mice per group) (Figure 2B, left graph). The frequency of mEPSCs was also not changed (*t* test, $p = .4133$) (Figure 2B, middle graph), and likewise the time constant of mEPSC decay was not changed (*t* test, $p = .4466$) (Figure 2B, right graph). The reduction in the amplitude of mIPSCs observed in our experiments suggests that the deletion of NL2 leads to a postsynaptic impairment of GABAergic synapses in the LS, which could arise from a loss of the clustering of $GABA_A$ receptors ($GABA_A$Rs) on the postsynaptic membrane (20,33,34). To further explore the mechanism underlying the regulation of synaptic $GABA_A$Rs by NL2 in the LS, we performed immunohistochemical staining of LS sections for the γ2 subunit of $GABA_A$Rs, which is a common constituent of synaptic receptors (35) and associates with NL2 (34). The absence of NL2 reduced the size of γ2 puncta in the LS, reflecting declustering of this subunit (*t* test, $p < .001$, n = 24–26 sections, 3 mice per group) (Figure 2C). Moreover, the density of γ2 puncta was decreased (*t* test, $p < .001$) (Figure 2C), suggesting that $GABA_A$Rs containing this subunit were destabilized in the absence of NL2. To examine whether the number of inhibitory synapses was altered following NL2 deletion, we performed

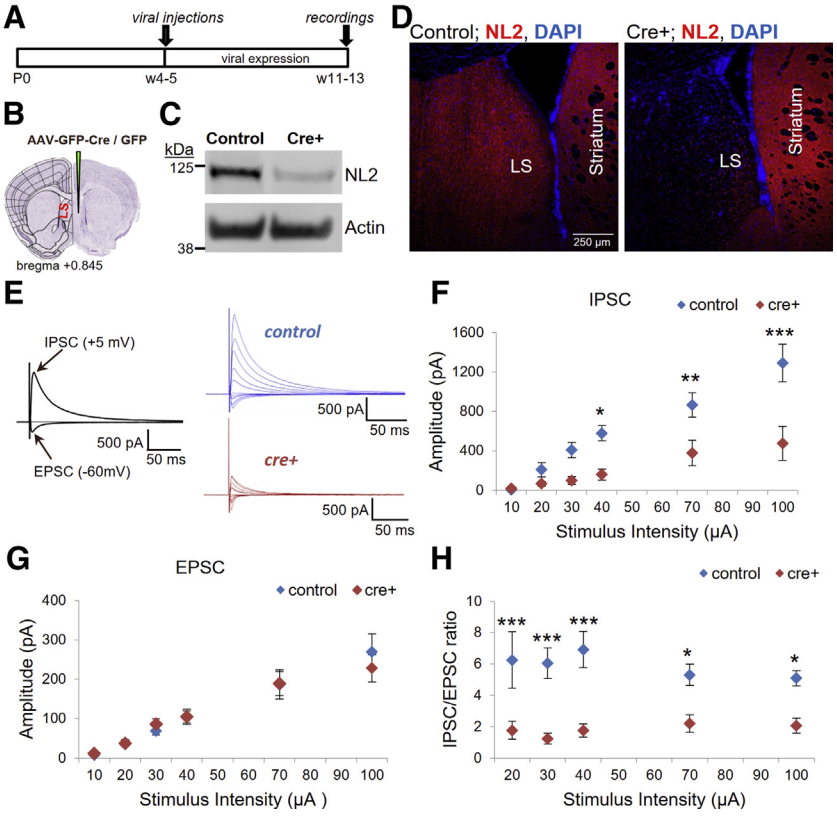

Figure 1. Conditional deletion of neuroligin-2 (NL2) in the lateral septum (LS) impairs inhibitory synaptic transmission. **(A)** Timeline for the experiments described in this figure. Virus expressing green fluorescent protein (GFP)–tagged Cre recombinase or GFP was injected in the LS of *Nlgn2*$^{fl/fl}$ animals at 4–5 weeks of age, followed by recordings 7–9 weeks later. **(B)** Nissl-stained coronal section of mouse brain (courtesy of Allen Brain Atlas), depicting the site of viral injection. **(C)** Western blot of lysates from control and Cre+ LS slices, immunoblotted with anti-NL2 or anti–β-actin antibody. **(D)** Immunohistochemical confirmation of NL2 deletion in the LS as shown by the absence of staining with NL2 antibody (in red) in Cre+ (right panel) and robust staining in control (left panel) sections. NL2 expression is selectively reduced in Cre+ LS with no change in the adjacent striatum. **(E)** Method of analysis of the amplitude of averaged inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) recorded from LS neurons (left panel). Sample averaged IPSCs and EPSCs recorded at different stimulation strengths from control (top right panel) and Cre+ (bottom right panel) neurons. **(F)** Amplitude of IPSCs recorded in control and Cre+ neurons at different stimulation intensities. There was marked reduction in IPSC amplitude in Cre+ cells. Two-way analysis of variance genotype × stimulation intensity interaction: $F_{5,144}$ = 4.099, p = .0016; genotype: $F_{1,144}$ = 37.01, $p < .0001$; stimulation intensity: $F_{5,144}$ = 20.51, $p < .0001$; Bonferroni's post hoc test: 10 µA, $p > .999$; 20 µA, $p > .999$; 30 µA, $p > .2665$; 40 µA, $p = .0206$; 70 µA, $p = .0045$; 100 µA, $p < .0001$; control: n = 10–13 cells, 4–5 mice; Cre+: n = 14 cells, 6 mice. **(G)** Amplitude of EPSCs recorded in control and Cre+ cells at different stimulation intensities was similar. Two-way analysis of variance genotype × stimulation intensity interaction: $F_{5,147}$ = 0.3489, p = .8823; genotype: $F_{1,147}$ = 0.05807, p = .8099; stimulation intensity: $F_{5,147}$ = 29.6, $p < .0001$; control: n = 10–13 cells, 4–5 mice; Cre+: n = 14 cells, 6 mice. **(H)** Ratio of IPSC to EPSC amplitude in control and Cre+ cells at different stimulation intensities. The ratio was strongly reduced in Cre+ cells. Two-way analysis of variance genotype × stimulation intensity interaction: $F_{4,119}$ = 0.8425, p = .5009; genotype: $F_{1,119}$ = 70.66, $p < .0001$; stimulation intensity: $F_{4,119}$ = 0.339, p = .8512; Bonferroni's post hoc test: 20 µA, p = .0007; 30 µA, p = .0003; 40 µA, $p < .0001$; 70 µA, p = .0212; 100 µA, p = .0250; control: n = 10–13 cells, 4–5 mice; Cre+: n = 14 cells, 6 mice. All graphs represent mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$. AAV, adeno-associated virus; DAPI, 4′,6-diamidino-2-phenylindole.

immunostaining for vesicular GABA transporter, which is a presynaptic marker of GABAergic synapses. Our results showed that there was no alteration in vesicular GABA transporter puncta size (*t* test, p = .0559, n = 26–29 sections, 3 mice per group) (Figure 2D) or density (*t* test, p = .2911) (Figure 2D), indicating that NL2 is unlikely to play a major role in the maintenance of GABAergic synapse number in the LS.

Impaired Responsiveness of LS Neurons to Stress in the Absence of NL2

Salient experiences can induce expression of IEGs in activated neurons (36). Multiple studies have shown that various behavioral challenges, including different forms of stress, increase the expression of c-Fos in the LS (31,37–42). However, the circuit dynamics that leads to the optimal behavioral activation of c-Fos in the LS is unknown. To understand how the impairment of inhibitory synaptic transmission following NL2 deletion in the LS affects the ability of LS neurons to be activated by stress, we performed an analysis of c-Fos after a stress challenge. *Nlgn2*$^{fl/fl}$ mice that had received intra-LS injections of AAV expressing Cre recombinase or control virus were subjected to ARS for 30 minutes, which was followed by harvesting of their brains and immunostaining for c-Fos on the brain sections (Figure 3A). Two-way ANOVA showed a significant effect for genotype × stress interaction ($F_{1,71}$ = 7.182, $p < .0091$), significant main effect for genotype ($F_{1,71}$ = 17.45, $p < .0001$), and significant main effect for stress ($F_{1,71}$ = 28.48, $p < .0001$) (n = 13–25 sections, 3–6 mice per group). Bonferroni's multiple comparisons test revealed that ARS increased the number of c-Fos–positive neurons in the LS of control animals ($p < .0001$) (Figure 3B, C), but not in animals with conditional deletion of NL2 (p = .4168) (Figure 3B, C). This resulted in a smaller number of c-Fos+ neurons following ARS in Cre+ LS compared with control LS ($p < .0001$) (Figure 3B, C). Our results thus indicate that reduced inhibitory synaptic transmission in the LS is associated with impaired response of LS neurons to ARS.

Conditional Deletion of NL2 in the LS Alters Anxiety-Related Avoidance Behavior

Next, we examined the consequences of conditional NL2 deletion in the LS on animal behavior. We focused our investigations on anxiety-related and depression-related behavior because previous lesion and pharmacological

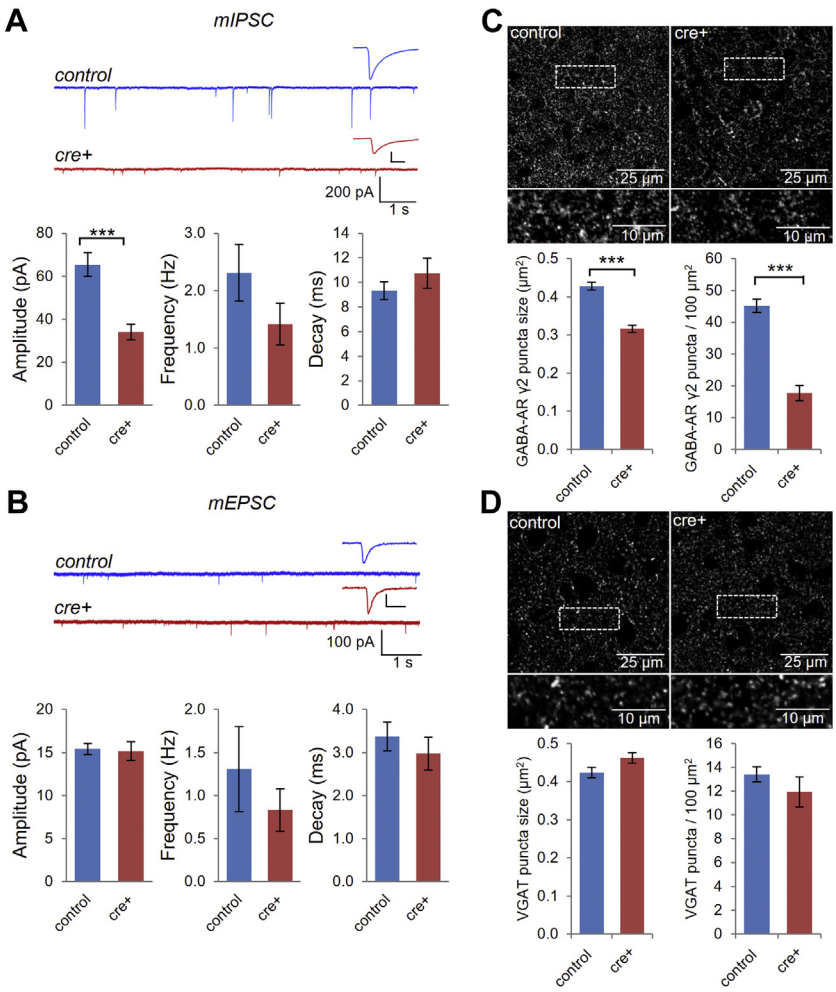

Figure 2. Neuroligin-2 (NL2) deletion in the lateral septum (LS) alters inhibitory synaptic transmission by a postsynaptic mechanism. **(A)** The recordings for miniature inhibitory postsynaptic currents (mIPSCs) show a reduction in mIPSC amplitude in Cre+ cells. Sample traces from control (blue, top) and Cre+ (red, bottom) neurons are shown above the graphs. Averaged mIPSCs for each condition are shown above their respective traces. The scale bars for averaged mIPSCs represent 10 ms (x-axis) and 20 pA (y-axis). The graphs depict that the amplitude of mIPSCs is reduced in the Cre+ neurons compared with the control neurons (left). The frequency (middle) and the time constant of decay (right) remain unchanged. **(B)** Sample traces of recordings for miniature excitatory postsynaptic currents (mEPSCs) in control (blue, top) and Cre+ (red, bottom) neurons are shown above the graphs. Averaged mEPSCs for each condition are shown above their respective traces. The scale bars for averaged mEPSCs represent 10 ms (x-axis) and 10 pA (y-axis). The graphs show that the amplitude (left), frequency (middle), and decay time constant (right) of mEPSCs are similar in control and Cre+ neurons. **(C)** Absence of neuroligin-2 in the LS reduces puncta size and puncta density of gamma-aminobutyric acid A receptor ($GABA_AR$) γ2 subunit. Sample confocal images showing γ2 immunostaining in the LS of control and Cre+ (top panels) group. Boxed regions in top panels are shown at higher magnification in bottom panels. Quantification of puncta size (left graph) and puncta density (right graph) are also shown. **(D)** Deletion of neuroligin-2 in the LS had no effect on the puncta size and puncta density of vesicular GABA transporter (VGAT). Sample confocal images showing VGAT immunostaining in the LS of control and Cre+ groups (top panels). Boxed regions in top panels are shown at higher magnification in bottom panels. Quantification of puncta size (left graph) and puncta density (right graph) are also shown. All graphs represent mean ± SEM. ***$p < .001$.

studies had revealed the importance of the LS in controlling these behaviors (8,10,11,43). We tested *Nlgn2*$^{fl/fl}$ mice that had received bilateral stereotaxic injections of AAV expressing GFP-tagged Cre recombinase (Cre+) or GFP (control) in the LS on the EPM, OFT, and TST (Figure 4A). Our experiments revealed that animals with conditional deletion of NL2 in the LS spent significantly more time exploring the open arms of the EPM compared with control animals (*t* test, $p = .0019$; $n = 10$ mice per group) (Figure 4B, left graph). The total distance traveled in the open arms (*t* test, $p = .0004$) (Figure 4B, middle graph) and the number of entries in the open arms (*t* test, $p = .0491$) (Figure 4B, right graph) were also significantly increased in Cre+ animals. In the OFT, the total distance traveled was similar in the two groups, indicating that the animals' locomotion was not altered by the absence of the NL2 in the LS (*t* test, $p = .1996$; $n = 10$ mice per group) (Figure 4C, left graph). Interestingly, the time spent in the center of the OFT remained unchanged in the Cre+ animals (*t* test, $p = .0522$) (Figure 4C, right graph), as was the distance traveled in the central square (*t* test, $p = .4578$) (Figure 4C, middle graph). Given that we saw a robust decrease in avoidance behavior in Cre+ animals in the EPM, it was surprising that the time spent in the center of the OFT was unaltered. Even though the exploration of both the open arms in the EPM and the central zone in the OFT is considered to measure risk avoidance behavior, unmatched results from the two tests are commonplace, and a meta-analysis of published studies found no concordance between the two methods (44). Also, such discrepancies were reported previously (but remained unexplained) in studies dealing with the analysis of the LS as well as NL2 function (7,21). One possible reason for the discrepancy may be that the EPM is a more sensitive test of anxiety because the elevated arms are inherently more anxiogenic than the OFT (45) and therefore more suitable to reveal deficits that are dependent on the activation of stress-responsive neuronal circuitry. We next examined depression-related behavior in our animals using the TST. We recorded the time animals spent immobile after being suspended upside down, as a measure of behavioral despair. The percentage of time mice spent immobile in the TST was unchanged between control and Cre+ animals (*t* test, $p = .5964$; $n = 10$ mice per group)

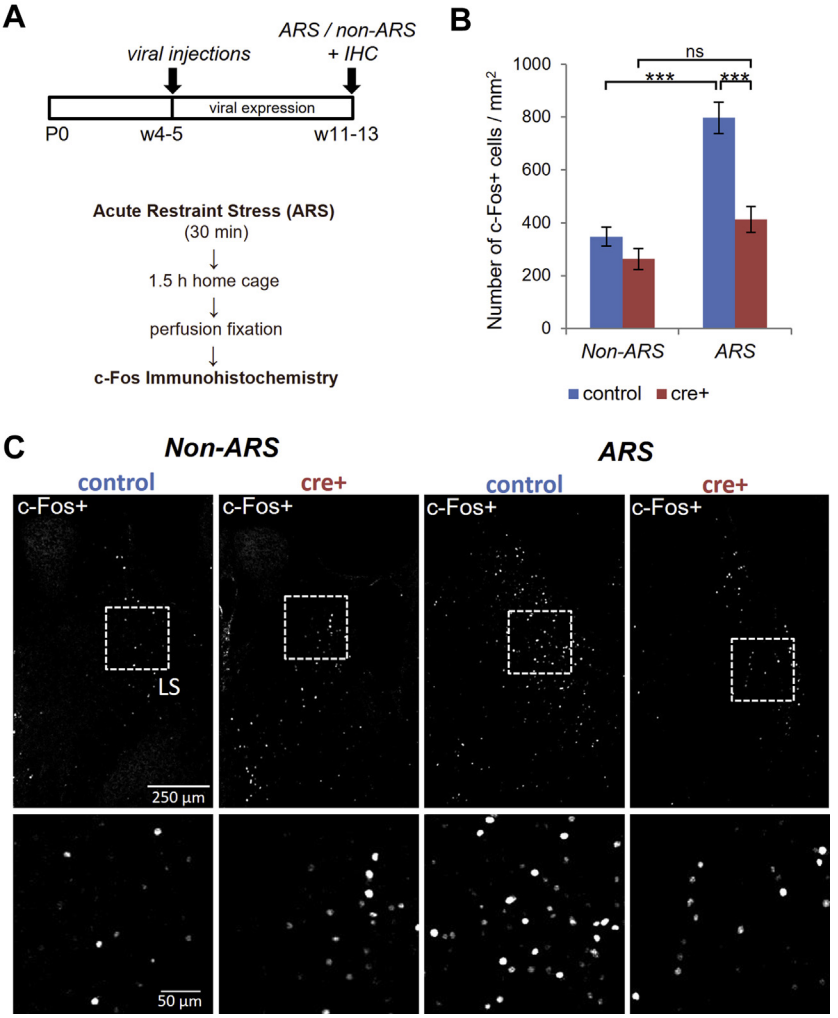

Figure 3. Impaired responsiveness of lateral septum (LS) neurons to stress in the absence of neuroligin-2. **(A)** Timeline of the behavioral challenge in the form of acute restraint stress (ARS) and c-Fos immunohistochemistry (IHC) performed in the LS. **(B)** Quantification of c-Fos+ cells in confocal stacks (region of interest 250 µm × 250 µm × 40 µm) from nonstressed condition (non-ARS) and following ARS in *Nlgn2*$^{fl/fl}$ animals that received intra-LS injection of control or Cre recombinase virus. **(C)** Sample confocal images showing c-Fos+ cells in the LS of control and Cre+ groups (top panels). Boxed regions in top panels are shown at higher magnification in bottom panels. All graphs represent mean ± SEM. ***$p < .001$. ns, not significant.

(Figure 4D). Overall, our behavior experiments showed that the conditional deletion of NL2 in the LS results in a selective decrease in avoidance behavior in the EPM without causing any change in depression-related behavioral despair in the TST.

Absence of NL2 in the LS Impairs Stress-Induced Neuronal Activation in the Lateral Hypothalamus and Anterior Hypothalamic Area

To understand how the E/I imbalance and impaired stress-induced activation in the LS in the absence of NL2 leads to reduced avoidance behavior, we examined the downstream target regions of LS projection neurons. We focused on the anterior hypothalamic area (AHA) and lateral hypothalamus (LH), two hypothalamic regions that receive prominent projections from the LS and have been implicated in controlling anxiety-related behavior (3,46,47). Conditional deletion of NL2 in the LS caused a strong reduction in the number of cells that expressed c-Fos in the LH following 30 minutes of ARS compared with control animals that received the same stress (Figure 5A–C). Two-way ANOVA revealed a statistically significant effect for genotype × stress interaction ($F_{1,69}$ = 5.607, p = .0207), significant main effect for genotype ($F_{1,69}$ = 12.7, p = .0007), and significant main effect for stress ($F_{1,69}$ = 30.37, $p < .0001$) (n = 14–21 sections, 4 mice per group). Bonferroni's post hoc test showed that ARS increased the number of c-Fos+ cells in the LH of control mice ($p < .0001$), but not in the mice with conditional NL2 deletion in the LS (p = .2166), resulting in a smaller number of c-Fos+ neurons in the LH following ARS in Cre+ mice compared with control mice (p = .0002). The conditional NL2 deletion in the LS also caused a decrease in c-Fos–expressing neurons following ARS in the AHA (two-way ANOVA for genotype: $F_{1,69}$ = 6.772, p = .0113; for stress: $F_{1,69}$ = 21.05, $p < .0001$; and for genotype × stress interaction: $F_{1,69}$ = 26.2, $p < .0001$; n = 14–21, 4 mice per group; with Bonferroni's post hoc test: control, non-ARS vs. ARS, $p < .0001$; Cre+, non-ARS vs. ARS, $p > .999$; ARS, control vs. Cre+, $p < .0001$) (Figure 5A–C). Interestingly, c-Fos response to ARS in the paraventricular hypothalamic nucleus (PVN), an important node in the hypothalamic-pituitary-adrenal axis,

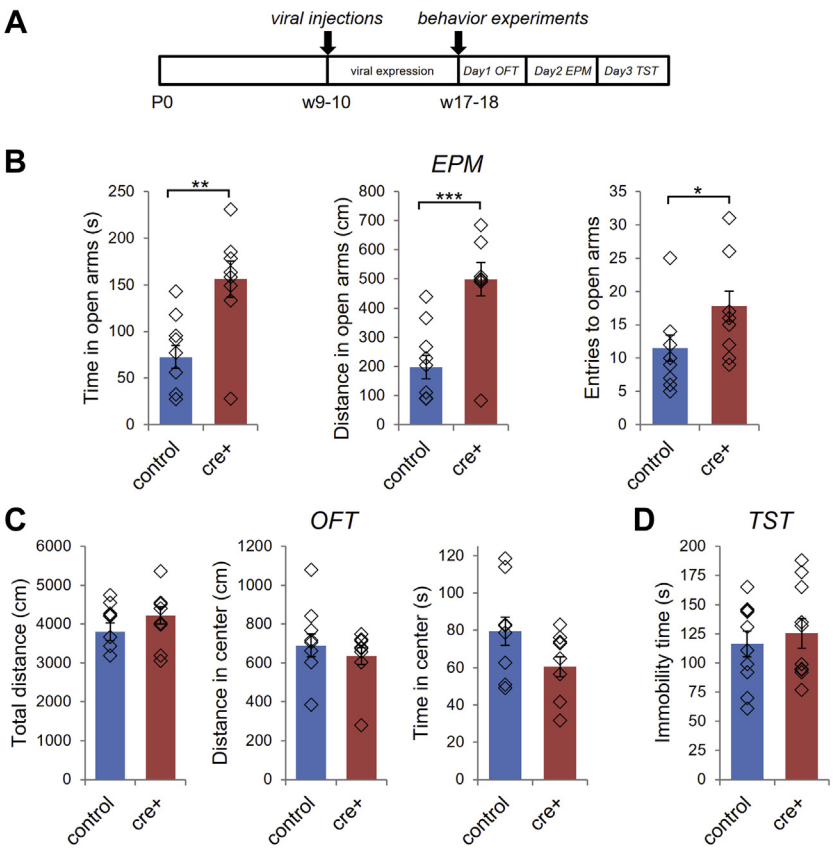

Figure 4. Conditional deletion of neuroligin-2 in the lateral septum reduces anxiety-related avoidance behavior. **(A)** Timeline for the behavior experiments following bilateral injections of control or Cre recombinase–expressing adeno-associated virus in the lateral septum of *Nlgn2*$^{fl/fl}$ mice. Animals were tested on 3 consecutive days in open field test (OFT), elevated plus maze (EPM), and tail suspension test (TST). **(B)** Quantification of behavioral parameters in EPM shows that mice with bilateral deletion of neuroligin-2 in the lateral septum spend more time in open arms (left panel), travel longer distances in open arms (middle panel), and make more entries to open arms (right panel). **(C)** Quantification of OFT data shows no difference between the two groups of mice in the total distance traveled (left panel), distance traveled in the central square (of one fourth the total area), and the time spent in the center (right panel). **(D)** Quantification of immobility time in TST shows no difference between the two groups of animals. All graphs represent mean ± SEM. Individual value from each animal is represented by an open quadrilateral. *$p < .05$, **$p < .01$, ***$p < .001$.

remained unaffected by the deletion of NL2 in the LS (two-way ANOVA revealed a significant main effect only for stress: $F_{1,69} = 140$, $p < .0001$; $n = 14–21$, 4 mice per group; with Bonferroni's post hoc test: control, non-ARS vs. ARS, $p < .0001$; Cre+, non-ARS vs. ARS, $p < .0001$; ARS, control vs. Cre+, $p > .999$) (Figure 5A–C). Even though PVN does not receive direct projections from the LS, acute optogenetic manipulation of LS projections to the AHA have been shown to affect PVN neurons and circulating corticosteroid levels (3). Our experiments that involve more chronic manipulations of LS neuronal output suggest that the dependence of PVN neuronal activation on LS is redundant at longer timescales and is likely compensated by homeostatic regulation of myriad inputs that reach the PVN (48). Instead, our results suggest that altered E/I balance in the LS has an uncompensated effect on stress-induced activation of neurons in the LH and AHA. In addition, our findings suggest a positive association between c-Fos expression in a subset of LH and/or AHA neurons and mounting of avoidance behavior in anxiety.

DISCUSSION

The conditional deletion of NL2 in the LS in mature mouse brain allowed us to reach the following conclusions. First, NL2 is required for proper function of inhibitory synapses on LS neurons. Second, optimal function of inhibitory synaptic transmission and E/I balance at synapses on LS neurons is required for efficient responsiveness of LS and downstream hypothalamic neurons (in LH and AHA) to behavioral stress. Finally, stress-induced activation of LS neurons leading to IEG expression is positively correlated with execution of avoidance behavior in an anxiety-inducing situation.

Our electrophysiological data demonstrate that NL2 is required for the maintenance of postsynaptic function at GABAergic synapses in the LS. The strong reduction in mIPSC amplitude along with decreases in $GABA_AR$ subunit $\gamma 2$ observed in our experiments after NL2 deletion supports this conclusion. As we did not observe major effects of NL2 deletion on mIPSC frequency or on vesicular GABA transporter puncta, NL2 seems dispensable for presynaptic function or maintenance of synapse number in the LS. The trend toward a decrease in mIPSC frequency in our experiments is likely secondary to robust reduction of mIPSC amplitude causing a proportion of events to fall below the threshold of detection. The lack of effect of NL2 deletion on excitatory synaptic transmission in our experiments is congruent with most published results from other brain regions (20,21,26,29,49). However, a recent study showed that the absence of NL2 in cortical astrocytes reduced excitatory synaptic transmission on cortical neurons (50). As we used a cytomegalovirus promoter to drive Cre recombinase expression in the LS for our electrophysiology experiments, which would have deleted NL2 in glia, our results indicate that astrocytic NL2 is unlikely to

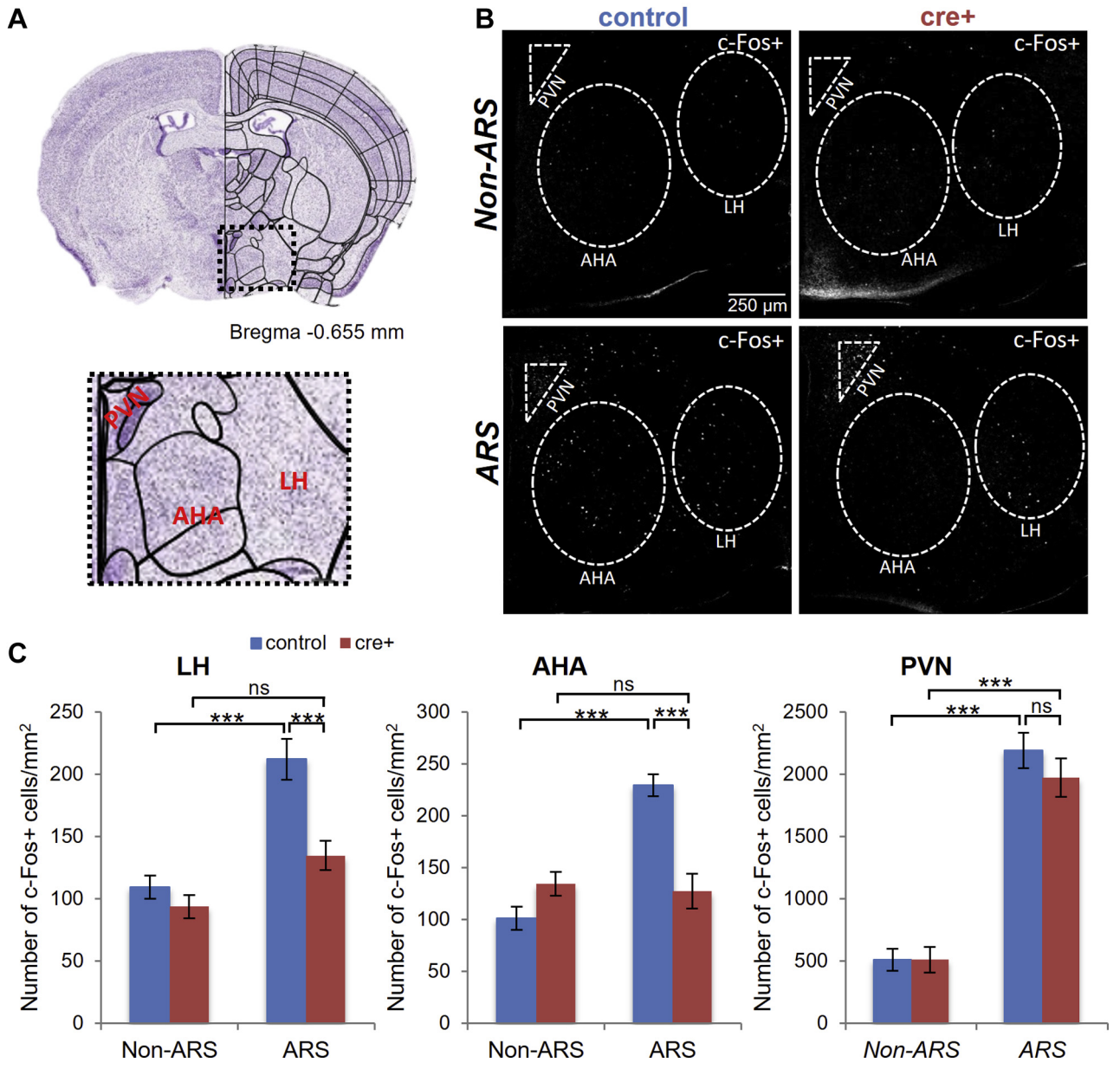

Figure 5. The absence of neuroligin-2 in the lateral septum (LS) blocks the stress-induced activation of neurons in LS projection areas in the hypothalamus. **(A)** Nissl-stained coronal section of mouse brain (courtesy of Allen Brain Atlas) at lower (top panel) and higher (bottom panel) magnification, depicting the locations of lateral hypothalamus (LH), anterior hypothalamic area (AHA), and paraventricular hypothalamic nucleus (PVN) at which c-Fos–expressing cells were quantified. **(B)** Sample confocal images showing c-Fos expression in LH, AHA, and PVN in nonstressed condition (non–acute restraint stress [ARS]) and following ARS in mice injected in the LS with control virus (control) or virus expressing Cre recombinase (Cre+). Regions of interest were manually drawn on each image stack according to the stereotaxic atlas. **(C)** The density of c-Fos+ cells in nonstressed condition (non-ARS) and following ARS in confocal stacks for the three different areas analyzed. The absence of neuroligin-2 in the LS prevented the increase in the number of c-Fos+ cells in LH and AHA following ARS but had no effect in the PVN. All graphs represent mean ± SEM. ***$p < .001$. ns, not significant.

control the maintenance of excitatory synaptic transmission in this brain region.

The selective effect of NL2 deletion on inhibitory synaptic transmission resulted in E/I imbalance in the LS. When we explored the consequences of this imbalance on behavior-induced neuronal activation, we observed a reduced c-Fos expression in the LS triggered by ARS. This important finding indicates that intact inhibition in the LS is required for optimal stress-induced neuronal activation. The understanding of the mechanisms underlying this apparent paradoxical result requires further investigation. We propose that by reducing the inhibition on LS neurons, the deletion of NL2 increases the background activity in the LS, which triggers the neuronal network to undergo normalization (51) or downscaling (52),

leading to the elevation of the thresholds for generating action potentials and c-Fos induction. In addition, there could be a disruption of synchronous rhythms between the LS and connected regions (5). It is also possible that NL2 has a more direct effect on c-Fos expression, independent of its control of inhibitory synaptic transmission. We believe that this is unlikely to be responsible for our findings because deletion of NL2 in the LS did not alter basal c-Fos levels and produced impairment of stress-induced c-Fos expression in downstream hypothalamic nuclei containing intact NL2. It must be emphasized here that c-Fos is one of many IEGs that are activated by salient experience and whose induction is an indirect measure of neuronal activation (53). Future experiments involving measurements of LS neuronal activity in vivo in awake-behaving animals (for example, using calcium-sensitive or voltage-sensitive indicators) would allow direct examination of stress-induced activation of LS neurons. Nonetheless, in agreement with our results, previous work in cerebral cortex has shown that E/I balance is crucial for multiple aspects of cortical sensory processing, such as gain control and stimulus selectivity (54,55). Acute elevation of E/I ratio in prefrontal cortex through optogenetic means impaired information transmission within cortical circuitry and severely affected social behavior (12). Also, a chronic alteration of E/I balance in prefrontal cortex by conditional deletion of NL2 caused a decrease in c-Fos activation in response to behavioral stimuli (21). Our results from the LS suggest that E/I balance is similarly fundamental for the processing of experience-induced input in this subcortical structure, even though it consists solely of GABAergic neurons and hence has a clearly different intrinsic circuit organization compared with neocortex.

The local NL2 deletion provided us a good tool to examine the behavioral consequences of E/I imbalance in the LS. We found that animals with loss of NL2 in the LS showed reduced anxiety in the EPM with normal locomotor activity and depression-related behavior. This further strengthens a body of evidence linking the LS with anxiety-related behavior (10,56). Interestingly, the injection of the $GABA_AR$ agonist muscimol in the LS has been reported to increase animals' open arm exploration in the EPM (8). This is intriguing because of our observation that reduced inhibitory transmission on NL2 deletion also leads to the same behavioral effect. The combined evidence thus indicates that an alteration in E/I balance in the LS in either direction results in identical phenotype in the form of reduced anxiety-related behavior. Our results showed that E/I imbalance in the LS had a selective effect on open arm exploration in the EPM with no alteration in animals' performance in the OFT and TST, the latter being an assay of depression-related behavior that tests behavioral despair. This is interesting in light of the documented role of the LS in regulating behavioral despair (43). The selective behavioral effect in our experiments indicates that some functions of a brain region (and the circuits in which it participates) are more sensitive to E/I imbalance than others, which could partly explain why patients with autism and schizophrenia have restricted behavioral phenotypes, even in the presence of mutations of universally important proteins. It should be noted that our behavioral assays were done with naïve animals that were not subjected beforehand to stressful experience. It will be interesting to examine in future experiments whether exposure of animals to acute or chronic stress uncovers an intra-LS role of NL2 in determining affective behavior in the OFT and TST.

How does reduced responsiveness of the LS to behavioral stimuli result in reduced anxiety-related behavior? We addressed this question by assaying the stress-induced activation of c-Fos in the principal LS projection areas in the hypothalamus. Our results showed that ARS resulted in reduced c-Fos activation in the LH and AHA when NL2 was deleted from the LS. This suggests that these hypothalamic areas were not optimally engaged by the behavioral stimulus in the face of reduced c-Fos activation in the upstream LS. The AHA has long been considered a critical component of the behavioral defense system and shown to regulate aspects of anxiety-related behavior and hypothalamic-pituitary-adrenal axis, in part by sending projections to the PVN (3,46,57). The role of LH in anxiety is also beginning to be appreciated. A recent study demonstrated that ventral hippocampal excitatory projections to the LH were anxiogenic, as optogenetic activation of the terminals in the LH increased avoidance behavior and their optogenetic inhibition reduced open arm avoidance in the EPM (47). The optogenetic activation of axon terminals from bed nucleus of the stria terminalis neurons (presumably inhibitory) within LH reduced open arm avoidance, without affecting other aspects of anxiety, such as rate of breathing (58). Also, the pharmacological manipulation of the LH affected rats' performance in EPM, but not defensive burying in the shock-probe test (46). In addition to supporting the role of the LH in anxiety, these studies suggest that the LH may be involved in regulating specific aspects of anxiety, particularly avoidance behavior. Our finding showing an association between reduced stress-induced c-Fos activation in the LH and decreased open arm avoidance strengthens this link. Direct testing of a causal relationship between the two will require experiments involving in vivo optogenetics and/or chemogenetics. Our results support a model (Supplemental Figure S5) in which anxiety cells in the ventral hippocampus control the avoidance behavior–regulating LH neurons by a direct projection as well as through an indirect disinhibition pathway involving LS neurons. In the absence of NL2, the stress-induced activation of LS neurons is impaired (hence reduced c-Fos in the LS), which may prevent optimal disinhibition of avoidance-controlling hypothalamic neurons (hence reduced c-Fos in the LH), resulting in increased open arm exploration.

Our findings have relevance to understanding the neural circuitry disturbances that arise from altered E/I balance in various neuropsychiatric disorders. Future work will test whether behavioral manipulations, such as chronic stress, modulate NL2 levels in the LS to modify inhibitory transmission, E/I balance, and behavior.

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REFERENCES

1. Shin S, Pribiag H, Lilascharoen V, Knowland D, Wang XY, Lim BK (2018): Drd3 signaling in the lateral septum mediates early life stress-induced social dysfunction. Neuron 97:195–208.e196.
2. Wong LC, Wang L, D'Amour JA, Yumita T, Chen G, Yamaguchi T, *et al.* (2016): Effective modulation of male aggression through lateral septum to medial hypothalamus projection. Curr Biol 26:593–604.
3. Anthony TE, Dee N, Bernard A, Lerchner W, Heintz N, Anderson DJ (2014): Control of stress-induced persistent anxiety by an extra-amygdala septohypothalamic circuit. Cell 156:522–536.
4. Bielsky IF, Hu SB, Ren X, Terwilliger EF, Young LJ (2005): The V1a vasopressin receptor is necessary and sufficient for normal social recognition: A gene replacement study. Neuron 47:503–513.
5. Carus-Cadavieco M, Gorbati M, Ye L, Bender F, van der Veldt S, Kosse C, *et al.* (2017): Gamma oscillations organize top-down signalling to hypothalamus and enable food seeking. Nature 542:232–236.
6. Risold PY, Swanson LW (1997): Connections of the rat lateral septal complex. Brain Res Brain Res Rev 24:115–195.
7. Parfitt GM, Nguyen R, Bang JY, Aqrabawi AJ, Tran MM, Seo DK, *et al.* (2017): Bidirectional control of anxiety-related behaviors in mice: Role of inputs arising from the ventral hippocampus to the lateral septum and medial prefrontal cortex. Neuropsychopharmacology 42:1715–1728.
8. Trent NL, Menard JL (2010): The ventral hippocampus and the lateral septum work in tandem to regulate rats' open-arm exploration in the elevated plus-maze. Physiol Behav 101:141–152.
9. Gray JA, McNaughton N (2000): The Neuropsychology of Anxiety, 2nd ed. New York: Oxford University Press.
10. Menard J, Treit D (1996): Lateral and medial septal lesions reduce anxiety in the plus-maze and probe-burying tests. Physiol Behav 60:845–853.
11. Radulovic J, Ruhmann A, Liepold T, Spiess J (1999): Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: Differential roles of CRF receptors 1 and 2. J Neurosci 19:5016–5025.
12. Yizhar O, Fenno LE, Prigge M, Schneider F, Davidson TJ, O'Shea DJ, *et al.* (2011): Neocortical excitation/inhibition balance in information processing and social dysfunction. Nature 477:171–178.
13. Shu Y, Hasenstaub A, McCormick DA (2003): Turning on and off recurrent balanced cortical activity. Nature 423:288–293.
14. Sudhof TC (2008): Neuroligins and neurexins link synaptic function to cognitive disease. Nature 455:903–911.
15. Ichtchenko K, Nguyen T, Sudhof TC (1996): Structures, alternative splicing, and neurexin binding of multiple neuroligins. J Biol Chem 271:2676–2682.
16. Krueger DD, Tuffy LP, Papadopoulos T, Brose N (2012): The role of neurexins and neuroligins in the formation, maturation, and function of vertebrate synapses. Curr Opin Neurobiol 22:412–422.
17. Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004): Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell 119:1013–1026.
18. Varoqueaux F, Jamain S, Brose N (2004): Neuroligin 2 is exclusively localized to inhibitory synapses. Eur J Cell Biol 83:449–456.
19. Chanda S, Hale WD, Zhang B, Wernig M, Sudhof TC (2017): Unique versus redundant functions of neuroligin genes in shaping excitatory and inhibitory synapse properties. J Neurosci 37:6816–6836.
20. Poulopoulos A, Aramuni G, Meyer G, Soykan T, Hoon M, Papadopoulos T, *et al.* (2009): Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. Neuron 63:628–642.
21. Liang J, Xu W, Hsu YT, Yee AX, Chen L, Sudhof TC (2015): Conditional neuroligin-2 knockout in adult medial prefrontal cortex links chronic changes in synaptic inhibition to cognitive impairments. Mol Psychiatry 20:850–859.
22. Roberts JL, Hovanes K, Dasouki M, Manzardo AM, Butler MG (2014): Chromosomal microarray analysis of consecutive individuals with autism spectrum disorders or learning disability presenting for genetic services. Gene 535:70–78.
23. Sun C, Cheng MC, Qin R, Liao DL, Chen TT, Koong FJ, *et al.* (2011): Identification and functional characterization of rare mutations of the neuroligin-2 gene (NLGN2) associated with schizophrenia. Hum Mol Genet 20:3042–3051.
24. Parente DJ, Garriga C, Baskin B, Douglas G, Cho MT, Araujo GC, *et al.* (2017): Neuroligin 2 nonsense variant associated with anxiety, autism, intellectual disability, hyperphagia, and obesity. Am J Med Genet A 173:213–216.
25. Heshmati M, Aleyasin H, Menard C, Christoffel DJ, Flanigan ME, Pfau ML, *et al.* (2018): Cell-type-specific role for nucleus accumbens neuroligin-2 in depression and stress susceptibility. Proc Natl Acad Sci U S A 115:1111–1116.
26. Babaev O, Botta P, Meyer E, Muller C, Ehrenreich H, Brose N, *et al.* (2016): Neuroligin 2 deletion alters inhibitory synapse function and anxiety-associated neuronal activation in the amygdala. Neuropharmacology 100:56–65.
27. Blundell J, Tabuchi K, Bolliger MF, Blaiss CA, Brose N, Liu X, *et al.* (2009): Increased anxiety-like behavior in mice lacking the inhibitory synapse cell adhesion molecule neuroligin 2. Genes Brain Behav 8:114–126.
28. Chen CH, Lee PW, Liao HM, Chang PK (2017): Neuroligin 2 R215H mutant mice manifest anxiety, increased prepulse inhibition, and impaired spatial learning and memory. Front Psychiatry 8:257.
29. Jiang DY, Wu Z, Forsyth CT, Hu Y, Yee SP, Chen G (2018): GABAergic deficits and schizophrenia-like behaviors in a mouse model carrying patient-derived neuroligin-2 R215H mutation. Mol Brain 11:31.
30. Sheehan TP, Chambers RA, Russell DS (2004): Regulation of affect by the lateral septum: Implications for neuropsychiatry. Brain Res Brain Res Rev 46:71–117.
31. Duncan GE, Knapp DJ, Breese GR (1996): Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res 713:79–91.
32. Risold PY, Swanson LW (1996): Structural evidence for functional domains in the rat hippocampus. Science 272:1484–1486.
33. Panzanelli P, Fruh S, Fritschy JM (2017): Differential role of GABAA receptors and neuroligin 2 for perisomatic GABAergic synapse formation in the hippocampus. Brain Struct Funct 222:4149–4161.
34. Yamasaki T, Hoyos-Ramirez E, Martenson JS, Morimoto-Tomita M, Tomita S (2017): GARLH family proteins stabilize GABAA receptors at synapses. Neuron 93:1138–1152.e1136.
35. Mody I, Pearce RA (2004): Diversity of inhibitory neurotransmission through GABA(A) receptors. Trends Neurosci 27:569–575.
36. Sheng M, Greenberg ME (1990): The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4:477–485.
37. Duncan GE, Johnson KB, Breese GR (1993): Topographic patterns of brain activity in response to swim stress: Assessment by 2-deoxyglucose uptake and expression of Fos-like immunoreactivity. J Neurosci 13:3932–3943.
38. Timofeeva E, Huang Q, Richard D (2003): Effects of treadmill running on brain activation and the corticotropin-releasing hormone system. Neuroendocrinology 77:388–405.

39. Frenois F, Cador M, Caille S, Stinus L, Le Moine C (2002): Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. Eur J Neurosci 16:1377–1389.
40. Campeau S, Watson SJ (1997): Neuroendocrine and behavioral responses and brain pattern of c-fos induction associated with audiogenic stress. J Neuroendocrinol 9:577–588.
41. Kollack-Walker S, Watson SJ, Akil H (1997): Social stress in hamsters: Defeat activates specific neurocircuits within the brain. J Neurosci 17:8842–8855.
42. Liu J, Nickolenko J, Sharp FR (1994): Morphine induces c-fos and junB in striatum and nucleus accumbens via D1 and N-methyl-D-aspartate receptors. Proc Natl Acad Sci U S A 91:8537–8541.
43. Singewald GM, Rjabokon A, Singewald N, Ebner K (2011): The modulatory role of the lateral septum on neuroendocrine and behavioral stress responses. Neuropsychopharmacology 36:793–804.
44. Mohammad F, Ho J, Woo JH, Lim CL, Poon DJJ, Lamba B, *et al.* (2016): Concordance and incongruence in preclinical anxiety models: Systematic review and meta-analyses. Neurosci Biobehav Rev 68:504–529.
45. Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P (2002): Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behav Brain Res 134:49–57.
46. Hakvoort Schwerdtfeger RM, Menard JL (2008): The lateral hypothalamus and anterior hypothalamic nucleus differentially contribute to rats' defensive responses in the elevated plus-maze and shock-probe burying tests. Physiol Behav 93:697–705.
47. Jimenez JC, Su K, Goldberg AR, Luna VM, Biane JS, Ordek G, *et al.* (2018): Anxiety cells in a hippocampal-hypothalamic circuit. Neuron 97:670–683.e676.
48. Bains JS, Wamsteeker Cusulin JI, Inoue W (2015): Stress-related synaptic plasticity in the hypothalamus. Nat Rev Neurosci 16:377–388.
49. Jedlicka P, Hoon M, Papadopoulos T, Vlachos A, Winkels R, Poulopoulos A, *et al.* (2011): Increased dentate gyrus excitability in neuroligin-2-deficient mice in vivo. Cereb Cortex 21:357–367.
50. Stogsdill JA, Ramirez J, Liu D, Kim YH, Baldwin KT, Enustun E, *et al.* (2017): Astrocytic neuroligins control astrocyte morphogenesis and synaptogenesis. Nature 551:192–197.
51. Heeger DJ (1992): Normalization of cell responses in cat striate cortex. Vis Neurosci 9:181–197.
52. Turrigiano G (2011): Too many cooks? Intrinsic and synaptic homeostatic mechanisms in cortical circuit refinement. Annu Rev Neurosci 34:89–103.
53. Yap EL, Greenberg ME (2018): Activity-regulated transcription: Bridging the gap between neural activity and behavior. Neuron 100:330–348.
54. Isaacson JS, Scanziani M (2011): How inhibition shapes cortical activity. Neuron 72:231–243.
55. Haider B, McCormick DA (2009): Rapid neocortical dynamics: Cellular and network mechanisms. Neuron 62:171–189.
56. Pesold C, Treit D (1992): Excitotoxic lesions of the septum produce anxiolytic effects in the elevated plus-maze and the shock-probe burying tests. Physiol Behav 52:37–47.
57. Canteras NS (2002): The medial hypothalamic defensive system: Hodological organization and functional implications. Pharmacol Biochem Behav 71:481–491.
58. Kim SY, Adhikari A, Lee SY, Marshel JH, Kim CK, Mallory CS, *et al.* (2013): Diverging neural pathways assemble a behavioural state from separable features in anxiety. Nature 496:219–223.