Impaired Hippocampal Neuroligin-2 Function by Chronic Stress or Synthetic Peptide Treatment is Linked to Social Deficits and Increased Aggression

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Neuroligins (NLGNs) are cell adhesion molecules that are important for proper synaptic formation and functioning, and are critical regulators of the balance between neural excitation/inhibition (E/I). Mutations in NLGNs have been linked to psychiatric disorders in humans involving social dysfunction and are related to similar abnormalities in animal models. Chronic stress increases the likelihood for affective disorders and has been shown to induce changes in neural structure and function in different brain regions, with the hippocampus being highly vulnerable to stress. Previous studies have shown evidence of chronic stress-induced changes in the neural E/I balance in the hippocampus. Therefore, we hypothesized that chronic restraint stress would lead to reduced hippocampal NLGN-2 levels, in association with alterations in social behavior. We found that rats submitted to chronic restraint stress in adulthood display reduced sociability and increased aggression. This occurs along with a reduction of NLGN-2, but not NLGN-1 expression (as shown with western blot, immunohistochemistry, and electron microscopy analyses), throughout the hippocampus and detectable in different layers of the CA1, CA3, and DG subfields. Furthermore, using synthetic peptides that comprise sequences in either NLGN-1 (neurolide-1) or NLGN-2 (neurolide-2) involved in the interaction with their presynaptic partner neurexin (NRXN)-1, intra-hippocampal administration of neurolide-2 led also to reduced sociability and increased aggression. These results highlight hippocampal NLGN-2 as a key molecular substrate regulating social behaviors and underscore NLGNs as promising targets for the development of novel drugs for the treatment of dysfunctional social behaviors.

INTRODUCTION

Substantial evidence highlights the synaptic cell adhesion molecules neuroligins (NLGNs) and neurexins (NRXNs) as critical regulators of the neural excitation/inhibition (E/I) balance (Levinson and El-Husseini, 2005; Südhof, 2008). The trans-synaptic complex formed by postsynaptically expressed NLGNs with their presynaptic partners, NRXNs, has been critically implicated in the maintenance and function of excitatory glutamatergic and inhibitory gamma-aminobutyric acid (GABA)-ergic synapses (Südhof, 2008). Among the four members of the NLGN family, NLGN-1 and NLGN-2 are typically associated with either excitatory (NLGN-1) or inhibitory (NLGN-2) synapses (Graf *et al*, 2004; Chih *et al*, 2005).

Alterations in the balance between neural E/I have been proposed to underlie behavioral dysfunctions in several psychiatric disorders, such as schizophrenia and autism spectrum disorders, characterized by marked deficits in social interactions and communication (Harrison and Weinberger, 2005; Südhof, 2008). This view is supported by clinical evidence reporting mutations in genes encoding for NLGN and NRXNs (Sun *et al*, 2011; Gauthier *et al*, 2011). Furthermore, mice exhibiting mutations in NLGNs were shown to display alterations in the E/I balance as well as

abnormal social behaviors (Tabuchi et al, 2007; Hines et al, 2008; Jamain et al, 2008; Blundell et al, 2010).

This genetic evidence is in line with the hypothesis that pathological social behaviors can emerge from the disruption in the E/I balance occurring in relevant neural circuits during critical periods of development (Gogolla et al, 2009). However, a recent report (Yizhar et al, 2011) showed that the acute alteration of this balance in adulthood can also have immediate effects: the acute elevation of the E/I balance in the prefrontal cortex in mice, by optogenetic facilitation of glutamatergic activity, was shown to reduce social exploration (Yizhar et al, 2011). These findings raise the possibility that not only genetic factors, but also life experiences or treatments alter the expression and/or function of key molecular pathways that control the levels of excitatory and/or inhibitory synapses. These changes could underlie alterations in social behaviors occurring in adulthood.

In rodents, chronic stress is possibly the best characterized example of environmentally induced social avoidance (Toth and Neumann, 2013), and emerging evidence indicates that it can also increase aggressive behavior (Wood et al, 2008). Chronic stress has also been shown to induce changes in neural structure and function in different brain regions, with the hippocampus being highly vulnerable to stress (McEwen, 2005). Importantly, in the hippocampus, chronic stress is thought to lead to an increase in the E/I balance as indicated by enhanced synaptic excitation (Karst and Joëls, 2003), increased levels of glutamate transporters (GLTs) (Reagan et al, 2004), and a reduced number of parvalbumin-immunoreactive GABAergic interneurons (Hu et al, 2010). A role for cell adhesion molecules in the structural and functional effects of chronic restraint stress has been substantiated in studies focusing on NCAM (and its polysialylated form, PSA-NCAM) and L1 following chronic stress (Sandi, 2004; Bisaz et al, 2011; Gilabert-Juan et al, 2011). We selected the 3-week chronic restraint stress paradigm in rats, as it has been shown to increase aggressive behavior against homecage mates (Wood et al, 2003, 2008), as well as for its well-characterized effects in the literature at different levels of analysis, including morphological, electrophysiological, and molecular levels, particularly with regards to the hippocampus (Pavlides et al, 2002; Stewart et al, 2005; Donohue et al, 2006; McEwen, 2012).

In this study, we aimed to investigate whether chronic stress would lead to changes in the expression levels of NLGN-1 and NLGN-2 in specific hippocampal subfields in association with alterations in social behavior (sociability and aggression). We hypothesized that chronic restraint stress would lead to reduced hippocampal NLGN-2 levels, with either increased or unchanged NLGN-1 levels. The hypothesis regarding NLGN-2 was based on the following observations: (i) NLGN-2-deficient mice display increased hippocampal excitability (Jedlicka et al, 2011) and (ii) reduced ultrasonic vocalizations (Wöhr et al, 2012); and (iii) hippocampus-specific overexpression of NLGN-2 led to inhibited aggressive behavior (Kohl et al, 2013). Regarding NLGN-1: (i) the fact that NLGN-1 overexpression leads to increased synaptic excitation in the hippocampus (Dahlhaus et al, 2010) led us to speculate that increased NLGN-1 expression occurs following exposure to chronic stress; however, (ii) the lack of evidence for alterations in

sociability or aggressive behaviors from genetic studies manipulating expression levels of this gene led us to hypothesize a lack of changes in NLGN-1 expression following chronic stress. Using a combination of behavioral, biochemical, and morphological approaches, we obtained results in support of these hypotheses that led us to further postulate that pharmacological interference with NLGN-2, but not NLGN-1, function in the hippocampus would alter social and aggressive behaviors. To investigate this, we performed a follow-up pharmacological study to determine the impact of interfering with NLGN function on social behaviors through intra-hippocampal microinfusion of synthetic peptides derived from either NLGN-1 (neurolide-1) (Gjørlund et al, 2012) or NLGN-2 (neurolide-2).

MATERIALS AND METHODS

Animals

Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC) and the Cantonal Veterinary Authorities (Vaud, Switzerland). Male Sprague–Dawley rats (Charles River Laboratories; Lyon, France) weighing 250 g at the start of the experiments were pair-housed under light- (12 h light/dark cycle; lights on at 0700 hours) and temperature (22 ± 2 °C)-controlled conditions. Food and water were freely available. With the exception of the resident–intruder test (performed from 0800–2000 hours), all experiments were conducted between 0830 and 1400 hours to minimize the influence of hormonal fluctuations. All animals were handled for 2 min/day for the 3 d preceding the first behavioral test or surgery. The animals used for western blot, immunohistochemistry, or electron microscopy analyses were killed 1 day after stress.

Chronic Restraint Stress

Rats were carefully wrapped in cloth to prevent injuries from the restraint. Animals were restrained 6 h/day for 21 days in wire mesh restrainers (26 × 26 cm) in their homecage between 1000 and 1600 hours. Body weight was measured every other day during the stress protocol starting at day 1. There was no indication of wounds or injuries to the rats.

Social Behaviors

The following tests were applied to evaluate different aspects of social behaviors: the three-chambered social approach, the resident–intruder, and the social encounter in a neutral cage tests. All behaviors were scored by a behavioral expert blinded to experimental conditions. The social approach test was performed 1 day after the stress paradigm and was performed to evaluate the animals' preference to approach a juvenile conspecific or an object (see Supplementary Materials and Methods for further details). Two weeks after the social approach, test animals were tested for aggression in the resident–intruder test. The effect of intra-hippocampal infusion of neurolide-1 and/or neurolide-2 on social behavior was assessed in the social approach test and the social encounter in a neutral cage test (see Supplementary Materials and Methods for further details).

Western Blots

Protein analyses were performed to investigate NLGN expression in the synaptoneurosomal compartments and total homogenates from hippocampal CA1, CA3, and DG and also in the frontal cortex as control region (see Supplementary Figure S1, Supplementary Figure S2 and Supplementary Materials and Methods for further details and for validation of the NLGN-2 antibody).

Immunohistochemistry

Immunofluorescent staining against NLGN-2 allowed for a detailed analysis of the protein in subregions of the hippocampal CA1, CA3, and DG (see Supplementary Materials and Methods for further details). We found that NLGN-2 colocalized with gephyrin that was expected, as both are expressed at GABA-ergic neurons. Furthermore, omission of the primary antibody or incubation of the primary antibody with a specific NLGN-2-blocking peptide prevented a NLGN-2 signal (Supplementary Figure S3).

Electron Microscopy Analyses

Electron microscopy enabled us to visualize and analyze ImmunoGold-positive labeling for NLGN-2 in the hippocampus (see Supplementary Materials and Methods for further details).

Measurement of Hippocampal Volume

Measurements of the volume of the whole hippocampus were made using the Cavalieri principle (Sousa et al, 1997; Popov et al, 2004, see Supplementary Materials and Methods for further details).

The NLGN-Derived Peptides, Neurolide-1, and Neurolide-2

The neurolide-1 peptide (SEGNRWSNSTKGLFQRA) was previously described and validated (Gjørlund et al, 2012). The neurolide-2 peptide (HSEGLFQRA) was newly developed for the current study. As no NLGN-2-derived peptide was available, we developed neurolide-2. Although there is no structural data for the NLGN-2/NRXN-1 complex, the ectodomain of NLGN-2 shares >70% sequence identity with the ectodomain of NLGN-1 (Gjørlund et al, 2012), and the crystal structure of the extracellular cholinesterase-like domain from NLGN-2 has been solved (Koehnke et al, 2008). Thus, we designed the neurolide-2 peptide based on sequence and structure homology between homologs sequence motifs in NLGN-2 and NLGN-1, respectively (Figure 4a–c), assuming that if the neurolide-1 motif is involved in NLGN-1 interaction with NRXN-1β (Gjørlund et al,, 2012), so it might be the neurolide-2 motif. The difference between these two motifs is that neurolide-1 includes a sequence corresponding to splice site B in NLGN-1, which increases affinity of the protein to NRXN-1β binding. Splice site B is absent in NLGN-2. Thus, the difference between the sequences of neurolide-2 and neurolide-1 reflects the fundamental difference in structural

(and therefore functional) modifications between NLGN-2 and NLGN-1.

The neurolide-2 peptide (HSEGLFQRA) was synthesized as a tetramer composed of four monomers coupled to a lysine backbone. Scrambled versions (FRGEASLQH, QAS-FELRHG, and FEASHRLQG) and reversed form (ARQFL-GESH) of 2NLGN2d peptide were synthesized in the same way by Schafer-N (Copenhagen, Denmark). The peptides were synthesized using the solid-phase Fmoc protection strategy, and purity was estimated to be $\geqslant 80\%$ by high-performance liquid chromatography. The peptides were reconstituted in sterile distilled water, and the concentrations were determined by measuring the absorbance at 205 nm. Human recombinant NLGN-2 was obtained from R&D Systems (Abingdon, UK).

The concentration for the in vitro assay for neurite outgrowth and for the in vivo microinfusion experiments were selected based on a cell survival assay (see Supplementary Materials and Methods for further details on neurolide-1, as well as Supplementary Figure S4).

Neurite Outgrowth

Hippocampal neurons were obtained on embryonic day 19 from Wistar rat embryos (Charles River Laboratories, Kisselegg, Germany) as described previously (Kraev et al, 2011). Neurite outgrowth was determined as previously described (Kolkova et al, 2000) (see Supplementary Materials and Methods for further details).

Plasma Corticosterone Analyses

Plasma samples (dilution 1/40) were assayed by a commercially available enzyme-linked immunoabsorbent assay (Corticosterone EIA Kit, Enzo Life Sciences, Lausen, Switzerland). The intra-assay coefficient of variation was 8% and 6.6% for low- and high concentrations, respectively. The inter-assay coefficient of variation was 13.1% and 7.8% for low- and high concentrations, respectively.

Surgery and Pharmacological Infusions

Rats subjected to pharmacological experiments were bilaterally implanted with 5-mm stainless steel guide cannulae (Plastics One, Roanoke, VA, USA) aimed at the dorsal hippocampus Bilateral simultaneous microinfusions of either the NLGN-1-derived peptide, termed neurolide-1 (Gjørlund et al, 2012), or the NLGN-2-derived peptide, termed neurolide-2 (dose for each peptide: 2.5 µg in 1 µl vehicle) were delivered at 0.5 µl/min over 2 min. The injector remained in place for 1 min additionally to allow local drug diffusion. Animals were infused once per day over 5 consecutive days before behavioral experiments. Thereafter, animals were killed by i.p. pentobarbital injection, and correct cannulae placement was verified with Evans blue and histology (see Supplementary Materials and Methods for further details).

Open-Field and Morris Water Maze

To investigate the specificity of intra-hippocampal neurolide-2 infusion on social behaviors, we assessed animals'

behavior after neurolide-2 infusion in the open-field (anxiety and locomotion) and Morris water maze (cognitive integrity). For methodological details see Supplementary Materials and Methods.

Statistical Analysis

Data are expressed as the mean ± SE. Behavioral observations from the social approach test and corticosterone data were analyzed using a two-way repeated measures (RM) analysis of variance (ANOVA) (Yang et al, 2011) followed by Bonferroni post hoc tests where appropriate. The in vitro data were analyzed using one-way RM ANOVA followed by the Bonferroni post hoc tests where appropriate. Two-sample comparisons were analyzed using the two-tailed Student's t-test. Data were considered statistically significant when $p < 0.05$.

RESULTS

Chronic Stress Reduces Social Investigation and Increases Aggressive Behavior

Body weight gain was reduced by chronic stress (data not shown), confirming the stressful nature of the restraint protocol. In addition, stress affected exploration in the social approach task (two-way RM ANOVA, main effect of stress $F_{1,26} = 7.35$, $p = 0.01$, controls: $n = 15$, stressed: $n = 13$, Figure 1a). Post hoc tests showed that stress reduced investigation of the juvenile ($t = 4.16$, $p < 0.001$) but not the object ($t = 0.31$, $p > 0.05$). Locomotor activity did not differ between controls and stressed animals (Figure 1b; $t = 0.94$, df = 25, $p = 0.36$). The same rats were tested in the resident–intruder test 14 days afterwards. Resident rats that had experienced chronic stress were overly aggressive against their matched intruder as compared with control residents (Figure 1c; $t = 2.43$, df = 25, $p = 0.02$). The enhanced aggression exhibited by stressed animals is reflected by the components that comprise total offensive behavior (Figure 1d–f; offensive upright: $t = 2.88$, df = 25, $p = 0.008$; keeping down: $t = 2.27$, df = 25, $p = 0.03$; and a nonsignificant tendency for lateral threat: $t = 1.59$, df = 25, $p = 0.12$). Moreover, stressed rats tended to bite their opponent more often than controls (Figure 1g; control: 2.8 ± 1.0; stressed: 5.7 ± 1.2; $t = 1.88$, df = 25, $p = 0.07$).

Chronic Stress Leads to Decreased Expression of NLGN-2 Throughout the Hippocampus

Western blot analyses from synaptoneurosomal fractions indicated a reduction in NLGN-2, but not NLGN-1, protein levels throughout the different hippocampal subfields CA1 (NLGN-1: $t = 0.92$, df = 10, $p = 0.38$ $n = 6$/group, Figure 2a; NLGN-2: $t = 2.48$, df = 10, $p = 0.03$, $n = 6$/group, Figure 2e), CA3 (NLGN-1: $t = 0.67$, df = 9, $p = 0.52$, control: $n = 5$, stress: $n = 6$, Figure 2b; NLGN-2: $t = 2.48$, df = 9, $p = 0.035$, control: $n = 6$, stress: $n = 5$, Figure 2f) and DG (NLGN-1: $t = 0.08$, df = 16, $p = 0.94$, $n = 9$/group, Figure 2c; NLGN-2: $t = 3.34$, df = 10, $p = 0.007$, $n = 6$/group, Figure 2g). The chronic stress-induced downregulation of NLGN-2 was specific to the hippocampus, as we found that stress did not affect expression of NLGN-2 (or NLGN-1) in the frontal

cortex (NLGN-1: $t = 0.45$, df = 9, $p = 0.67$, control: $n = 6$, stress: $n = 5$, Figure 2d; NLGN-2: $t = 0.81$, df = 10, $p = 0.44$, $n = 6$/group, Figure 2h). Furthermore, expression levels of NLGN-1 and NLGN-2 in the total fraction from hippocampus or frontal cortex were not affected by stress (Supplementary Figure S2). Validation of the NLGN-2 antibody for the western blot and immunohistochemical analyses is presented in Supplementary Figure S1 and S3.

Reduced levels of hippocampal NLGN-2 in chronic stress-treated animals were confirmed by immunohistochemistry as described below. We found reduced NLGN-2 expression in different layers throughout the CA1 (stratum oriens, $t = 1.04$, df = 6, $p = 0.34$; stratum pyramidale: $t = 0.57$, df = 6, $p = 0.59$, stratum radiatum, $t = 2.46$, df = 6, $p = 0.049$; stratum lacunosum-moleculare: $t = 2.84$, d = 6, $p = 0.03$, Figure 3a), CA3 (stratum oriens, $t = 3.12$, df = 6, $p = 0.02$; stratum pyramidale: $t = 2.64$, df = 6, $p = 0.04$; stratum radiatum: $t = 2.63$, df = 6, $p = 0.04$; stratum lacunosum-moleculare: $t = 1.60$, df = 6, $p = 0.16$, Figure 3b), and DG (granular cell layer: $t = 2.81$, df = 6, $p = 0.03$; molecular cell layer: $t = 2.32$, df = 6, $p = 0.06$, Figure 3c).

Using electron microscopy, we were able to confirm that the density of total hippocampal NLGN-2 was reduced in stressed animals as compared with controls ($t = 2.52$, df = 6, $p = 0.045$, Figure 3d). An example of ImmunoGold-labeled particles for NLGN-2 is shown at an inhibitory synapse (Figure 3e). We found that total hippocampal volume was not affected by stress ($t = 0.48$, df = 14, $p = 0.64$, Figure 3f).

In Vitro Characterization of the NLGN-2-Derived Peptide: Effects on Neurite Outgrowth

As the only existing and characterizing peptide was neurolide-1 peptide (Gjørlund et al, 2012), we developed here the neurolide-2 peptide based on sequence and structure homology between homologs sequence motifs in NLGN-2 and NLGN-1, respectively (Figure 4a–c). For its validation, we first performed an in vitro study in primary hippocampal cell cultures to check different peptide concentrations in cell survival (see Supplementary Materials and Methods and Supplementary Figure S4). We, then, proceeded to assess the effect of neurolide-2 in neurite outgrowth in hippocampal neurons. A treatment effect on neurite outgrowth was observed (one-way RM ANOVA, $p < 0.0001$) only with neurolide-2 ($t = 4.36$, $p < 0.01$; Figure 4d). The neuritogenic effect of neurolide-2, as well as of the whole NLGN-2 ectodomain, was abrogated by knocking down NRXN-1β (Figure 4e–f), indicating that in single-neuronal cultures, neuritogenic activity of the peptide required NRXN-1β expression, and that the neurolide-2 peptide might have a potential to interfere with NLGN-2-NRXN-1β binding. This result shows that the peptide is biologically active and, accordingly, potentially effective to disrupt NLGN-2-NRXN-1β interactions.

Infusion of a NLGN-2-, but not NLGN-1-, Derived Peptide into the Hippocampus Leads to Reduced Social Investigation and Increased Aggression

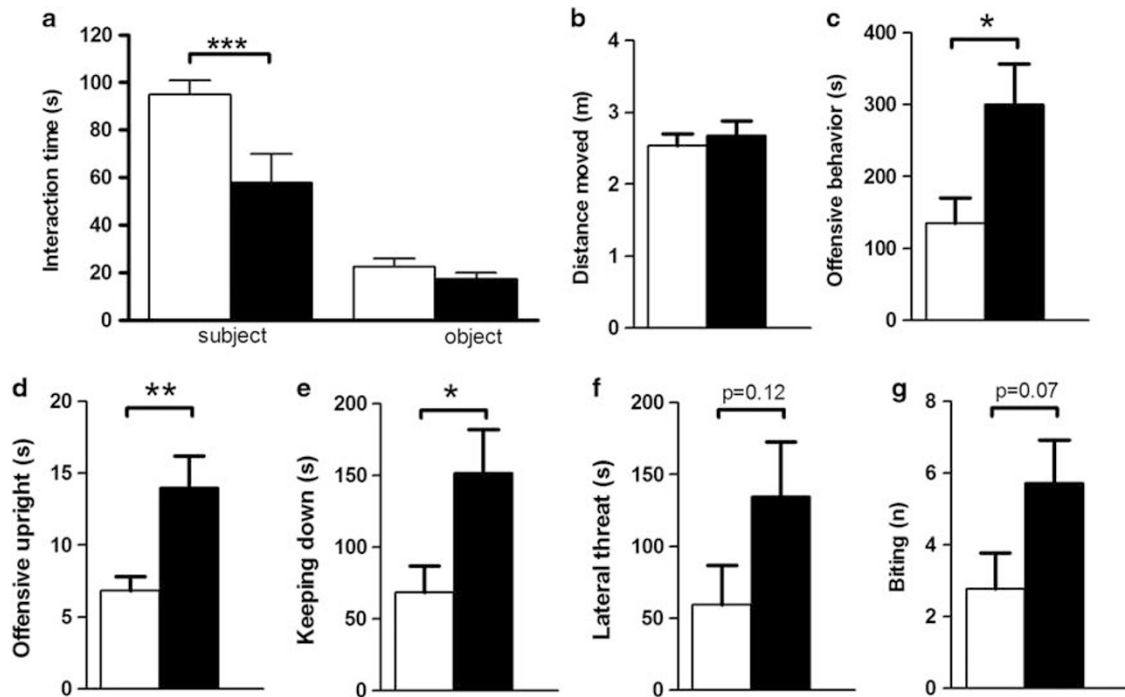First, we found that infusion of neurolide-1 in the hippocampus did not affect social investigation (two-way

Figure 1 Effects of chronic restraint stress on sociability and aggressive behavior. Investigatory behavior for a juvenile and an object (***$p<0.001$, controls: $n=15$, stressed: $n=13$ (a). Locomotor activity during the social approach task (b). Total aggressive behavior expressed in the resident–intruder test (*$p<0.05$) (c); offensive upright (**$p<0.01$) (d); keeping down (*$p<0.05$) (e); lateral threat (f) and biting (g). White bars indicate controls whereas the black bars indicate stressed animals. Error bars represent SEM.

RM ANOVA, main treatment effect, $F_{1,10}=0.02$, $p=0.88$, $n=6$/group, Figure 5a). However, neurolide-2 infusion did reduce social investigation as compared with vehicle-treated animals (two-way RM ANOVA, main treatment effect, $F_{1,17}=4.65$, $p=0.046$. *post hoc* tests: $p<0.01$, vehicle: $n=6$ and neurolide-2: $n=7$, Figure 5b). None of the peptides affected overall locomotor activity (neurolide-1: $t=0.55$, df $=12$, $p=0.59$, $n=7$ and neurolide-2: $t=0.53$, df $=10$, $p=0.61$, $n=6$, Figure 5c).

Neurolide-2 treatment led to increased aggressive behavior when animals ($n=7$/group) were exposed to an unfamiliar male in a neutral cage ($t=2.66$, df $=12$, $p=0.02$, Figure 5d). In line with the results on stressed animals in the resident–intruder task, animals treated with neurolide-2 tended to show increased amounts of offensive upright ($t=1.70$, df $=12$, $p=0.12$, Figure 5e), keeping down ($t=2.17$, df $=12$, $p=0.05$, Figure 5f), although the duration of lateral threat was low and not different between the two groups ($t=0.28$, df $=12$, $p=0.78$, Figure 5g). The infusion of neurolide-2 into the hippocampus did not affect peripheral corticosterone levels either at baseline or immediate after the social encounter (two-way RM ANOVA, main effect of social encounter, $F_{1,39}=42.6$, $p<0.001$, but no effect of drug, $F_{1,39}=0$, $p=0.97$, $n=13$ for vehicle/homecage and $n=10$ for all other groups, Figure 5h). Importantly, the effectiveness of neurolide-2 hippocampal treatment seems to be quite specific for social behaviors, as in the open field we found that locomotor activity or anxiety-like behavior were unaffected (locomotor activity: $t=0.44$, df $=11$, $p=0.67$ and anxiety-like behavior: $t=1.49$, df $=11$, $p=0.16$, vehicle: $n=6$, neurolide-2: $n=7$) (see Supplementary Figure S5). Moreover, spatial learning in the water maze was also not affected by neurolide-2 infusion (Supplementary Figure S6).

DISCUSSION

This study shows that chronic restraint stress in adult rats leads to reduced sociability and increased aggression along with a reduction of NLGN-2, but not NLGN-1, expression throughout the hippocampus that was detectable in different layers of the CA1, CA3, and DG subfields. The decrease in NLGN-2 was specific to the synaptoneurosomal compartment, as NLGN-2 protein levels were not affected in the total fraction. These results led us to speculate that interfering with NLGN-2 function in the hippocampus of adult rats would affect social behaviors.

Using synthetic peptides that comprise sequences in either NLGN-1 (neurolide-1) or NLGN-2 (neurolide-2) involved in the interaction with their presynaptic partner NRXN-1 (Gjørlund et al, 2012), we found that intra-hippocampal administration (over 5 consecutive days) of neurolide-2 in adult rats reduced sociability and increased aggression. Together with our previous findings showing that viral-induced overexpression of NLGN-2 in the adult hippocampus leads to decreased aggression (Kohl et al, 2013), these results highlight hippocampal NLGN-2 as a key molecular substrate regulating social behaviors. In an earlier study, however, constitutive NLGN-2 knockout mice did not differ from controls in social interactions (Blundell et al, 2009). We suspect that during development, compensatory mechanisms are at work in the NLGN-2
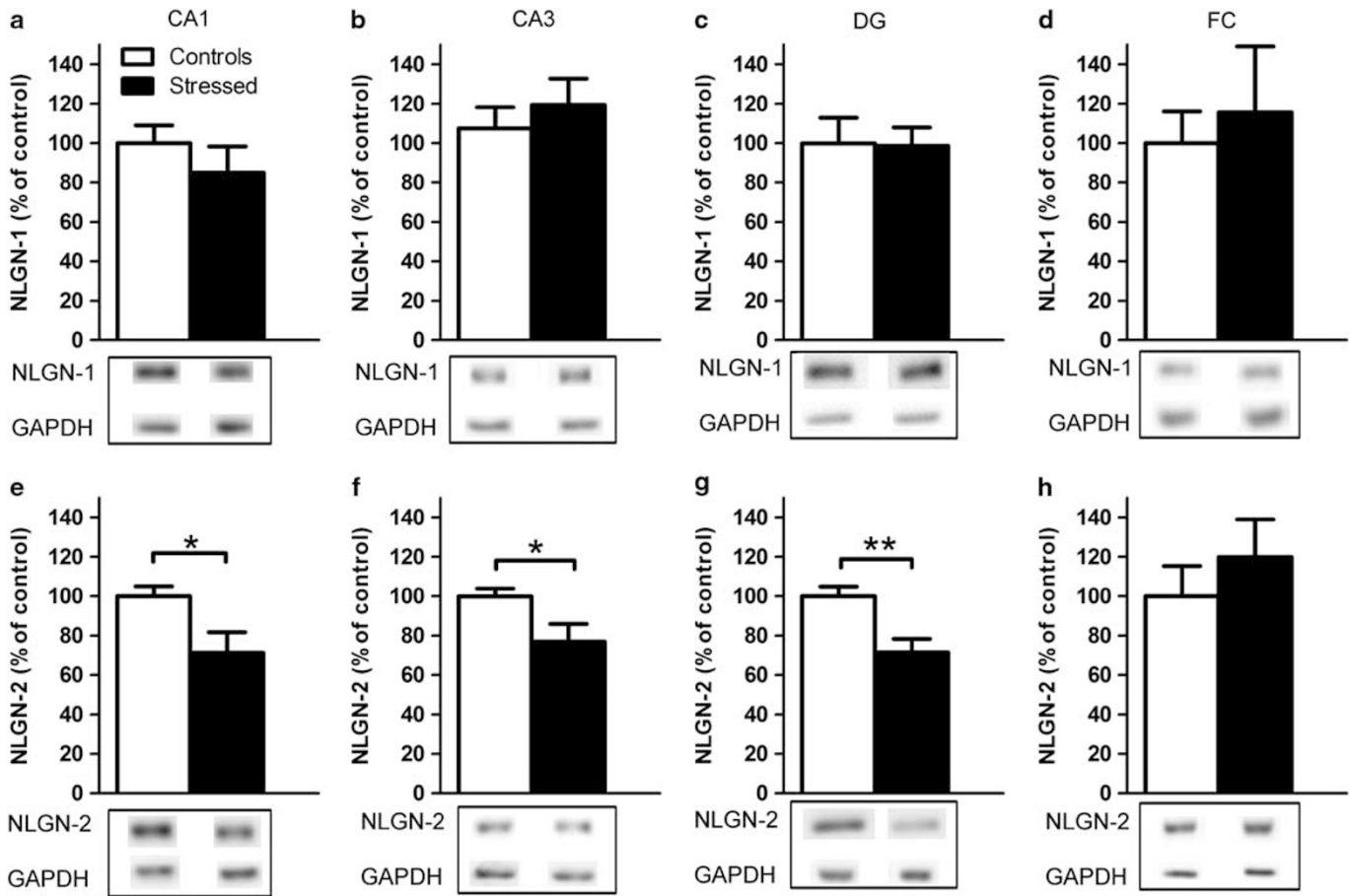
Figure 2 Effects of chronic restraint stress on hippocampal protein neuroligin-1 (NLGN-1) and neuroligin-2 (NLGN-2) expression. Synaptoneurosomal protein fractions of NLGN-1 (a–d) and NLGN-2 (e–h) in the hippocampal CA1 (a and e), ($n=6$/group); CA3 (b and f), ($n=5–6$/group); dentate gyrus (c and g), ($n=9$/group); and in the frontal cortex (d and h), ($n=5–6$/group) (*$p<0.05$, **$p<0.01$). Error bars represent SEM.

knockout mice that may have masked the expression of the social impairments in these animals.

The reduction in social exploration observed following exposure to chronic stress was not because of a general decrease in locomotor activity, which is in agreement with the literature (Kleen et al, 2006). These results are in line with the finding of social avoidance in rats exposed to a mild chronic stress protocol of the same length as here (21 days) (Kompagne et al, 2008) and with mounting evidence indicating reduced sociability following sustained exposure to stress (Toth and Neumann, 2013). The increased aggression that we find in stressed animals in a resident–intruder test extends previous reports, indicating increased aggression toward homecage mates (Wood et al, 2008).

The mechanisms whereby chronic restraint stress alters individuals' social interactions are probably multiple. Chronic restraint stress is known to induce neural remodeling in several brain regions, notably including the hippocampus, but also the prefrontal cortex and amygdala (Leuner and Shors, 2012; McEwen, 2012). A growing body of evidence suggests that the hippocampus is critically involved in the regulation of social interactions (Kogan et al, 2000; Uekita and Okanoya, 2011) and aggressive behaviors (Maaswinkel et al, 1997; Uekita and Okanoya,

2011). In agreement with the literature (Lee et al, 2009), we found evidence for a marginal (2.2%), and nonsignificant reduction in hippocampal volume in chronically stressed rats. Importantly, chronic stress is known to induce neural remodeling, including dendritic atropy and changes in synaptic morphology, throughout the hippocampus (Sousa et al, 2000; Bessa et al, 2009; Stewart et al, 2005; Donohue et al, 2006; McEwen, 2012).

Substantial evidence highlights changes in the neural circuit E/I balance as an underlying mechanism for social dysfunction (Harrison and Weinberger, 2005; Südhof, 2008). Importantly, different lines of evidence indicate that chronic stress might lead to an increase in the E/I balance in the hippocampus. In particular, chronic stress was shown (i) to enhance glutamate transmission and synaptic excitation in different hippocampal subfields (Karst and Joëls, 2003; Joëls et al, 2004); (ii) to increase expression levels of the GLTs-1 and -1b (Reagan et al, 2004) and synaptobrevin/vesicle-associated membrane protein 2, a synaptic vesicle molecule involved in neurotransmitter—notably glutamate—release (Gao et al, 2006); and (iii) chronic stress was also shown to lead to a reduction in extracellular GABA levels (Grønli et al, 2007), as well as in the number of parvalbumin-immunoreactive GABA-ergic interneurons (Hu et al, 2010).
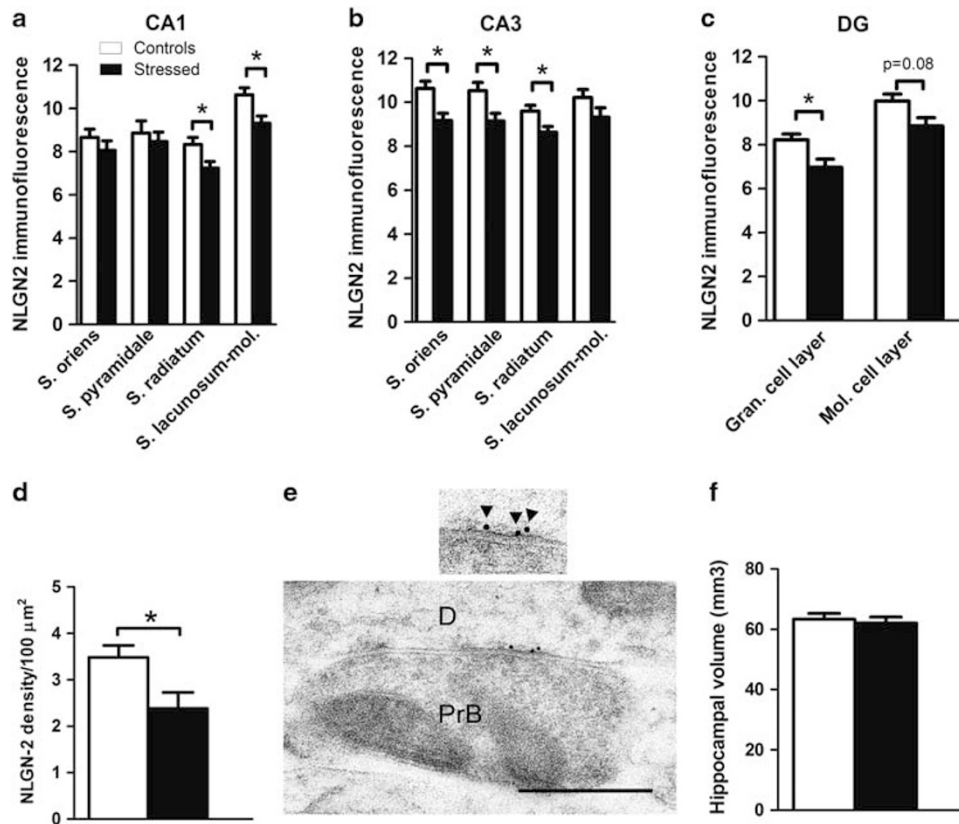
Figure 3 Effects of chronic restraint stress on hippocampal neuroligin-2 (NLGN-2) expression in the hippocampus as revealed by immunofluorescence and electron microscopy. Immunofluorescence staining for NLGN-2 was employed to investigate the effects of chronic stress in subregions of the hippocampal CA1 (a); CA3 (b); and dentate gyrus (*$p < 0.05$) (c). Hippocampal NLGN-2 density is measured by electron microscopy (*$p < 0.05$) (d). ImmunoGold labeling (as indicated by arrowheads, see insert) for NLGN-2 at an inhibitory synapse (D), dendrite, PrB, presynaptic bouton, and the scale bar measures 0.5 μm) (e). Total hippocampal volume in controls and stressed animals (f) ($n = 4$/group). Error bars represent SEM.

Altogether, these observations provide a picture of changes in the glutamatergic and GABA-ergic systems induced by chronic (restraint, in most cases) stress that, along with the electrophysiological evidence indicated above, strongly indicate a shift in the balance of the hippocampal E/I ratio toward increased excitation. Our findings showing a decreased expression of NLGN-2 throughout the hippocampus are congruent with an increased E/I ratio in stressed animals. However, given the numerous examples of stress-induced glutamatergic changes (see above), the fact that no changes were observed in the expression levels of NLGN-1 raises several questions. One possibility is that the role of this isoform in the reorganization of the glutamatergic changes induced by chronic stress occurs at earlier time points, which are not captured by this study. Alternatively, NLGN-1 might not be involved with, instead, other mechanisms being responsible. One interesting candidate is NLGN-3, as it has been implicated in both glutamatergic and GABA-ergic synapses (Budreck and Scheiffele, 2007). Non-synapse-specific mechanisms, such as the described stress-induced changes in glial glutamate transporters (Reagan et al, 2004), could also be involved. Nevertheless, the observed reduction in NLGN-2 expression might be sufficient to account for the described changes in the E/I balance. In support of this view is the fact that whereas the overexpression of NLGN-2 was found to

increase synaptic contacts and shifted the E/I balance downwards, overexpression of NLGN-1 did not affect these parameters (Hines et al, 2008).

The reduction that we find in NLGN-2 following chronic stress was detected both in synaptoneurosomes, in electron microscope images, and also in immunohistochemical preparations that revealed the widespread reduction throughout the different hippocampal subfields and layers (except for stratum oriens and stratum pyramidale in CA1 and stratum lacunosum-moleculare in CA3). Several studies have reported structural (Donohue et al, 2006) and molecular (Reagan et al, 2004; Gao et al, 2006) changes coinciding with the same subregions where we detect differences in NLGN-2 expression. However, it is notable that NLGN-2 was unaltered in the stratum lacunosum-moleculare of the CA3, one of the layers to which the distal apical dendritic trees of the pyramidal neurons project and where stress-induced atrophy is detected (Magariños and McEwen, 1995).

Therefore, our results illustrate that a life experience, such as chronic stress, known to elevate the hippocampal E/I ratio, leads simultaneously to a reduction in NLGN-2 expression throughout the hippocampus and to alterations in social behaviors. Yizhar et al, (2011) showed that elevating this ratio in the prefrontal cortex can induce immediate deficits in sociability. Importantly, our
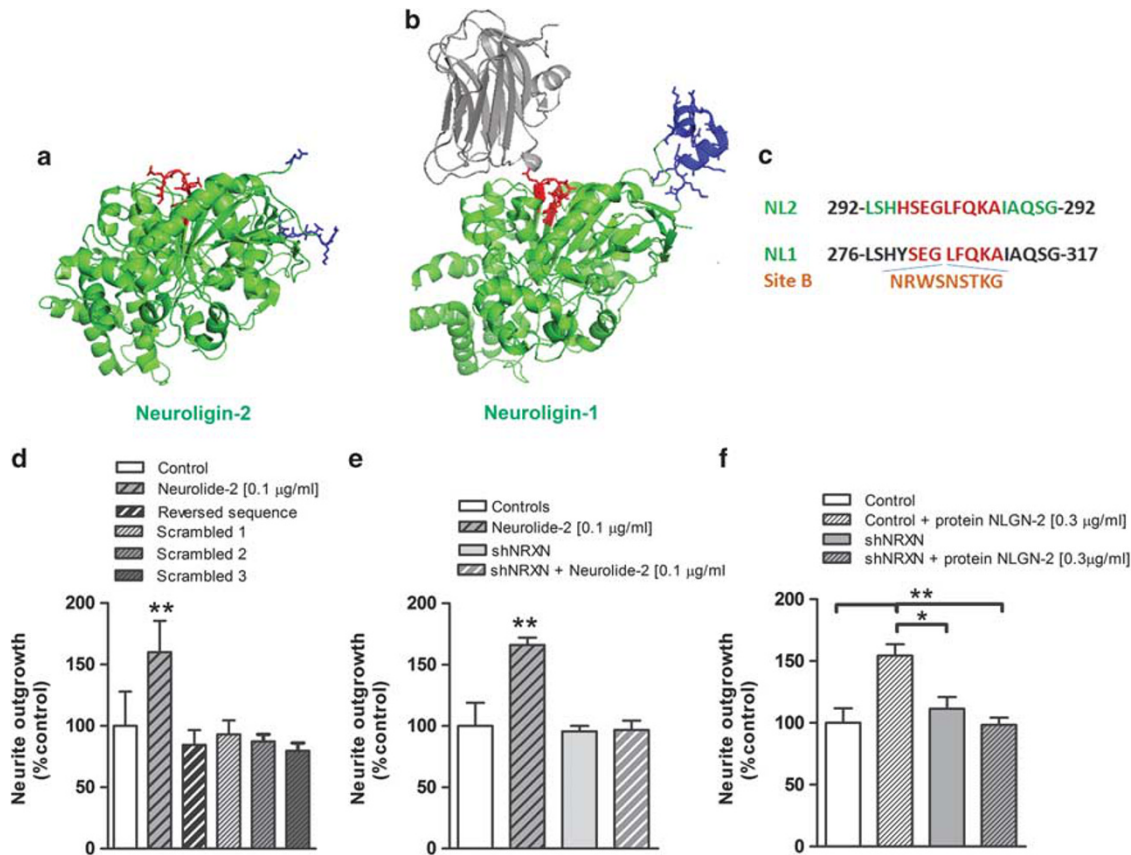
Figure 4 Design of Neurolide-2, a neuroligin-2 (NLGN-2)-derived peptide. Model of NLGN-2 (PDB identification 3BL8) (a). Localization of the neurolide-2 sequence motif is shown in red. Localization of splice site A is shown in blue. (b) Model of the NLGN-1 (green)/neurexin (NRXN)-1β (gray) complex (PDB identification 3VKF). Localization of part of the neurolide-1 sequence motif (without splice site B) is shown in red. Localization of splice site A is shown in blue. (c) Sequence motif of neurolide-2 is shown in red (top, UniProtKB/Swiss-Prot identification Q69ZK9). Sequence motif of neurolide-1 (bottom, UniProtKB/Swiss-Prot identification Q62765 is shown in red. It includes splice site B (brown). The *in vitro* effects of neurolide-2 on neurite outgrowth as compared with the reversed, or a scrambled sequence (d). Shown is the effect of neurexin (NRXN)-knockdown on neurolide-2- and NLGN-2 protein-induced neurite outgrowth (*$p < 0.05$, **$p < 0.01$) (e and f). Error bars represent SEM.

pharmacological experiments showing that intra-hippocampal infusions of the synthetic peptide neurolide-2, but not neurolide-1, lead to similar social alterations as found following chronic restrain stress. This further supports a role for hippocampal NLGN-2 in the regulation of social behaviors (Kohl *et al*, 2013). These peptides are biologically active, as shown by their ability to stimulate neurite outgrowth in dissociated neuronal cultures, when NLGNs are not involved in trans-synaptic binding with NRXN. Our findings showing increased aggression following stress when reduced NLGN-2 expression is observed as well as following the infusion of neurolide-2, which is in fact a synthetic fragment of the NLGN-2 molecule, might appear counterintuitive. However, it is important to emphasize that the neurolide-2 peptide was designed to coincide with the exact sequence motifs in NLGN-2 that are involved in the interaction with NRXN-1β (see Figure 4), thus not being a full mimetic of the entire NLGN-2 protein. For example, neurolide-2 lacks critical sequences in the NLGN-2 part in interaction with the plasma membrane and intracellular compartment. As proposed for other synthetic peptides targeting specific cell adhesion molecules (Foley *et al*, 2000; Cambon *et al*, 2003; Kraev *et al*, 2011), when given *in vivo*, these peptides can disrupt

endogenous cell–cell molecular interactions and eventually display antagonistic effects. In this case, NRXN-NLGN complexes for the specific NLGNs were targeted. Therefore, our data on NLGN-2 reduction following chronic stress and neurolide-2 infusions seems to be congruent in suggesting that impaired NLGN-2 function in the hippocampus is associated with increased aggression. Importantly, we also showed here that the NLGN-derived peptides neither altered locomotor activity, anxiety-like behaviors, or spatial learning nor affected basal or activated plasma corticosterone responses, thereby excluding non-specific stress effects in the observed results on social behaviors obtained with neurolide-2.

Our study highlights for the first time NLGN-2 as a molecular target broadly affected by chronic stress exposure in the hippocampus, and suggests that impaired hippocampal NLGN-2 function might be instrumental in the development of alterations in sociability and aggression. In addition to emphasizing the role of NLGN-2 in the hippocampus (Kohl *et al*, 2013), these findings add to a growing body of data highlighting NLGN-2 in the regulation of social behaviors (Hines *et al*, 2008; Wöhr *et al*, 2012). The effectiveness of neurolide-2 to affect social behaviors underscores NLGNs as promising targets for the
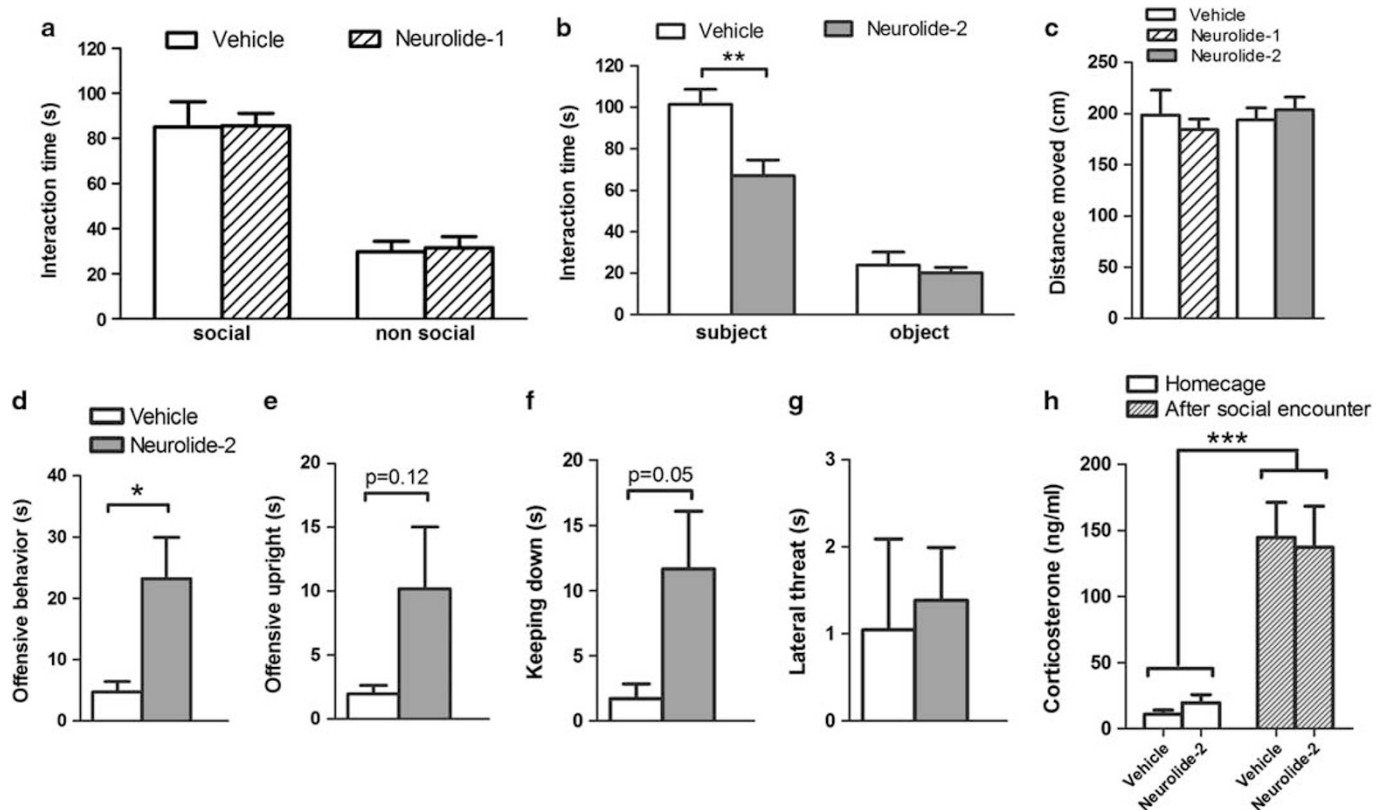
Figure 5 Effects of Neurolide-1 or Neurolide-2 infused in the dorsal hippocampus on social behavior, locomotion, and peripheral corticosterone. Investigation of a juvenile conspecific or inanimate object after treatment with neurolide-1 (a) or neurolide-2 in the dorsal hippocampus (**$p < 0.01$; $n = 6–7$/group) (b). Locomotor activity during the social approach task ($n = 6–7$/group) (c). In a subsequent social encounter, we measured total offensive behavior (*$p < 0.05$; $n = 7$/group) (d), offensive upright (e), keeping down (f), and lateral threat (g). Plasma corticosterone levels were taken when the animals were in their homecage and immediately after social interaction ($n = 13$ for controls under homecage conditions and $n = 10$ for the other groups, ***$p < 0.001$) (h). Error bars represent SEM.

development of novel drugs ideally capable of improving social behaviors.

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