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Archival Report

Gephyrin Palmitoylation in Basolateral Amygdala Mediates the Anxiolytic Action of Benzodiazepine

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ABSTRACT

BACKGROUND: Benzodiazepines (BZDs) have been used to treat anxiety disorders for more than five decades as the allosteric modulator of the gamma-aminobutyric acid A receptor ($GABA_AR$). Little is known about other mechanisms of BZDs. Here, we describe how the rapid stabilization of postsynaptic $GABA_AR$ is essential and sufficient for the anxiolytic effect of BZDs via a palmitoylation-dependent mechanism.

METHODS: Palmitoylated proteins in the basolateral amygdala (BLA) of rats with different anxious states were assessed by a biotin exchange protocol. Both pharmacological and genetic approaches were used to investigate the role of palmitoylation in anxiety behavior. Electrophysiological recording, reverse transcription polymerase chain reaction, Western blotting, and coimmunoprecipitation were used to investigate the mechanisms.

RESULTS: Highly anxious rats were accompanied by the deficiency of gephyrin palmitoylation and decreased the synaptic function of $GABA_AR$ in the BLA. We then identified that the dysfunction of DHHC12, a palmitoyl acyltransferase that specifically palmitoylates gephyrin, contributed to the high-anxious state. Furthermore, diazepam, as an anxiolytic drug targeting $GABA_ARs$, was found to increase gephyrin palmitoylation in the BLA via a $GABA_AR$-dependent manner to activate DHHC12. The anxiolytic effect of diazepam was nearly abolished by the DHHC12 knockdown. Specifically, similar to the effect of BZD, the overexpression of DHHC12 in the BLA exerted a significant anxiolytic action, which was prevented by flumazenil.

CONCLUSIONS: Our results support the view that the strength of inhibitory synapse was controlled by gephyrin palmitoylation in vivo and proposes a previously unknown palmitoylation-centered mode of BZD's action.

Keywords: Antianxiety, Benzodiazepine, DHHC12, $GABA_AR$, Gephyrin, Palmitoylation

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Anxiety disorders are a category of mental disorders and produce heavy social burdens worldwide. Although the precise mechanism of anxiety remains largely unknown, previous studies have revealed that anxiety is closely associated with hyperexcitability of the basolateral amygdala (BLA) (1–3). Gamma-aminobutyric acid A receptor ($GABA_AR$), which inhibits the sensitivity of the primary output neurons in the BLA, is the most important target for many anxiolytic drugs (4).

Benzodiazepines (BZDs), the prototypic wide-spectrum anxiolytic agent used clinically for more than 50 years, have been considered as allosteric modulators of $GABA_ARs$. It has long been assumed that the pharmacological effects of BZDs are mediated through direct binding with the ligand via increasing affinity of $GABA_AR$ for GABA (5) or inducing conformational changes (6,7). It is noteworthy that BZDs also alter the availability of postsynaptic receptor. Long-term treatment with BZDs reduces the number of $GABA_ARs$ (8,9), which may lead to tolerance. Conversely, recent findings have revealed that a rapid increase in postsynaptic $GABA_ARs$ is an adaptive response to BZD binding. For instance, the BZD full agonist diazepam (DZP) rapidly reduces lateral diffusion of $GABA_ARs$ and increases their synaptic stabilization and clustering in cultured neurons (10,11). Flumazenil, a drug that is generally believed to antagonize the sedative-hypnotic actions of BZDs only at the classical BZD-binding domain in $GABA_AR$, rapidly decreases surface expression of $GABA_AR$ (12). Considering that the surface stability of $GABA_AR$ on the primary output neurons of the BLA contributes to the relief of anxiety (13), this rapid adaptation of postsynaptic $GABA_AR$ may be another anxiolytic mechanism of BZD.

It has been reported that BZDs stabilize $GABA_ARs$ by changing their interaction with gephyrin (14–16), a multifunctional scaffolding protein in the formation of the postsynaptic scaffold at inhibitory synapses (17). Recently, it was found that the interaction between $GABA_AR$ and gephyrin is controlled by palmitoylation (18), a reversible lipid modification that attaches a 16-carbon palmitic acid to one or more cysteine residues of targets. Palmitoylation governs protein function in many aspects such as protein traffic, membrane association, and cellular signaling transduction (19–22). In the

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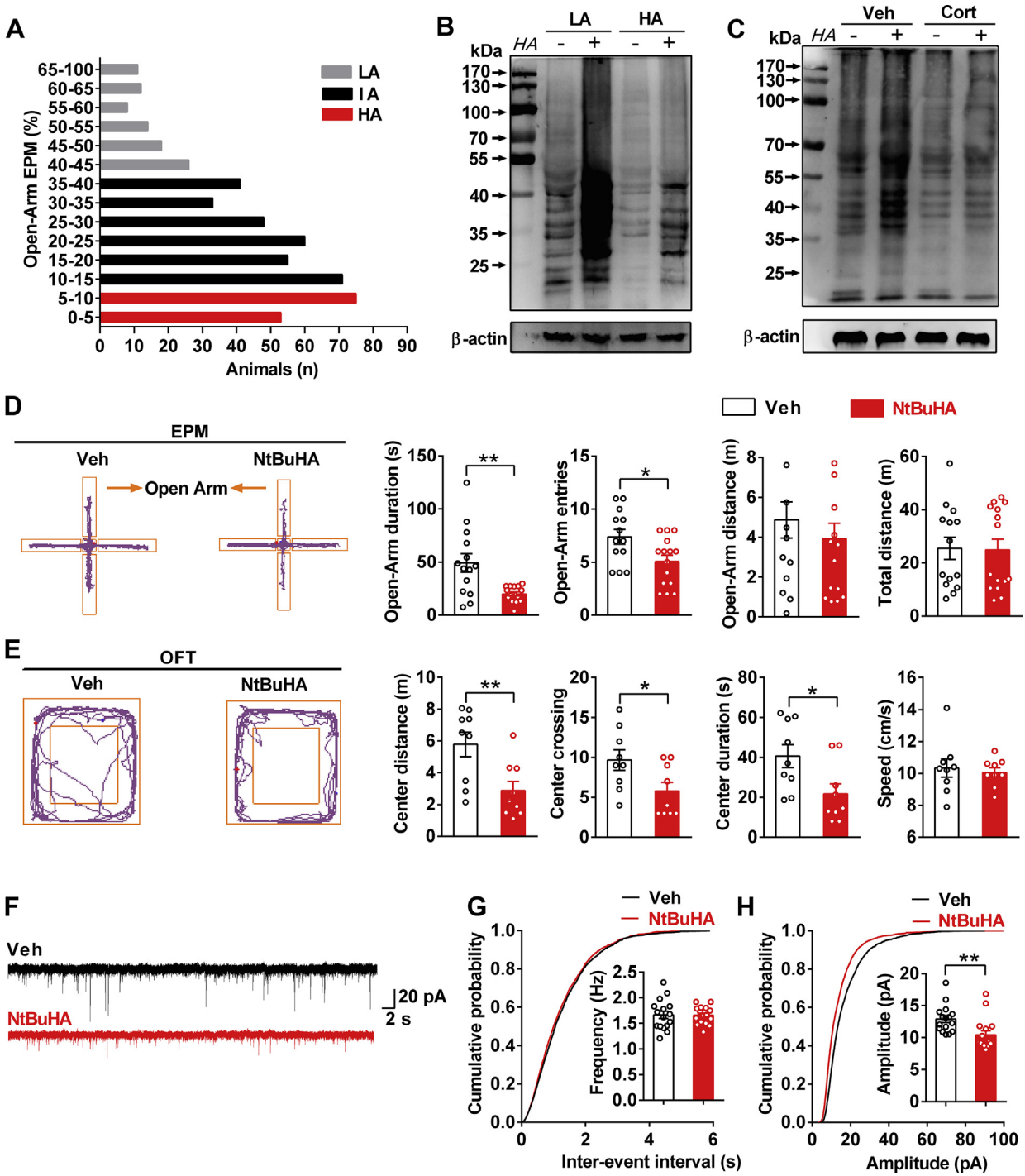

Figure 1. Deficiency in protein palmitoylation in the basolateral amygdala (BLA) generates high-anxiety behavior in rats. **(A)** Division of rats into high-anxiety (HA) ($n = 128$), intermediate-anxiety (IA) ($n = 308$), and low-anxiety (LA) ($n = 89$) groups based on the duration of their open arm avoidance in the elevated plus maze (EPM). **(B)** Overall palmitoylation within the lysates from the BLA of LA and HA rats. Representative Western blots of streptavidin-labeled protein from a paired LA rat and HA rat are shown. **(C)** Overall palmitoylation within the lysates from the BLA of rats exposed to chronic corticosterone (Cort). Representative Western blots of streptavidin-labeled protein from a paired control rat and corticosterone-treated rat are shown. **(D)** Representative maps showing time spent in the open and closed arms of the EPM. *N*-(tert-butyl)hydroxylamine hydrochloride (NtBuHA), a hydroxylamine (*HA*) derivative that rapidly cleaved thioester linkage of palmitoylated proteins, decreased time spent in the open arms and reduced number of entries into the open arms ($n = 14$–15 rats per group, two-tailed *t* test, $^{*}p < .05$ and $^{**}p < .01$ vs. vehicle [Veh] group). **(E)** Representative maps showing time spent in the central distance of open field test (OFT). Anxiogenic action of NtBuHA in the OFT for 5 minutes, including reduced central distance and crossings ($n = 9$ rats per group, two-tailed *t* test, $^{*}p < .05$ and $^{**}p < .01$ vs. Veh group), is shown. **(F–H)** NtBuHA decreased gamma-aminobutyric acid A receptor–mediated miniature inhibitory postsynaptic currents in the BLA neurons. **(F)** Representative miniature inhibitory postsynaptic current recording in the BLA neurons. **(G, H)** Cumulative probabilities and average miniature inhibitory postsynaptic current frequencies and amplitudes from the Veh-treated and NtBuHA-treated groups ($n = 16$ cells from 8 rats per group, two-tailed *t* test, $^{**}p < .01$ vs. Veh group). Error bars represent mean ± SEM.

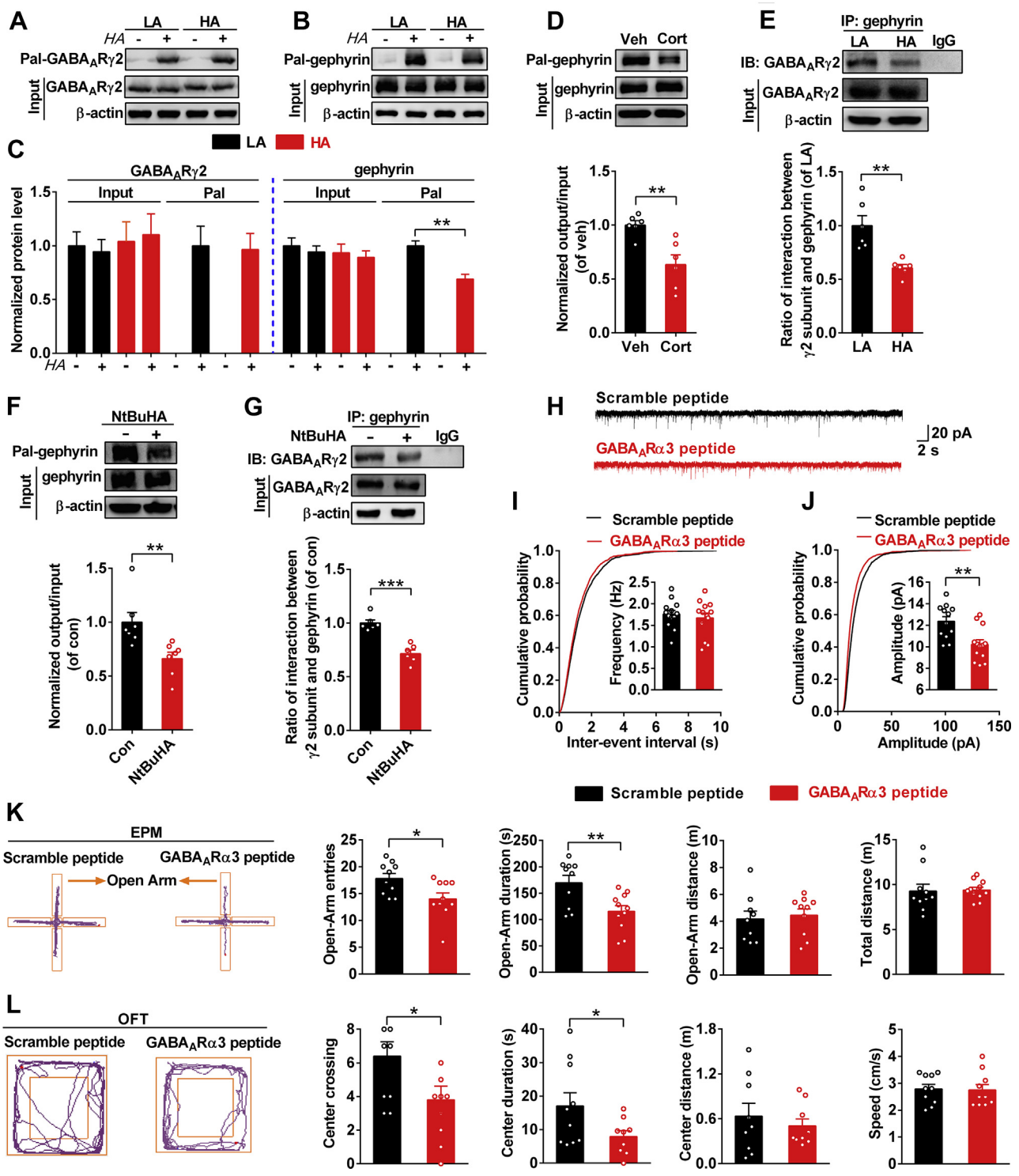

Figure 2. Impaired gephyrin–gamma-aminobutyric acid A receptor ($GABA_AR$) interaction by hypopalmitoylation is sufficient for expression of high trait anxiety. **(A–C)** Acyl-biotin exchange labeling was performed on brain extracts from the basolateral amygdala (BLA) of low-anxiety (LA) and high-anxiety (HA) rats, followed by purification of labeled proteins by streptavidin beads. Palmitoylation of indicated neural proteins was assessed by immunoblotting. HA status selectively reduced palmitoylation of gephyrin but not $GABA_AR$ γ2 (n = 5 rats per group, two-tailed t test, $^{**}p < .01$ vs. LA group). **(D)** Corticosterone (Cort)-exposed rats displayed hypopalmitoylation of gephyrin in the BLA (n = 6 per group, two-tailed t test, $^{**}p < .01$ vs. vehicle [Veh] group). **(E)** Impaired gephyrin–$GABA_AR$ interaction is indicated by the reduced coimmunoprecipitation of $GABA_AR$ γ2 protein with gephyrin antibody in the HA rats (n = 6 per group, two-tailed t test, $^{**}p < .01$ vs. LA group). **(F)** *N*-(tert-butyl)hydroxylamine hydrochloride (NtBuHA)-treated rats displayed hypopalmitoylation of gephyrin in the

brain, palmitoylation plays important roles in development, synaptic plasticity, and the membrane cluster of various receptors (23–25). Despite its fundamental physiological function, very little is known about the role of palmitoylation in emotional disorders. Under physiological conditions, the level of palmitoylation is finely tuned by palmitoyl acyltransferases (PATs) and palmitoyl protein thioesterases (PPTs). One of the PATs, DHHC12 (Asp-His-His-Cys (DHHC)12), palmitoylates gephyrin and affects its function. The palmitoylation of gephyrin seems to be a $GABA_AR$ activity–dependent process (18). Thus, it may serve as a downstream pathway of BZD to support the availability of GABA–BZD receptor at synapses (26). Although it has been well recognized that the formation of GABA–BZD receptor complex requires exogenous ligand, some studies also indicate that an endogenous BZD-like agent, such as endozepine, can bind to the GABA–BZD receptor complex in the brain (27–31). Here, we identified that DHHC12-controlled gephyrin palmitoylation in the BLA mediated a novel mechanism underlying the anxiolytic action of BZDs.

METHODS AND MATERIALS

Detailed materials and methods are provided in the Supplement.

Materials

The experiments were conducted in male Sprague Dawley rats aged 8 or 9 weeks weighing 220 to 250 g (Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China). The use of animals for the experimental procedure was in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All the agents were purchased from commercial suppliers.

Behavioral Testing

All behavioral tests were conducted in adult rats. Anxiety was measured by the elevated plus maze (EPM) (32) and open field test (OFT) (33). According to the total time spent in the open arm in the EPM test, rats were classified into three groups (34,35): the low-anxiety (LA) group, the intermediate-anxiety (IA) group, and the high-anxiety (HA) group. The LA group was classified as the time spent in the open arm at the top 40%, and the HA group was classified as the time spent in the open arm at the bottom 10% (Supplemental Figure S1).

Electrophysiological Recording

Male Sprague Dawley rats (8–9 weeks) were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally [i.p.]), decapitated, and brain dissected. Miniature inhibitory postsynaptic current (mIPSC) recordings were made using a submersion chamber with patch electrodes (3–5 MΩ resistance) filling a solution containing 140 mM K-gluconate, 8 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.2, 290–320 mOsm) and a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) at room temperature. See details in the Supplement.

Molecular Biology Experiments

The BLA tissue was dissected, homogenized, and analyzed using standard Western blotting, real-time polymerase chain reaction, and coimmunoprecipitation procedures as in our previous reports (36–38) with minor modifications. Primers are described in Supplemental Table S2. Palmitoylation was assessed by acyl-biotin exchange assays as described previously (39,40) with minor modifications (Supplemental Figure S2).

Stereotaxic Surgery

The rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and then mounted in a stereotaxic apparatus. For knockdown or overexpression of DHHC12, green fluorescent protein (GFP)-tagging lentiviral expressed specific short hairpin RNA (shRNA) against DHHC12 or adeno-associated virus (AAV) overexpressed DHHC12 (1.0 μL per side; GeneChem, Shanghai, China) was stereotaxically delivered directly into the BLA region of adult rats. See details in the Supplement.

Statistical Analysis

All the analyses were employed using SPSS 18.0 software (SPSS Inc., Chicago, IL). The results are presented as mean ± SEM. All statistical results and tests used are included in the figure legends. Required sample size was estimated based on prior experience. The independent-samples *t* test, the one-way analysis of variance (ANOVA), or the two-way ANOVA was used as appropriate. The post hoc test was used to assess the least significant difference test for one-way ANOVAs. The Bonferroni post hoc test was used to evaluate isolated comparisons for two-way ANOVAs.

RESULTS

Deficiency in Palmitoylation Signal in the BLA Generates a High-Anxious State in Rats

We first sought to determine the correlation between anxiety status and palmitoylation in the BLA. According to the duration in the open arm in the EPM test, the rats were divided into three groups (Figure 1A and Supplemental Figure S1): the

BLA (n = 7 per group, two-tailed t test, $^{**}p < .01$ vs. Veh group). **(G)** Hypopalmitoylation by NtBuHA decreased the coimmunoprecipitation of $GABA_AR$ γ2 protein with gephyrin antibody in the BLA (n = 6 per group, two-tailed t test, $^{***}p < .001$ vs. control [Con] group). **(H–J)** Impairing gephyrin–$GABA_AR$ interaction reduced miniature inhibitory postsynaptic currents in the pyramidal neurons of the BLA. **(H)** Representative miniature inhibitory postsynaptic currents recording in the BLA pyramidal neurons. **(I, J)** Cumulative probabilities and average miniature inhibitory postsynaptic current frequencies and amplitudes from the scramble peptide group and $GABA_AR$ α3 peptide group (n = 13 cells from 10 rats per group, two-tailed t test, $^{**}p < .01$ vs. scramble peptide group). **(K, L)** Impaired gephyrin–$GABA_AR$ interaction in the BLA is sufficient for expression of high trait anxiety. $GABA_AR$ α3 peptide, a gephyrin-binding $GABA_AR$-derived peptide that disrupts their interactions, significantly decreased the exploration of rats in the open arms of the elevated plus maze (EPM) **(K)** (n = 10–11 rats per group, two-tailed t test, $^{*}p < .05$ and $^{**}p < .01$ vs. scramble peptide group) and in the central field of the open field test (OFT) **(L)** (n = 9–10 rats per group, two-tailed t test, $^{*}p < .05$ vs. scramble peptide group). Error bars represent mean ± SEM. *HA*, hydroxylamine; IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation.

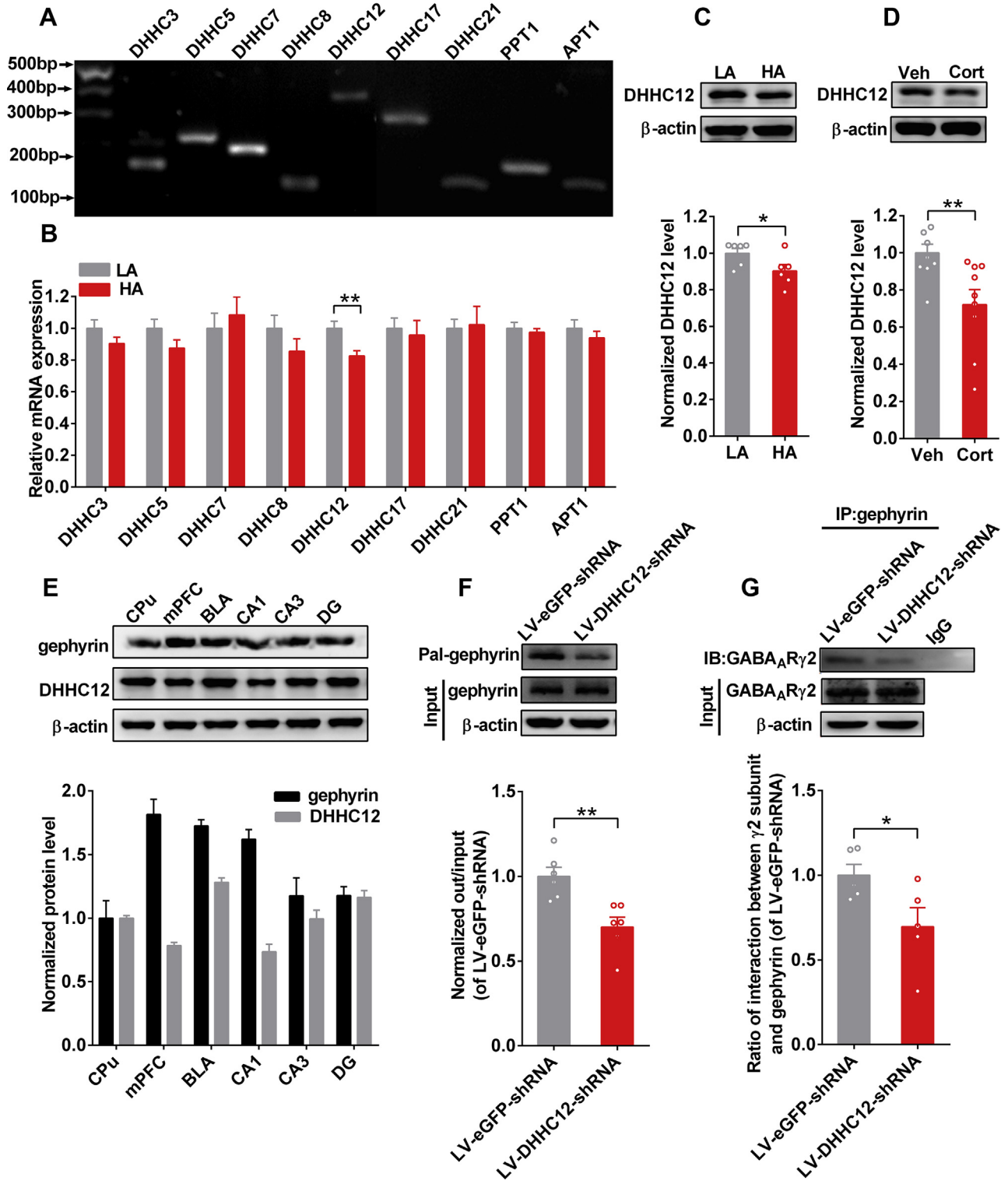

Figure 3. Dysfunction of DHHC12, the main enzyme that controls gephyrin palmitoylation, mediates the impaired gephyrin–gamma-aminobutyric acid A receptor ($GABA_AR$) interaction of high-anxiety (HA) rats. **(A)** Identification of DHHCs (Asp-His-His-Cys) (DHHC3, DHHC5, DHHC7, DHHC8, DHHC12, DHHC17, and DHHC21) and palmitoyl protein thioesterases (PPTs) (PPT1 and acyl protein thioesterase 1 [APT1]) by polymerase chain reaction with reverse transcription. **(B)** Quantification of the messenger RNA (mRNA) level of DHHCs and PPTs in the basolateral amygdala (BLA) of low-anxiety (LA) and HA rats by quantitative polymerase chain reaction with reverse transcription ($n = 8$ per group, two-tailed t test, $^{**}p < .01$ vs. LA group). **(C)** Dysregulated DHHC12 expression in HA

LA group (n = 89), the IA group (n = 308), and the HA group (n = 128). We found that there was a significant reduction in the content of palmitoylated proteins in the BLA of HA rats (Figure 1B). Consistent with the tendency in HA rats, in another anxiety model (41,42) (Supplemental Figure S3) corticosterone exposure significantly downregulated the palmitoylated protein level in the BLA (Figure 1C). To determine whether the decrease in palmitoylation contributed to the high trait anxiety, we used a nontoxic chemical tool, *N*-(tert-butyl)-hydroxylamine (NtBuHA), a hydroxylamine derivative that rapidly cleaves thioester linkage in palmitoylated proteins. We found that NtBuHA dose-dependently reduced the palmitoylated protein level in the BLA after incubation of BLA slices for 60 minutes. In addition, microinjection of NtBuHA (1.0 mM) into the BLA decreased palmitoylation in vivo and open arm exploration in the EPM (Supplemental Figure S4A–D). Such reduced open arm exploration was evident in terms of both open arm duration (Figure 1D) (19.90 ± 1.910 seconds of NtBuHA group, 49.09 ± 8.634 seconds of vehicle group, n = 14 or 15, $p < .01$) and open arm entries (Figure 1D) (5.067 ± 0.5812 of NtBuHA group, 8.357 ± 1.170 of vehicle group, n = 14 or 15, $p < .05$). The increase in open arm exploration was not due to an increase in locomotor activity (Figure 1D). A similar anxiogenic effect of NtBuHA was observed in the OFT (Figure 1E).

Considering that $GABA_AR$ on the primary neurons in the BLA is critical for the relief of anxiety behaviors, we examined the effects of NtBuHA on $GABA_AR$-mediated mIPSCs. Whole-cell patch-clamp recordings were obtained from BLA neurons that were identified on the basis of their size, pyramid-like shape, and firing patterns in response to depolarizing current pulses. Compared with the vehicle group, there was a substantial reduction of amplitude of mIPSCs in the NtBuHA-treated group, but without difference in the frequency of mIPSCs (Figure 1F–H), indicating that the inhibition of palmitoylation may disrupt the inhibitory synaptic transmission via a postsynaptic mechanism.

Disruption of Gephyrin–$GABA_AR$ Interaction by Hypopalmitoylation Is Sufficient to Induce High Trait Anxiety

Considering that the $GABA_AR$ $\gamma2$ subunit can be palmitoylated (43), the palmitoylation of the $GABA_AR$ $\gamma2$ subunit was measured by acyl-biotin exchange assays (Supplemental Figure S2). However, there was no significant difference between the LA and HA groups (Figure 2A). Next, the palmitoylation sites were predicted by software (CSS-Palm 4.0; http://csspalm.biocuckoo.org). The results indicated that the residues on other major subunits of $GABA_AR$, including the C260 of α_1, C8, C261 of α_2, C191 and C286 of α_3, and C23 of β_2, may also serve as potential palmitoylation sites. Thus, we detected their palmitoylation by acyl-biotin exchange assays and found little difference between the HA and LA groups (Supplemental Figure S5). It is well known that gephyrin is the major instructive molecule at inhibitory synapses.to cluster $GABA_AR$. We found that the palmitoylated gephyrin was significantly decreased in the BLA of HA rats (Figure 2B, C) (n = 5, $p < .01$) and corticosterone-induced animal model of anxiety (Figure 2D) (n = 6, $p < .01$). The palmitoylated gephyrin was reduced by NtBuHA (Figure 2F) (n = 7, $p < .01$), which may underlie the anxiogenic effect of NtBuHA.

Palmitoylation of gephyrin is essential for the control of receptor clustering and plasticity of GABAergic synapses (18). Thus, we asked whether $GABA_AR$ levels were altered in complex with gephyrin under the hypopalmitoylation condition. Samples were immunoprecipitated with the anti-gephyrin antibody and then immunoblotted with an anti-$GABA_AR$ $\gamma2$ antibody. We found that the coimmunoprecipitated $\gamma2$ subunit was significantly decreased by NtBuHA treatment (Figure 2G) (n = 6, $p < .001$), indicating a critical role of palmitoylation in $GABA_AR$ clustering. A similar reduction of gephyrin–$GABA_AR$ $\gamma2$ association was observed in the HA rats (Figure 2E) (n = 6, $p < .01$). Given that the reduced association between gephyrin and $GABA_AR$ $\gamma2$ may contribute to anxiety-like behavior induced by hypopalmitoylation, we used a reported gephyrin-binding $GABA_AR$-derived peptide (44), $GABA_AR$ $\alpha3$ peptide, to disrupt the interaction between gephyrin and $GABA_AR$ (Supplemental Figure S6). In the whole-cell recordings, we observed that $GABA_AR$ $\alpha3$ peptide significantly reduced the amplitude (Figure 2H, J) (n = 13, $p < .01$), but not the frequency (Figure 2I), of mIPSCs in BLA neurons. Compared with the scramble peptide group, microinjection of $GABA_AR$ $\alpha3$ peptide into the BLA significantly decreased open arm exploration in the EPM (Figure 2K), including decreased open arm duration (n = 9–10, $p < .01$) and open arm entries (n = 9–10, $p < .05$), which was similar to the effect of NtBuHA. The reduced open arm exploration was not due to a decrease in locomotor activity, as demonstrated by the similar total distance (Figure 2K). A similar anxiogenic effect was observed in the OFT. Peptide-treated rats spent less time in the center area (Figure 2L) (n = 9–10, $p < .05$), but without differences in the speed (Figure 2L) in the OFT.

Genetic Knockdown of DHHC12 Decreases the Association of Gephyrin With $GABA_AR$ in the BLA of Rats

It has been demonstrated that PATs catalyze palmitoylation of multiple targets in vivo (45), and depalmitoylation is catalyzed by palmitoyl protein thioesterases such as PPT1 and APT1. As shown in Figure 3A and Supplemental Figure S7A, except for DHHC12, the specific gephyrin-palmitoylating enzyme (18), there was no significant difference in other PATs and PPTs between the LA and HA groups. Comparing with the LA group,

rats. Representative Western blots and quantification of DHHC12 in the BLA of LA and HA rats (n = 6 per group, two-tailed t test, $^*p < .05$ vs. LA group) are shown. **(D)** Chronic corticosterone (Cort) exposure decreased DHHC12 expression in the BLA in rats (n = 9 per group, two-tailed t test, $^{**}p < .01$ vs. vehicle [Veh] group). **(E)** Representative immunoblots and quantification of DHHC12 and gephyrin within the caudate putamen (CPu), medial prefrontal cortex (mPFC), BLA, CA1, CA3, and dentate gyrus (DG) (n = 6 per group). **(F, G)** Knockdown of DHHC12 by lentiviral (LV) vector-mediated short hairpin RNA (shRNA) decreased the interaction between $GABA_AR$ $\gamma2$ and gephyrin (n = 5 per group, two-tailed t test, $^*p < .05$ vs. LV-enhanced green fluorescent protein [eGFP]-shRNA group) and reduced gephyrin palmitoylation (n = 6 per group, two-tailed t test, $^{**}p < .01$ vs. LV-eGFP-shRNA group). Error bars represent mean ± SEM. IgG, immunoglobulin G.

the level of DHHC12 messenger RNA was decreased significantly in HA rats (Figure 3B) (82.50 ± 3.45% of LA group, $n = 8$, $p < .01$). Similarly, a decreased level of DHHC12 protein was observed in the BLA of HA rats (Figure 3C) (90.28 ± 3.48% of LA group, $n = 6$, $p < .05$) and corticosterone-exposed rats (Figure 3D) (72.10 ± 8.139% of vehicle group, $n = 9$, $p < .01$). Furthermore, we found a relatively higher tendency in the expression of DHHC12, not gephyrin, in the BLA compared with other brain areas, including caudate putamen, medial prefrontal cortex, CA1, CA3, and dentate gyrus (Figure 3E).

We hypothesized that DHHC12 dysfunction may lead to the reduced gephyrin–$GABA_AR$ interaction. Next, lentiviral vector–mediated shRNA was delivered into the BLA to knock down DHHC12 gene expression (Supplemental Figure S8). Consistent with previous findings (18), the rats with DHHC12 knockdown exhibited a decrease in gephyrin palmitoylation compared with the lentiviral vector-enhanced GFP (eGFP)-shRNA group (Figure 3F) ($n = 6$, $p < .01$). We found that the association of $GABA_AR$ $\gamma 2$ subunit with gephyrin was reduced (Figure 3G) ($n = 5$, $p < .05$). Furthermore, it was found that knockdown of DHHC12 reduced the surface level of $GABA_AR$ subunits in the BLA (Supplemental Figure S9). These results confirmed that the reduction of DHHC12 may be involved with the decreased gephyrin–$GABA_AR$ association in the high trait anxiety.

DHHC12-Mediated Gephyrin Palmitoylation Is Required for the Anxiolytic Action of DZP

Considering that gephyrin palmitoylation is regulated by $GABA_AR$ activity (18), we asked whether DHHC12-mediated gephyrin palmitoylation was involved in the effects of BZDs. Interestingly, DZP (1 mg/kg, i.p., once) rapidly elevated the palmitoylated protein level in the BLA (Figure 4A) at 30 minutes after administration. It was also shown that DZP increased the palmitoylated gephyrin level in the BLA (Figure 4B) (154.40 ± 6.835% of vehicle group, $n = 6$, $p < .001$). The acyltransferase activity of DHHC12 could be detected by its autopalmitoylation, and we found that DZP rapidly triggered DHHC12 autopalmitoylation in the BLA (Supplemental Figure S10A) (157.7 ± 14.89% of vehicle group, $n = 8$, $p < .01$), indicating higher PAT activity.

To clarify the role of $GABA_AR$ in the DZP-mediated DHHC12 activation, bicuculline, the classic $GABA_AR$ antagonist, was used to abolish the increased $GABA_AR$ activity followed by DZP action. At 20 minutes after DZP administration, the rats were quickly decapitated and then moved to ice-cold oxygenated artificial cerebrospinal fluid. Coronal bilateral slices containing the BLA were cut by a vibratome in artificial cerebrospinal fluid and then transferred to a holding chamber with artificial cerebrospinal fluid to incubate with bicuculline (20 μM) or vehicle for 30 minutes. The blockade of $GABA_AR$ prevented the sustained effect of DZP on DHHC12 autopalmitoylation (Figure 4C), indicating that the DZP-mediated DHHC12 activation is $GABA_AR$ dependent.

Consistent with previous reports in the cell model (11), DZP promoted the association of $GABA_AR$ with gephyrin in vivo, as shown in Supplemental Figure S10C ($n = 4$, $p < .01$). Furthermore, it was found that DHHC12 knockdown prevented the effect of DZP on their associations (Figure 4D) ($n = 4$, $p < .01$), indicating that the DZP-induced increase in gephyrin–$GABA_AR$ interaction is mediated through DHHC12-dependent palmitoylation.

Then, the effect of DHHC12 knockdown on the anxiolytic action of DZP was examined. In the behavior test, the DHHC12-$shRNA^{+/-}$ DZP group (gray column) exhibited increased anxiety behaviors compared with the DHHC12-$shRNA^{-/-}$ DZP group (white column), including fewer open arm entries (Figure 4E) ($n = 7$–8, $p < .05$), shorter open arm duration (Figure 4E) ($n = 8$, $p < .05$), and shorter open arm distances (Figure 4E) ($n = 8$, $p < .05$), indicating a critical role of DHHC12 in anxiety behavior. A similar anxiogenic effect was observed in the OFT (Figure 4F) ($n = 8$, $p < .05$). After DZP injection (1 mg/kg, i.p., once), it exerted a significant anxiolytic action only in the DHHC12-$shRNA^{-/+}$ DZP group (red column), including more open arm entries (Figure 4E) ($n = 7$–8, $p < .05$), longer open arm duration (Figure 4E) ($n = 8$, $p < .05$), and longer open arm distances (Figure 4E) ($n = 8$, $p < .05$) in the EPM as well as more central crossings (Figure 4F) ($n = 8$, $p < .01$), central time (Figure 4F) ($n = 8$, $p < .05$), and central distance (Figure 4F) ($n = 8$, $p < .05$) in the OFT. Compared with the DHHC12-$shRNA^{+/-}$ DZP group (Figure 4E, F, gray column), DZP (1 mg/kg, i.p., once) (Figure 4E, F, black column) exerted little anxiolytic effect in DHHC12 knockdown rats. The rats in the DHHC12-$shRNA^{+/-}$ DZP group (red column) exhibited decreased anxiety behaviors compared with those in the DHHC12-$shRNA^{-/-}$ DZP group (white column), whereas the rats in the DHHC12-$shRNA^{+/+}$ DZP group (black column) exhibited the same behavior changes as those in the DHHC12-$shRNA^{+/-}$ DZP group (gray column). These results suggest that DHHC12 is essential for the anxiolytic action of DZP.

Overexpression of DHHC12 in the BLA Exerts a BZD-like Anxiolytic Action

The results described above indicated that DHHC12-mediated gephyrin palmitoylation may be a functional consequence of BZD-$GABA_AR$ formation. Thus, we wondered whether overexpression of DHHC12 may produce a BZD-like action. The effect of AAV-mediated DHHC12 overexpression in the BLA (Supplemental Figure S11) on anxiety was assessed in the EPM and OFT (Figure 5A). Similar to the effect of DZP, DHHC12 overexpression increased gephyrin palmitoylation in the BLA (Supplemental Figure S12). Compared with the vehicle AAV-eGFP group, AAV-DHHC12 significantly increased the amplitude, but not the frequency, of mIPSCs in BLA neurons (Figure 5B–D) ($n = 10$–12, $p < .01$). Overexpression of DHHC12 in the BLA significantly increased the open arm exploration compared with the AAV-eGFP group, including more open arm entries (Figure 5E) ($n = 8$, $p < .05$), longer open arm duration (Figure 5E) ($n = 8$, $p < .05$), and longer open arm distances (Figure 5E) ($n = 8$, $p < .05$), indicating an anxiolytic action of DHHC12 overexpression. A similar anxiolytic effect was observed in the OFT. Overexpression of DHHC12 involved more time in the center area (Figure 5F) ($n = 8$, $p < .05$), but without differences in speed.

Our rationale for the above results is that if the DHHC12-mediated gephyrin palmitoylation confers an endogenous mimic of BZDs via increasing the availability of GABA-BZD

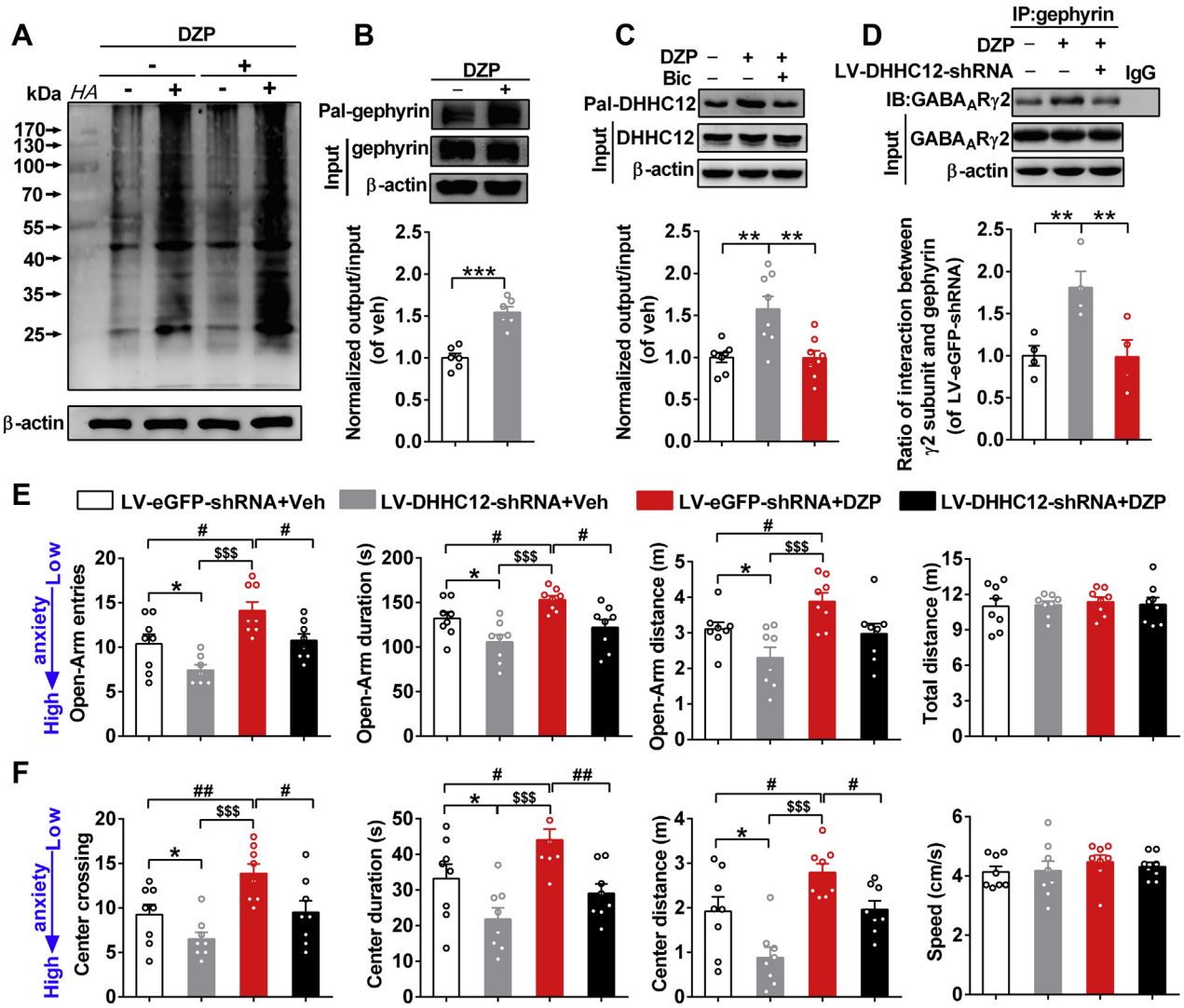

Figure 4. DHHC12-mediated gephyrin palmitoylation is required for the anxiolytic action of diazepam (DZP). **(A)** Representative Western blots of streptavidin-labeled protein from the basolateral amygdala. Compared with the vehicle (Veh)-treated group, DZP-treated rats (1 mg/kg, intraperitoneally) rapidly displayed significantly higher levels of biotin-labeled proteins, indicating an increase in overall palmitoylation. DZP also significantly increased the palmitoylation of gephyrin **(B)** (n = 6 per group, two-tailed t test, $^{***}p < .001$ vs. Veh group). **(C)** DZP (1 mg/kg, intraperitoneally) significantly increased the autopalmitoylation of DHHC12, which could be prevented by bicuculline (Bic) (20 μM) incubation (n = 8 per group, two-tailed t test, $^{*}p < .01$ vs. Veh group). **(D)** Coimmunoprecipitation test indicating that DZP rapidly promoted the association between gephyrin and gamma-aminobutyric acid A receptor ($GABA_AR$) γ2, which was prevented by the DHHC12 knockdown. **(E, F)** Knockdown of DHHC12 by lentiviral (LV) vector-mediated short hairpin RNA (shRNA) significantly increased anxiety behavior and abolished the effects of DZP (1 mg/kg, intraperitoneally, n = 8 rats per group, two-way analysis of variance with Bonferroni posttests, $^{*}p < .05$ vs. LV-enhanced green fluorescent protein [eGFP]-shRNA + Veh group, $^{\#}p < .05$ and $^{\#\#}p < .01$ vs. LV-eGFP-shRNA + DZP group, $^{\$\$\$}p < .001$ vs. LV-DHHC12-shRNA + Veh group). The elevated plus maze and open field test were performed at day 15 after microinjection of LV-eGFP-shRNA or LV-DHHC12-shRNA into the basolateral amygdala. Error bars represent mean ± SEM. *HA*, hydroxylamine; IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation.

receptor, then this effect should be inhibited by specific blockade of GABA-BZD receptor. Flumazenil, a specific antagonist of BZD receptor without affecting physiological $GABA_AR$, was used in the following experiments. Flumazenil (10 mg/kg, i.p., once) exerted little effect on anxiety behavior in the AAV-eGFP group, but in DHHC12-overexpressed rats flumazenil nearly abolished the anxiolytic action of DHHC12, including fewer open arm entries (Figure 5E) (n = 8, $p < .01$), shorter open arm duration (Figure 5E) (n = 8, $p < .01$), and shorter open arm distance (Figure 5E) (n = 8–9, $p < .01$) in the EPM as well as fewer central crossings (Figure 5F) (n = 8–9, $p < .01$) , shorter central time (Figure 5F) (n = 8, $p < .01$), and shorter central distance (Figure 5F) (n = 8, $p < .05$) in the OFT.

DISCUSSION

In this study, we demonstrated that deficits in palmitoylation resulted in an increase in anxiety behavior via impairing the

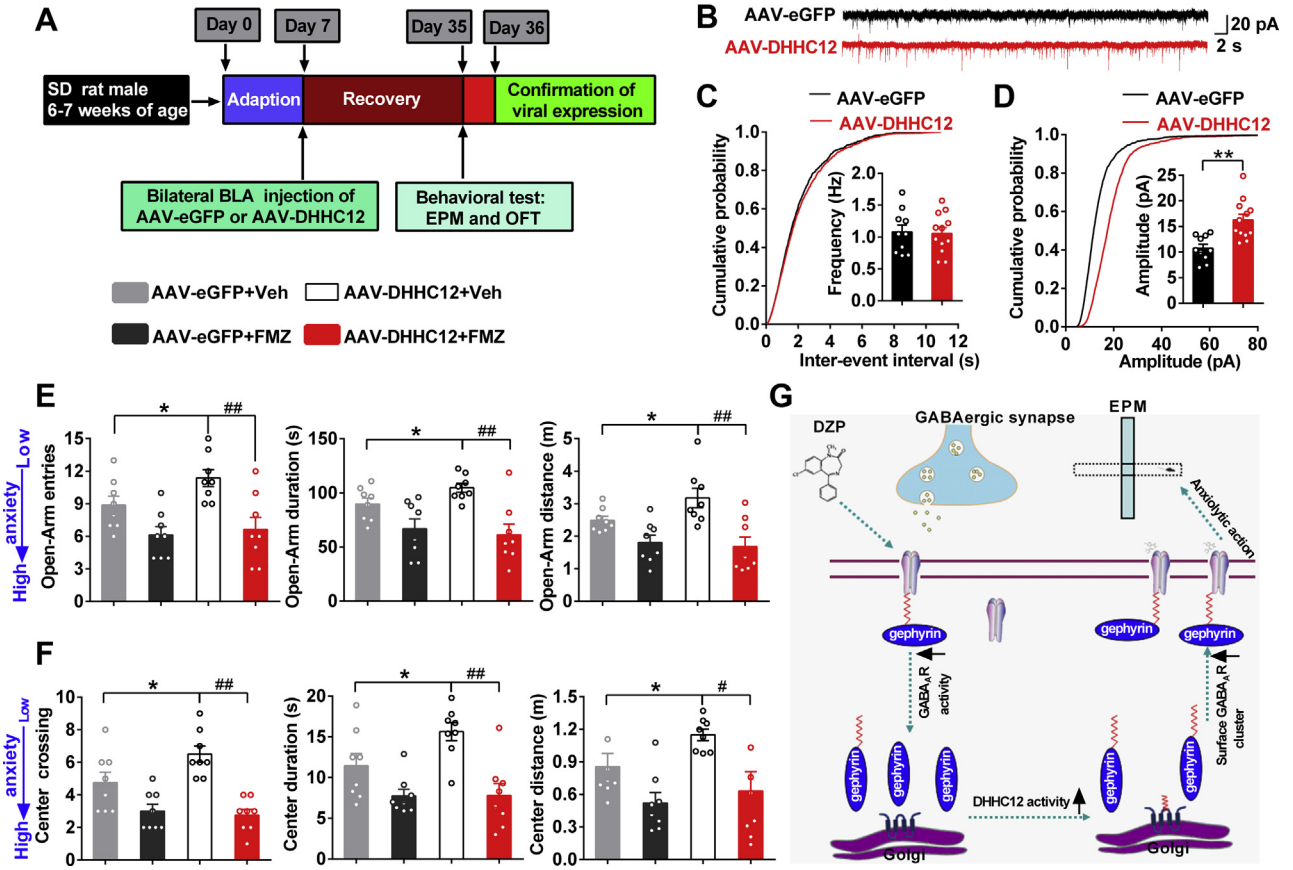

Figure 5. Overexpression of DHHC12 in the basolateral amygdala (BLA) exerts a benzodiazepine-like anxiolytic action. **(A)** Schematic of experimental design. Adeno-associated virus (AAV)-enhanced green fluorescent protein (eGFP) or AAV-DHHC12 was stereotaxically injected into the BLA of adult rats and 4 weeks later was tested in a battery of behavioral tests. **(B)** Representative miniature inhibitory postsynaptic currents recording in the BLA pyramidal neurons. **(C, D)** Cumulative probabilities and average miniature inhibitory postsynaptic current frequencies and amplitudes from the AAV-eGFP group and AAV-DHHC12 group (n = 10–12 cells from 6 rats per group, two-tailed t test, $^{**}p < .01$ vs. AAV-eGFP group). **(E, F)** The anxiolytic action of DHHC12 overexpression was abolished by flumazenil (FMZ). Rats that overexpressed DHHC12 spent more time in the elevated plus maze (EPM) compared with the AAV-eGFP group, including more open arm entries, longer open arm duration, and longer open arm distances, which was abolished by FMZ **(E)** (n = 8 rats per group, two-way analysis of variance with Bonferroni posttests, $^{*}p < .05$ vs. AAV-eGFP + Veh group, $^{\#\#}p < .01$ vs. AAV-DHHC12 + Veh group). A similar effect was observed in the open field test (OFT) **(F)** (n = 8 rats per group, two-way analysis of variance with Bonferroni posttests, $^{*}p < .05$ vs. AAV-eGFP + Veh group, $^{\#}p < .05$, $^{\#\#}p < .01$ vs. AAV-DHHC12 + Veh group). **(G)** Schematic model for the role of palmitoylation in the benzodiazepine-like anxiolytic action. Diazepam (DZP) binds to its site on the receptor and increases $GABA_AR$ activity, followed by activity-dependent DHHC12 autopalmitoylation, which triggers gephyrin palmitoylation and stabilizes surface gamma-aminobutyric acid A receptor ($GABA_AR$) cluster to confer a sustained anxiolytic action. Error bars represent mean ± SEM.

association of gephyrin with $GABA_AR$ in the BLA. The lower level of palmitoylated gephyrin in highly anxious rats was consistent with the decreased expression of DHHC12. We also found that DZP increased DHHC12 activity in the BLA and triggered gephyrin palmitoylation, which may contribute to its anxiolytic effects. Interestingly, the anxiolytic effect of DHHC12 overexpression was blocked by flumazenil, a specific antagonist of BZD-GABA receptor, indicating that DHHC12 may represent an endogenous anxiolytic mechanism (Figure 5G).

Our finding provides important in vivo evidence for the critical function of gephyrin palmitoylation in the stabilization of $GABA_ARs$. Inhibitory GABAergic interneurons in the BLA releases GABA and acts on $GABA_ARs$ of the pyramidal neuron (46–48). At inhibitory synapses, the scaffolding protein gephyrin is required for the synaptic localization of $GABA_AR$ (13–17). We found that although the expression of gephyrin in the BLA was not altered, its function was impaired under anxiety status. It should be noted that gephyrin depends on both actin microfilaments and microtubules for synaptic targeting and binds to the cytoplasmic loop of many targets (49–52) such as the glycine receptor β subunit (53) and ena/VASP (54). To verify the role of gephyrin–$GABA_AR$ signaling in anxiety, we used a reported $GABA_AR$ α3 subunit–derived peptide that specifically disrupted the interactions between gephyrin and $GABA_AR$ (44,55). Previous studies have demonstrated that the α3 subunits display the highest in vitro gephyrin affinity of all $GABA_AR$ subunits (56). Blocking the gephyrin–α3 interaction also disrupts the interactions of the α1 and α2 subunits with gephyrin because the binding motifs of

these α subunits are conserved (57). We found that injection of α3 subunit–derived peptide into the BLA aggravated anxiety behavior. These results support the hypothesis that the surface stability of α2- and α3-containing $GABA_AR$ subtypes contributes to the etiology of anxiety.

Many currently available anxiolytic drugs act on the $GABA_AR$ by increasing GABA content or binding to $GABA_AR$. Alternatively, the anxiolytic drug could target the interaction between $GABA_AR$ and its intracellular scaffolding protein gephyrin to promote their association. Recently, it has been shown that the interaction between $GABA_AR$ and gephyrin is controlled by palmitoylation. Under physiological conditions, palmitoylation is finely regulated by PATs. One of the PATs, DHHC12, selectively palmitoylates gephyrin and affects its association with the postsynaptic membrane. Thus, promotion of DHHC12 activity may emerge as a potential target for the anxiolytic agent. BZDs have been used to treat anxiety disorders for more than five decades, and their effects are thought to be mediated by the modulation of single-channel activity, which means that BZDs bind to $GABA_AR$ at a high-affinity binding site located between the α and γ subunits, followed by an increase in affinity of the receptor. Here, we identified that DHHC12 may be a new target for BZD action. We found that DZP rapidly increased DHHC12 activity and gephyrin palmitoylation via a $GABA_AR$-dependent manner in the BLA, and DHHC12 knockdown nearly abolished the anxiolytic effect of DZP. The acute effect of DZP on anxiety by binding to $GABA_AR$, and its lasting anxiolytic effect by initiating a DHHC12-dependent structure consequence of $GABA_AR$ conformation, may constitute the basis of DZP action on anxiety.

The discovery of BZD sites on $GABA_AR$ leads to the hypothesis that there may exist an endogenous molecular basis of allosteric modulation of $GABA_AR$ such as endogenous ligands acting on BZD sites or endogenous GABA-BZD-like receptors. Altered conformational property of $GABA_AR$ may work as the functional basis of GABA-BZD-like receptors. DHHC12-mediated palmitoylation may affect $GABA_AR$ conformational property via promoting localization of gephyrin at synapse (18). We found that the functional consequence of DHHC12 overexpression was abolished by flumazenil, indicating that enhancing DHHC12 activity may mimic a GABA-BZD receptor-dependent action via promoting the formation or increasing the availability of endogenous GABA-BZD-like receptors. The precise mechanism requires further investigation.

Several questions remained to be verified. In the BLA, both interneurons and principal neurons are involved in the control of anxiety. Whether the decrease in gephyrin palmitoylation under high anxiety was located in the principal neurons needs to be clarified. It would be appropriate to investigate its location using specific antibodies for palmitoylated proteins. Although some targets have been screened, other targets may also be palmitoylated, which can be further evaluated by a palmitoylation-coupled proteomic assay.

In summary, our study provides evidence that gephyrin palmitoylation negatively controls anxiety behavior, which principally mediates the anxiolytic effect of BZD and represents another anxiolytic mechanism. These results also support the view that the strength of inhibitory synapses can be scaled via gephyrin palmitoylation and raise a potential therapeutic value of DHHC12-regulating compounds in the therapy of anxiety disorders.

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Z-CS performed most molecular and behavioral experiments and analyzed data. P-FW conceived the project, designed the experiments, and helped with methodology. Z-XX performed electrophysiological experiments and analyzed data. QD, W-HL, and T-LN performed stereotaxic surgeries. S-QZ, X-PY, and H-LZ performed quantitative polymerase chain reaction. J-JL, S-QG, L-HL, and Z-LH gave useful suggestions and discussed the manuscript. FW and J-GC supervised the project, designed the experiments, revised the manuscript, and supported funding acquisition.

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ARTICLE INFORMATION

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REFERENCES

1. McGaugh JL (2004): The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci 27:1–28.
2. Phelps EA, LeDoux JE (2005): Contributions of the amygdala to emotion processing: From animal models to human behavior. Neuron 48:175–187.
3. Rainnie DG, Asprodini EK, Shinnick-Gallagher P (1991): Inhibitory transmission in the basolateral amygdala. J Neurophysiol 66:999–1009.
4. Trincavelli ML, Da Pozzo E, Daniele S, Martini C (2012): The $GABA_A$-BZR complex as target for the development of anxiolytic drugs. Curr Top Med Chem 12:254–269.
5. Rogers CJ, Twyman RE, Macdonald RL (1994): Benzodiazepine and beta-carboline regulation of single $GABA_A$ receptor channels of mouse spinal neurones in culture. J Physiol 475:69–82.
6. Berezhnoy D, Baur R, Gonthier A, Foucaud B, Goeldner M, Sigel E (2005): Conformational changes at benzodiazepine binding sites of $GABA_A$ receptors detected with a novel technique. J Neurochem 92:859–866.

7. Williams DB, Akabas MH (2000): Benzodiazepines induce a conformational change in the region of the gamma-aminobutyric acid type A receptor α_1-subunit M3 membrane-spanning segment. Mol Pharmacol 58:1129–1136.
8. Jacob TC, Michels G, Silayeva L, Haydon J, Succol F, Moss SJ (2012): Benzodiazepine treatment induces subtype-specific changes in $GABA_A$ receptor trafficking and decreases synaptic inhibition. Proc Natl Acad Sci U S A 109:18595–18600.
9. Gravielle MC (2016): Activation-induced regulation of $GABA_A$ receptors: Is there a link with the molecular basis of benzodiazepine tolerance? Pharmacol Res 109:92–100.
10. Gouzer G, Specht CG, Allain L, Shinoe T, Triller A (2014): Benzodiazepine-dependent stabilization of $GABA_A$ receptors at synapses. Mol Cell Neurosci 63:101–113.
11. Levi S, Le Roux N, Eugene E, Poncer JC (2015): Benzodiazepine ligands rapidly influence $GABA_A$ receptor diffusion and clustering at hippocampal inhibitory synapses. Neuropharmacology 88:199–208.
12. Kuver A, Smith SS (2016): Flumazenil decreases surface expression of $\alpha_4\beta_2\delta$ $GABA_A$ receptors by increasing the rate of receptor internalization. Brain Res Bull 120:131–143.
13. Peng G, Yan Y, Zhu C, Wang S, Yan X, Lu L, *et al.* (2008): Borna disease virus P protein affects neural transmission through interactions with gamma-aminobutyric acid receptor-associated protein. J Virol 82:12487–12497.
14. Tretter V, Mukherjee J, Maric HM, Schindelin H, Sieghart W, Moss SJ (2012): Gephyrin, the enigmatic organizer at GABAergic synapses. Front Cell Neurosci 6:23.
15. Betz H (1998): Gephyrin, a major player in GABAergic postsynaptic membrane assembly? Nat Neurosci 1:541–543.
16. Jacob TC, Bogdanov YD, Magnus C, Saliba RS, Kittler JT, Haydon PG, *et al.* (2005): Gephyrin regulates the cell surface dynamics of synaptic $GABA_A$ receptors. J Neurosci 25:10469–10478.
17. Kneussel M (2002): Dynamic regulation of $GABA_A$ receptors at synaptic sites. Brain Res Brain Res Rev 39:74–83.
18. Dejanovic B, Semtner M, Ebert S, Lamkemeyer T, Neuser F, Luscher B, *et al.* (2014): Palmitoylation of gephyrin controls receptor clustering and plasticity of GABAergic synapses. PLoS Biol 12:e1001908.
19. Mitchell DA, Vasudevan A, Linder ME, Deschenes RJ (2006): Protein palmitoylation by a family of DHHC protein *S*-acyltransferases. J Lipid Res 47:1118–1127.
20. Linder ME, Deschenes RJ (2007): Palmitoylation: Policing protein stability and traffic. Nat Rev Mol Cell Biol 8:74–84.
21. Aicart-Ramos C, Valero RA, Rodriguez-Crespo I (2011): Protein palmitoylation and subcellular trafficking. Biochim Biophys Acta 1808:2981–2994.
22. Iwanaga T, Tsutsumi R, Noritake J, Fukata Y, Fukata M (2009): Dynamic protein palmitoylation in cellular signaling. Prog Lipid Res 48:117–127.
23. Fukata Y, Fukata M (2010): Protein palmitoylation in neuronal development and synaptic plasticity. Nat Rev Neurosci 11:161–175.
24. Thomas GM, Huganir RL (2013): Palmitoylation-dependent regulation of glutamate receptors and their PDZ domain-containing partners. Biochem Soc Trans 41:72–78.
25. el-Husseini A-D, Bredt DS (2002): Protein palmitoylation: A regulator of neuronal development and function. Nat Rev Neurosci 3:791–802.
26. Song M, Messing RO (2005): Protein kinase C regulation of $GABA_A$ receptors. Cell Mol Life Sci 62:119–127.
27. Rye DB, Bliwise DL, Parker K, Trotti LM, Saini P, Fairley J, *et al.* (2012): Modulation of vigilance in the primary hypersomnias by endogenous enhancement of $GABA_A$ receptors. Sci Transl Med 4:161ra151.
28. Ojima K, Matsumoto K, Watanabe H (1997): Flumazenil reverses the decrease in the hypnotic activity of pentobarbital by social isolation stress: Are endogenous benzodiazepine receptor ligands involved? Brain Res 745:127–133.
29. Christian CA, Herbert AG, Holt RL, Peng K, Sherwood KD, Pangratz-Fuehrer S, *et al.* (2013): Endogenous positive allosteric modulation of $GABA_A$ receptors by diazepam binding inhibitor. Neuron 78:1063–1074.
30. Rothstein JD, Guidotti A, Tinuper P, Cortelli P, Avoni P, Plazzi G, *et al.* (1992): Endogenous benzodiazepine receptor ligands in idiopathic recurring stupor. Lancet 340:1002–1004.
31. Cravatt BF, Prospero-Garcia O, Siuzdak G, Gilula NB, Henriksen SJ, Boger DL, *et al.* (1995): Chemical characterization of a family of brain lipids that induce sleep. Science 268:1506–1509.
32. Hogg S (1996): A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 54:21–30.
33. Prut L, Belzung C (2003): The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. Eur J Pharmacol 463:3–33.
34. Bi LL, Sun XD, Zhang J, Lu YS, Chen YH, Wang J, *et al.* (2015): Amygdala NRG1-ErbB4 is critical for the modulation of anxiety-like behaviors. Neuropsychopharmacology 40:974–986.
35. Hollis F, van der Kooij MA, Zanoletti O, Lozano L, Canto C, Sandi C (2015): Mitochondrial function in the brain links anxiety with social subordination. Proc Natl Acad Sci U S A 112:15486–15491.
36. Li YL, Wu PF, Chen JG, Wang S, Han QQ, Li D, *et al.* (2017): Activity-dependent sulfhydration signal controls *N*-methyl-D-aspartate subtype glutamate receptor-dependent synaptic plasticity via increasing d-serine availability. Antioxid Redox Signal 27:398–414.
37. Yu XW, Hu ZL, Ni M, Fang P, Zhang PW, Shu Q, *et al.* (2015): Acid-sensing ion channels promote the inflammation and migration of cultured rat microglia. Glia 63:483–496.
38. Gourley SL, Kiraly DD, Howell JL, Olausson P, Taylor JR (2008): Acute hippocampal brain-derived neurotrophic factor restores motivational and forced swim performance after corticosterone. Biol Psychiatry 64:884–890.
39. Wan J, Roth AF, Bailey AO, Davis NG (2007): Palmitoylated proteins: Purification and identification. Nat Protoc 2:1573–1584.
40. Han J, Zhang H, Wang S, Zhou J, Luo Y, Long LH, *et al.* (2016): Potentiation of surface stability of AMPA receptors by sulfhydryl compounds: A redox-independent effect by disrupting palmitoylation. Neurochem Res 41:2890–2903.
41. Magarinos AM, Orchinik M, McEwen BS (1998): Morphological changes in the hippocampal CA3 region induced by non-invasive glucocorticoid administration: A paradox. Brain Res 809:314–318.
42. Deroche V, Piazza PV, Maccari S, Le Moal M, Simon H (1992): Repeated corticosterone administration sensitizes the locomotor response to amphetamine. Brain Res 584:309–313.
43. Rathenberg J, Kittler JT, Moss SJ (2004): Palmitoylation regulates the clustering and cell surface stability of $GABA_A$ receptors. Mol Cell Neurosci 26:251–257.
44. Maric HM, Kasaragod VB, Haugaard-Kedstrom L, Hausrat TJ, Kneussel M, Schindelin H, *et al.* (2015): Design and synthesis of high-affinity dimeric inhibitors targeting the interactions between gephyrin and inhibitory neurotransmitter receptors. Angew Chem Int Ed Engl 54:490–494.
45. Globa AK, Bamji SX (2017): Protein palmitoylation in the development and plasticity of neuronal connections. Curr Opin Neurobiol 45:210–220.
46. Gean PW, Chang FC (1992): Pharmacological characterization of excitatory synaptic potentials in rat basolateral amygdaloid neurons. Synapse 11:1–9.
47. Smith BN, Dudek FE (1996): Amino acid-mediated regulation of spontaneous synaptic activity patterns in the rat basolateral amygdala. J Neurophysiol 76:1958–1967.
48. Smith KS, Rudolph U (2012): Anxiety and depression: Mouse genetics and pharmacological approaches to the role of $GABA_A$ receptor subtypes. Neuropharmacology 62:54–62.
49. Kirsch J, Betz H (1995): The postsynaptic localization of the glycine receptor-associated protein gephyrin is regulated by the cytoskeleton. J Neurosci 15:4148–4156.
50. Allison DW, Chervin AS, Gelfand VI, Craig AM (2000): Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: Maintenance of core components independent of actin filaments and microtubules. J Neurosci 20:4545–4554.
51. Giesemann T, Schwarz G, Nawrotzki R, Berhorster K, Rothkegel M, Schluter K, *et al.* (2003): Complex formation between the postsynaptic

scaffolding protein gephyrin, profilin, and Mena: A possible link to the microfilament system. J Neurosci 23:8330–8339.

52. Maas C, Tagnaouti N, Loebrich S, Behrend B, Lappe-Siefke C, Kneussel M (2006): Neuronal cotransport of glycine receptor and the scaffold protein gephyrin. J Cell Biol 172:441–451.
53. Sola M, Bavro VN, Timmins J, Franz T, Ricard-Blum S, Schoehn G, *et al.* (2004): Structural basis of dynamic glycine receptor clustering by gephyrin. EMBO J 23:2510–2519.
54. Bausen M, Fuhrmann JC, Betz H, O'Sullivan GA (2006): The state of the actin cytoskeleton determines its association with gephyrin: Role of ena/VASP family members. Mol Cell Neurosci 31:376–386.
55. Tretter V, Kerschner B, Milenkovic I, Ramsden SL, Ramerstorfer J, Saiepour L, *et al.* (2011): Molecular basis of the gamma-aminobutyric acid A receptor α_3 subunit interaction with the clustering protein gephyrin. J Biol Chem 286:37702–37711.
56. Maric HM, Hausrat TJ, Neubert F, Dalby NO, Doose S, Sauer M, *et al.* (2017): Gephyrin-binding peptides visualize postsynaptic sites and modulate neurotransmission. Nat Chem Biol 13:153–160.
57. Maric HM, Kasaragod VB, Hausrat TJ, Kneussel M, Tretter V, Stromgaard K, *et al.* (2014): Molecular basis of the alternative recruitment of $GABA_A$ versus glycine receptors through gephyrin. Nat Commun 5:5767.