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ARTICLE

Hippocampal mossy cell involvement in behavioral and neurogenic responses to chronic antidepressant treatment

Seo-Jin Oh[1] · Jia Cheng[2] · Jin-Hyeok Jang[1] · Jeffrey Arace[2] · Minseok Jeong[1] · Chang-Hoon Shin[1] · Jeongrak Park[1] · Junghee Jin[2] · Paul Greengard[2] · Yong-Seok Oh[1]

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Abstract

Most antidepressants, including selective serotonin reuptake inhibitors (SSRIs), initiate their drug actions by rapid elevation of serotonin, but they take several weeks to achieve therapeutic onset. This therapeutic delay suggests slow adaptive changes in multiple neuronal subtypes and their neural circuits over prolonged periods of drug treatment. Mossy cells are excitatory neurons in the dentate hilus that regulate dentate gyrus activity and function. Here we show that neuronal activity of hippocampal mossy cells is enhanced by chronic, but not acute, SSRI administration. Behavioral and neurogenic effects of chronic treatment with the SSRI, fluoxetine, are abolished by mossy cell-specific knockout of *p11* or *Smarca3* or by an inhibition of the p11/AnxA2/SMARCA3 heterohexamer, an SSRI-inducible protein complex. Furthermore, simple chemogenetic activation of mossy cells using Gq-DREADD is sufficient to elevate the proliferation and survival of the neural stem cells. Conversely, acute chemogenetic inhibition of mossy cells using Gi-DREADD impairs behavioral and neurogenic responses to chronic administration of SSRI. The present data establish that mossy cells play a crucial role in mediating the effects of chronic antidepressant medication. Our results indicate that compounds that target mossy cell activity would be attractive candidates for the development of new antidepressant medications.

Introduction

Major depressive disorder (MDD) is the most prevalent mental illness, of which lifetime prevalence is estimated to be as high as 16.2% in the United States. This disease encompasses various symptoms, including anhedonia, depressed mood, increased stress sensitivity, helplessness, apathy, shift towards negative emotions (sadness, emotional numbness, irritability, and anxiety), and cognitive deficits [1, 2]. Selective serotonin reuptake inhibitors (SSRIs) are the most widely used class of antidepressants [3], but SSRIs generally take several weeks to show therapeutic effects, despite their immediate effect on serotonin neurotransmission [3, 4]. This therapeutic delay suggests the existence of slow adaptive changes in neural circuits over a long-lasting period of drug treatment, possibly involving changes in gene expression and protein translation [5–7]. In addition, although significant progress has been made in treating depression by SSRIs, only a little more than half of severely depressed patients has been found to respond to this class of drugs [8]. Our knowledge of the molecular mechanisms underlying therapeutic responses to long-term treatment with SSRIs, as well as the side effects of the drugs, have yet to be established at the level of neuronal cell types.

Mossy cells reside in the hilus of the dentate gyrus and make synaptic connections with various types of cells, such as granule cells and basket cells [9–13]. Moreover, they are positioned to integrate numerous inputs mainly from multiple granule cells, CA3c pyramidal cells, as well as from the local GABAergic interneurons including basket cells and hilar perforant path-associated (HIPP) cells and then to propagate a signal over the longitudinal axis of the hippocampus [13–15].

These authors contributed equally: Seo-Jin Oh, Jia Cheng

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✉ Yong-Seok Oh
ysoh2040@dgist.ac.kr

[1] Department of Brain-Cognitive Science, Daegu-Gyeongbuk Institute of Science and Technology (DGIST), Hyenpung-myeon, Dalseong-gun, Daegu, Republic of Korea

[2] Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10065, USA

Of particular interest, recent studies have shown that mossy cells are highly enriched in selective subsets of monoamine receptors, proving mossy cells to be immediate regulatory targets of monoaminergic inputs to the hippocampus [12, 16, 17]. They also highly express type II glucocorticoid receptors, which sense the stress hormone, cortisol [18]. Mossy cells fire frequently in multiple place fields in various environments, in contrast to granule cells which fire sparsely [19–21]. The neuronal activity of mossy cells within the dentate gyrus circuitry is crucial for the proper function of the hippocampus [22]. Indeed, degeneration of mossy cells results in the dysregulation of granule cell excitability, which leads to abnormal behaviors such as elevated anxiety, and impaired pattern separation [23]. Despite the potential implication of the mossy cells in the affective disorders, as well as in the actions of monoaminergic drugs, a detailed analysis of the role of mossy cells in antidepressant responses has not yet been carried out.

p11 (S100A10) is a key factor involved in regulating affective state and in determining responses to antidepressants [24, 25]. It is downregulated in depressed humans and rodents and is induced by chronic but not by acute administration of antidepressants in rodents [24, 26, 27]. Global abolition of p11 in mice produces a depressive state [24]. Removal of p11 from specific classes of p11-enriched neurons produces a variety of depressive effects, depending on the subtype of p11-containing neurons that is targeted [28–37].

p11 was initially identified within a heterotetrameric complex where it forms with Annexin A2 (AnxA2) [38], and this complex form of p11 and AnxA2 can be induced in the hippocampal dentate gyrus by chronic treatment with an SSRI. Previous studies identified SMARCA3 as a binding partner of the p11/AnxA2 complex and both p11 and SMARCA3 are crucial for behavioral and neurogenic responses to chronic antidepressant administration [34]. Chronic SSRI administration increased the level of the heterohexameric complex of p11/AnxA2/SMARCA3 in the hippocampus [34]. In addition, p11 and SMARCA3 are highly enriched in hilar mossy cells and basket cells in the hippocampal dentate gyrus [34]. However, a cell type-specific role of the p11/AnxA2/SMARCA3 heterohexameric complex in antidepressant action has not yet been explored. Here we examine the role of mossy cells in mediating the actions of the antidepressant, fluoxetine. Results from this study have suggested that the heterohexameric complex of p11/AnxA2/SMARCA3 regulates the activity of mossy cells, which is required for behavioral and neurogenic responses to chronic antidepressant medication.

Materials and methods

Animal breeding

All procedures involving animals were approved by the Animal Care and Use Committee of the Daegu Gyeongbuk Institute of Science & Technology (DGIST, Korea) and by the Rockefeller University (USA) Institutional Animal Care and Use Committee. Transgenic mouse lines were used in this study: MC-Cre ([*Calcrl*]-Cre, JAX stock #023014), D2-Cre ([*Drd2*]-Cre, GENSAT, Clone #ER44), tdTomato Reporter (JAX stock #007908), p11 cKO ($p11^{(f/f);D2\text{-}Cre}$), Smarca3 cKO($Smarca3^{(f/f);D2\text{-}Cre}$, $Smarca3^{(f/f);MC\text{-}Cre}$). All the animal works were done at the laboratory animal resource center (LARC) of DGIST or the comparative bioscience center (CBC) of the Rockefeller University. The mouse breeding methods and housing conditions are described in Supplementary Materials and Methods.

Drug treatments

Details of drug treatments were included in Supplementary Materials and Methods.

Plasmid constructions and immunoprecipitation

All plasmid constructions and immunoprecipitation were performed as previously described [34]. See detailed methods in Supplementary Materials and Methods.

Stereotaxic surgery

Mice were anesthetized by intraperitoneal injection of avertin (250 mg/kg), and stereotaxic injection of AAV constructs was performed into the hippocampal hilus regions. (coordinates for dorsal: AP −2.1 mm, ML ±1.4 mm, DV −1.95 mm, ventral: AP −3.3 mm, ML ±2.7 mm, DV −3.6 mm). See detailed methods in Supplementary Materials and Methods.

Behavior assessments

Mood and anxiety-related behaviors (novelty suppressed feeding [NSF] test, tail suspension test [TST], elevated plus maze [EPM], and light/dark box [LDB] test) and locomotor activity (open field [OF] test) were tested as described in Supplementary Materials and Methods.

Chronic unpredictable mild stress paradigm

This paradigm was described in detail in Supplementary Materials and Methods.

Immunohistochemistry

All mice were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) in PBS and post fixed in the same solution overnight at 4 °C. The brains were coronally cut into 40-μm-thick sections with a Cryostat (CM3050S, Leica). Sections were processed using a free-floating procedure. Detailed description of antibody preparation, antigen retrieval, image acquisition, and quantification is provided in Supplementary Materials and Methods.

BrdU labeling and neurogenesis assay

For the neurogenesis assay, the mice were labeled with BrdU solution (200 mg/kg) for 3 h prior to sacrifice by perfusion. See detailed methods in Supplementary Materials and Methods.

Electrophysiological recordings of mossy cells

Slice preparation and electrophysiology were performed as described previously [39]. Experimental details are included in Supplementary Materials and Methods.

Data analysis and statistics

All data were presented as means ± SEM. Statistical analysis was conducted using GraphPad Prism Version 7.0a. Two group comparisons were done by two-tailed, unpaired or paired Student's *t*-test. Multiple group comparisons were assessed using a one-way or two-way ANOVA, followed by the appropriate post hoc test. Significance thresholds were as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

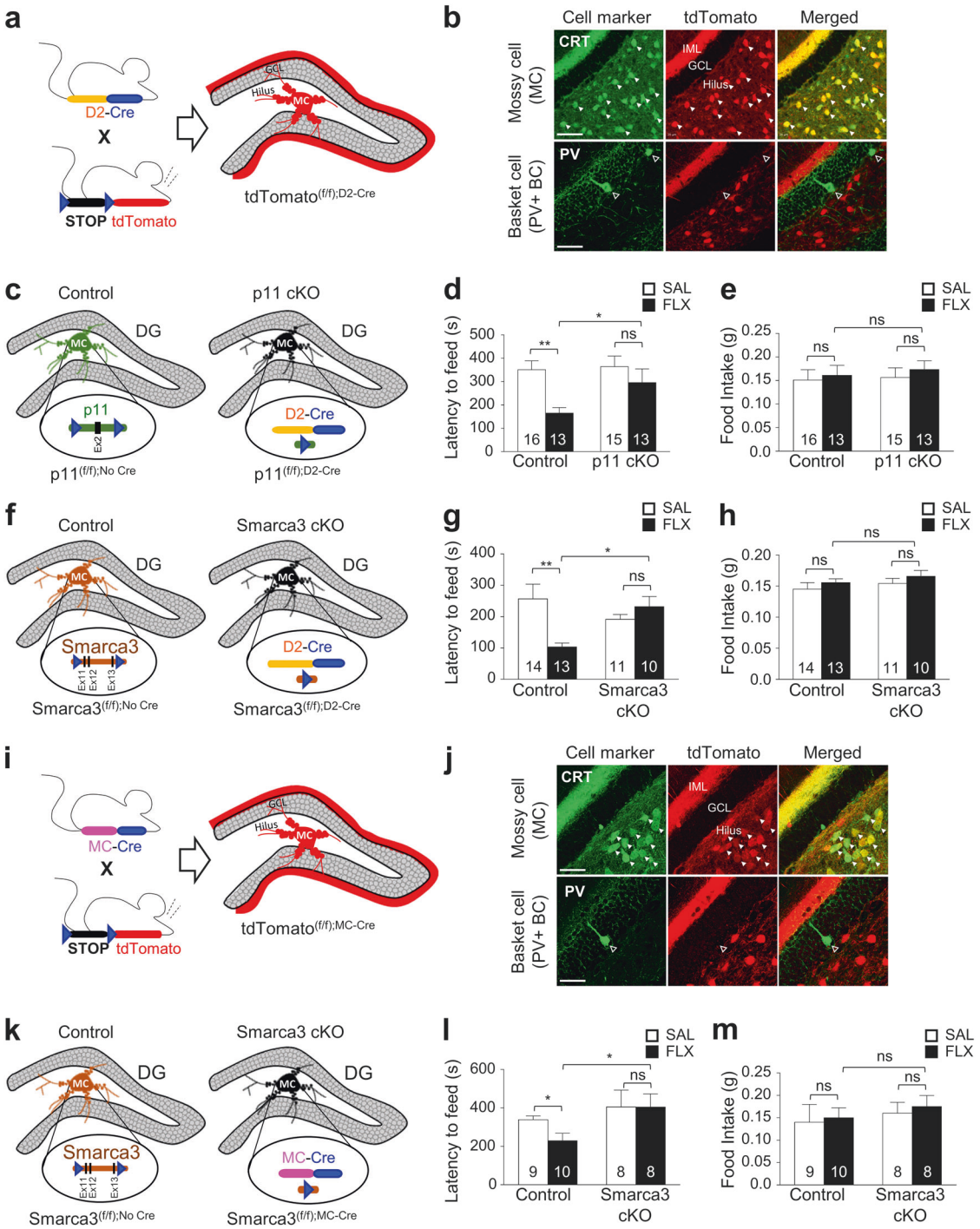
Effects of genetic deletion of *p11* or *Smarca3* in hippocampal mossy cells on behavioral responses to chronic SSRI administration

Previous studies identified SMARCA3 as a binding partner of the p11/AnxA2 complex and, using constitutive *Smarca3* KO mice, demonstrated its crucial role for neurogenic and behavioral responses to chronic antidepressant administration. Those studies also showed that p11 and SMARCA3 were highly enriched in hippocampal mossy cells [34]. However, a possible mossy cell-specific involvement of the p11/AnxA2/SMARCA3 complex in antidepressant actions was not studied. Therefore, we generated mossy cell-specific deletion of *p11* or *Smarca3* conditional KO mice to identify the cell type-specific role of the ternary complex. To investigate the possibility of a mossy cell-specific role of *p11* and/or *Smarca3* in the actions of antidepressants, we used a transgenic Cre driver line to target hippocampal mossy cells. According to previous reports, dopamine D2 receptor gene expression is limited to mossy cells in the dentate gyrus [40, 41]. We validated dopamine D2 receptor promoter driven Cre recombinase expression in a mossy cell-specific manner in the dentate gyrus by crossing a D2-Cre ([*Drd2*]-Cre) driver mouse line with a tdTomato reporter line (Fig. 1a, b). Then, we crossed them with a *p11* or *Smarca3* floxed conditional line to achieve specific deletion of *p11* or *Smarca3* in mossy cells (Fig. 1c, f, Figures S1a and S1b). After chronic administration of SSRI to a *p11* or *Smarca3* conditional KO (cKO) line, we carried out the novelty suppressed feeding test to investigate depression-like states. We found that the latency to approach food pellet was reduced in the control mice ($p11^{(f/f)}$ and $Smarca3^{(f/f)}$), but not in *p11* cKO ($p11^{(f/f);D2\text{-}Cre}$) or *Smarca3* cKO ($Smarca3^{(f/f);D2\text{-}Cre}$) mice (Fig. 1d, g), but that their home cage feeding level was unaffected (Fig. 1e, h). We further validated the effect of genetic deletion of *Smarca3* in the mossy cells using another type of mossy cells-specific Cre mouse line, MC-Cre (*[Calcrl]*-Cre) (Fig. 1i, j). This MC-Cre line was very specific in the hippocampus, but exhibited wide activity in both hilar mossy cells of dentate gyrus and some pyramidal cells of the CA3 region [23]. We deleted the *Smarca3* gene by crossing MC-Cre mice with *Smarca3* floxed conditional mice ($Smarca3^{(f/f)}$) (Fig. 1k) and conducted the novelty suppressed feeding test (NSF) and the tail-suspension test (TST) after chronic administration of SSRI. We found that the latency to feed in the NSF and the immobility in the TST were reduced in the control mice by fluoxetine treatment ($Smarca3^{(f/f)}$), but not in *Smarca3* cKO ($Smarca3^{(f/f);MC\text{-}Cre}$) (Fig. 1l, m, Figure S2a), without any significant change in locomotor activity (Figure S2b).

All of these data indicate that selective deletion of *p11* or *Smarca3* in dentate mossy cells affects the antidepressant responses. These results suggest that *p11* and *Smarca3* in mossy cells are involved in mediating the behavioral responses to chronic antidepressant treatment.

Effect of mossy cell-specific inhibition of the p11/AnxA2/SMARCA3 complex on neurogenic and behavioral responses to chronic antidepressant treatment

Although we evaluated the effects of genetic deletion of *p11* or *Smarca3* gene in mossy cells on antidepressant responses using cell type-specific KO mice, these transgenic approaches could not rule out the possibility that these behavioral changes are due to developmental defects or off-target deletion of *p11* or *Smarca3* gene in those cKO mice. Thus,



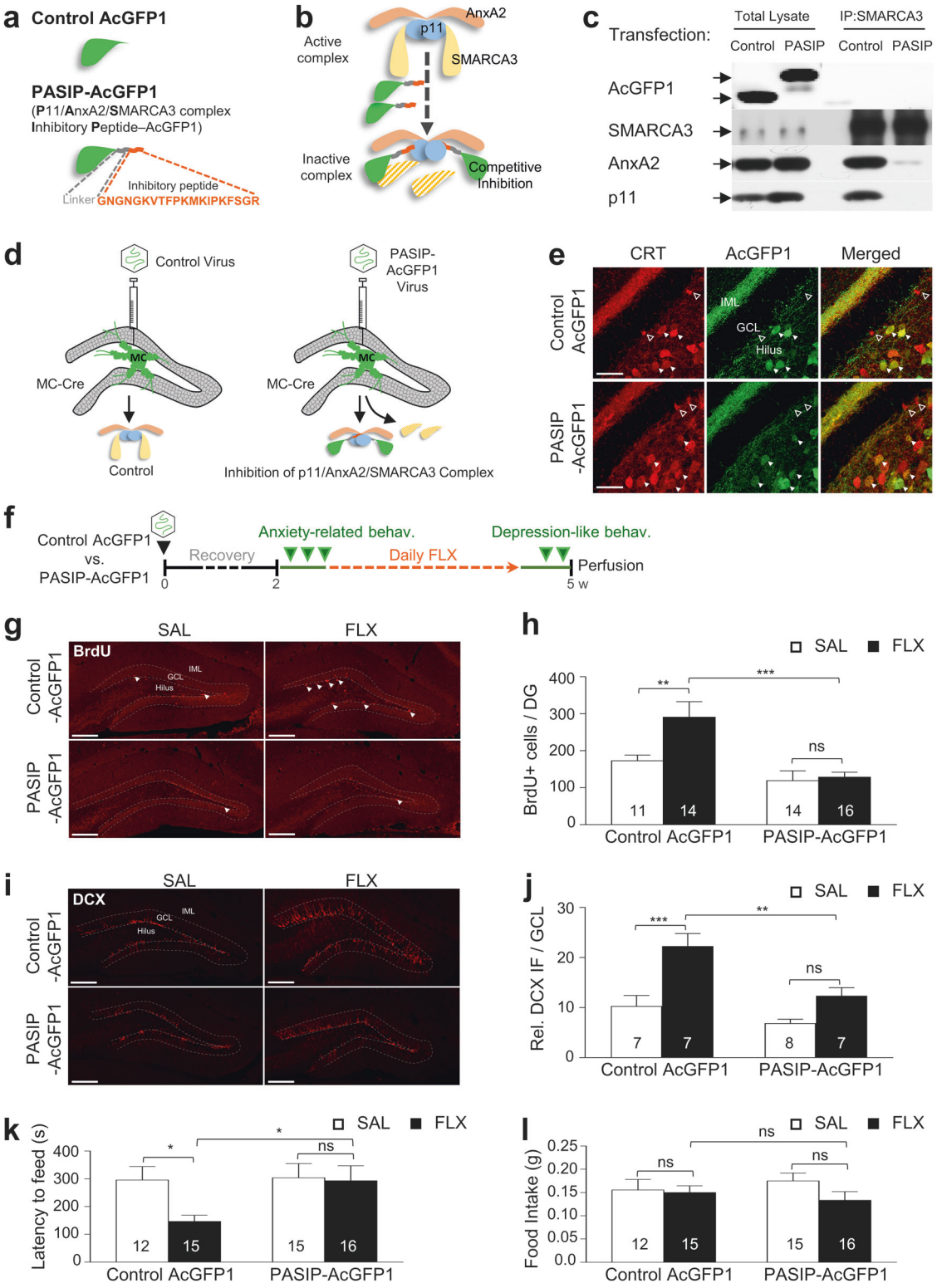
we further examined the neurogenic and behavioral effects of mossy cell-specific inhibition of the p11/AnxA2/SMARCA3 complex in adult mice. To this end, we developed recombinant constructs to inhibit the assembly of the p11/AnxA2/SMARCA3 complex. Our previous study showed that the p11/AnxA2 heterotetramer formed a stable heterohexameric complex with either SMARCA3 or AHNAK1, using very similar recognition principles. The short peptide derived from the binding region of SMARCA3 or AHNAK1 makes a stable complex with the p11/AnxA2 heterotetramer by occupying the hydrophobic binding pocket created on the surface of the P11/AnxA2 heterotetrameric complex [34]. Based on information about the co-crystalization structure of the ternary complex, we designed a recombinant inhibitor of the p11/AnxA2/SMARCA3 complex (P11/AnxA2/SMARCA3 complex inhibitory peptide-AcGFP1 fusion construct (PASIP-AcGFP1)) along with an inert control (control AcGFP1)

◀ **Fig. 1** Mossy cell-specific knockout of *p11* or *Smarca3* gene abolishes behavioral responses to chronic SSRI treatment. **a** Schematic design to confirm mossy cell-specific Cre recombination in the hippocampus of D2-Cre transgenic mice. **b** Co-localization of Cre-dependent reporter (tdTomato, red) with CRT, a mossy cell marker (green, filled arrowheads), but not with PV, a PV+ basket cell marker (green, open arrowheads). Scale bar, 25 µm. **c** Schematic illustration of genetic deletion of *p11* gene in mossy cells using a D2-Cre line. **d** Novelty suppressed feeding (NSF) test. Bar graphs showing latency to food pellet. Control and *p11* cKO group have genotype of $p11^{(f/f)}$ without Cre, and $p11^{(f/f)}$ with D2-Cre, respectively. Two-way ANOVA, [genotype × drug interaction $F(1,54) = 5.031$; $p = 0.0290$, genotype factor $F(1,54) = 7.435$; $p = 0.0086$, drug factor $F(1,54) = 6.728$; $p = 0.0122$], followed by the Turkey's post hoc test. **e** Assessment of the hunger level in control and *p11* cKO group using home cage feeding test right after NSF test of the same set of animals. **f** Schematic illustration of genetic deletion of *Smarca3* gene in mossy cells using a D2-Cre line. **g** NSF tests. Control and *Smarca3* cKO group have genotype of $Smarca3^{(f/f)}$ without Cre, and $Smarca3^{(f/f)}$ with D2-Cre, respectively. Two-way ANOVA, [genotype × drug interaction $F(1,44) = 9.111$; $p = 0.0042$, genotype factor $F(1,44) = 0.9715$; $p = 0.3297$, drug factor $F(1,44) = 3.094$; $p = 0.0855$], followed by the Turkey's post hoc test. **h** Assessment of the hunger level in control and *Smarca3* cKO group. **i** Schematic illustration to confirm mossy cell-specific Cre recombination in the hippocampus of MC-Cre transgenic mice. **j** Co-localization of Cre-dependent reporter (tdTomato, red) with CRT (green, filled arrowheads), but not with PV(green, open arrowheads). Scale bar, 25 µm. **k** Schematic illustration of genetic deletion of *Smarca3* gene in mossy cells using MC-Cre line. **l** The NSF test. Control and *Smarca3* cKO group have genotype of $Smarca3^{(f/f)}$ without Cre, and $Smarca3^{(f/f)}$ with MC-Cre, respectively. Two-way ANOVA, [genotype x drug interaction $F(1,31) = 4.334$; $p = 0.0489$, genotype factor $F(1,31) = 4.404$; $p = 0.00441$, drug factor $F(1,31) = 2.77$; $p = 0.3238$], followed by the Turkey's post hoc test. **m** The hunger level was assessed by home cage feeding test with the same set of animals. Data are represented as means ± SEM. Pair-wise comparison by post hoc test; $*p < 0.5$, $**p < 0.01$, $***p < 0.001$, ns, non-significant. GCL, granule cell layer; IML, inner molecular layer, CRT, calretinin; PV, parvalbumin; DG, dentate gyrus; MC, mossy cells; SAL, saline; FLX, fluoxetine. See also Figure S1, and S2

(Fig. 2a). We assumed that this recombinant inhibitor protein competes with endogenous SMARCA3 on the binding pocket and thus blocks the functional assembly of the active ternary complex, the p11/AnxA2/SMARCA3 heterohexamer (Fig. 2b). By conducting an in vitro co-immunoprecipitation assay, we confirmed that the recombinant PASIP-AcGFP1 construct was able to successfully block the assembly of the P11/AnxA2/SMARCA3 complex (Fig. 2c). We further generated Cre-dependent AAVs to deliver those PASIP-AcGFP1 constructs into the hippocampal mossy cells. Cre-dependent AAVs were injected locally into hilus regions of MC-Cre transgenic mice (Fig. 2d, Figures S3a and S3b). Because MC-Cre mice were known to display Cre-recombination in both dentate mossy cells and a minor pyramidal subpopulation in the CA3c region [23], we delivered recombinant AAV solution in the middle of the hilus region to avoid the unnecessary diffusion of viral particles. Our histological analysis confirmed that AcGFP1 signal was found exclusively in the hilus and the inner molecular layer (IML), where somas and axonal fibers of mossy cells localize, respectively, but not in the CA3 region where pyramidal cells localize (Figure S3c), ensuring the hippocampal subregion specificity. In addition, we confirmed that AcGFP1-fusion proteins (control AcGFP1, PASIP-AcGFP1) were specifically expressed in mossy cells, but not in other GABAergic interneurons, including PV+ basket cells and NPY + HIPP cells (Fig. 2e, Figure S3d), ensuring the cell type specificity in the dentate gyrus.

After preventing formation of the p11/AnxA2/SMARCA3 complex with mossy cell-specific expression of the PASIP-AcGFP1 construct, we examined the neurogenic and behavioral outcome of chronic administration of SSRI (Fig. 2f). It is well established that adult neurogenesis in the dentate gyrus is induced in response to chronic antidepressant treatment [42–44]. In control and PASIP-AcGFP1 mice, we assessed chronic fluoxetine-induced proliferation activity of neural progenitors. A significant increase in BrdU+ proliferating cells was observed in the subgranular zone (SGZ) of control AcGFP1 mice after chronic fluoxetine treatment, which was abolished in the SGZ of PASIP-AcGFP1 mice ($p = 0.73$) (Fig. 2g, h, Figures S4a and S4b). In addition, we analyzed the expression level of doublecortin (DCX), which represent a snapshot of newborn postmitotic cells undergoing neuronal differentiation and maturation [45]. Chronic fluoxetine administration increased the DCX immunofluorescence signal in the SGZ of control AcGFP1 mice, but the effect of fluoxetine was attenuated in the SGZ of PASIP-AcGFP1 mice (Fig. 2i, j, Figures S4c and S4d). We found that the inhibitory effect of PASIP-AcGFP1 expression on fluoxetine-induced neurogenic activities was observed consistently along the rostrocaudal axis of the dentate gyrus (Figures S4b and S4d).

We next investigated the possible functional significance of the p11/AnxA2/SMARCA3 complex in mossy cells for basal locomotor activity, anxiety-related behaviors and SSRI-induced behavioral changes. Control AcGFP1 and PASIP-AcGFP1 mice did not display a difference in basal locomotor activity when monitored using the OF test (Figure S5a). In addition, inhibition of the p11/AnxA2/SMARCA3 complex failed to show a consistent effect on multiple anxiety-related behaviors. Control AcGFP1 and PASIP-AcGFP1 mice showed a difference in the light/dark box [LDB] test ($p = 0.0064$) (Figure S5c), but not in the other two anxiety-related behaviors (thigmotaxis test ($p = 0.74$), Figure S5b; the elevated plus maze test [EPM] ($p = 0.587$), Figure S5d). In addition, they did not display any baseline difference in depression-like behavioral tests (tail suspension test [TST], Figure S5e; novelty-suppressed feeding [NSF] test, Fig. 2k). However, the latency to feed was decreased after chronic fluoxetine administration in control AcGFP1 mice, but not in PASIP-AcGFP1 mice



(Fig. 2k). Neither fluoxetine treatment nor inhibition of the p11/AnxA2/SMARCA3 complex caused any significant effect on home cage feeding (Fig. 2l), suggesting that this behavioral change was unlikely due to different hunger levels between comparison groups. The antidepressant-induced behavioral change observed in the NSF test was also observed in the TST (Figure S5e). Taken together, these data indicate that the p11/AnxA2/SMARCA3 complex in mossy cells may play a crucial role in behavioral changes after chronic treatment with SSRIs.

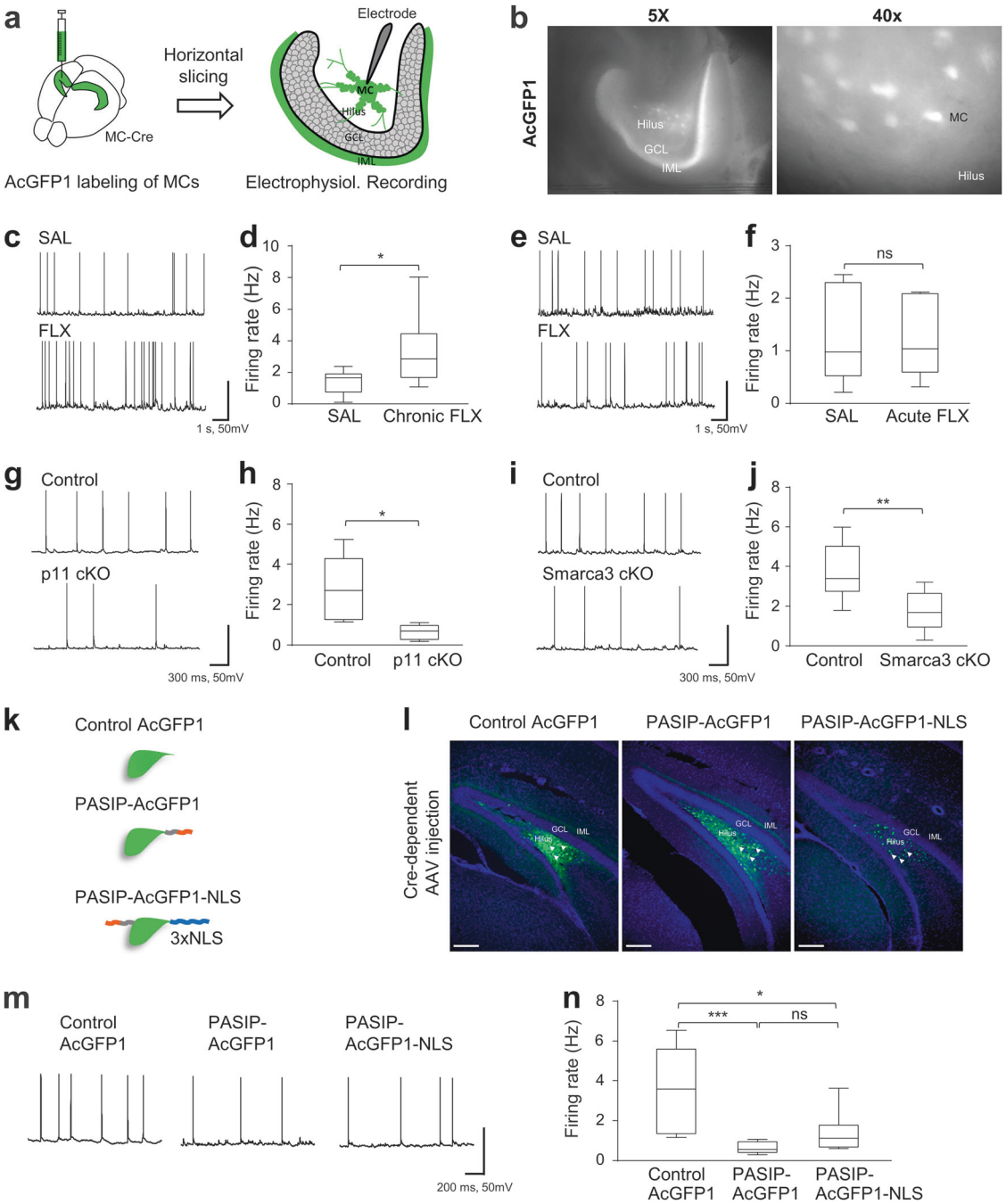
◀ **Fig. 2** Mossy cell-specific disruption of the p11/AnxA2/SMARCA3 complex silences neurogenic and behavioral responses to chronic SSRI treatment. **a** Molecular design for the p11/AnxA2/SMARCA3 complex-Inhibitory peptide (PASIP)-AcGFP1 fusion protein (PASIP-AcGFP1). **b** Schematic illustration of how the recombinant inhibitor (PASIP-AcGFP1) blocks the functional assembly of the p11/AnxA2/SMARCA3 heterohexameric complex. Upon dissociation from the complex, SMARCA3 becomes inactive. **c** In vitro pull-down assay demonstrates that PASIP-AcGFP1 disrupts the p11/AnxA2/SMARCA3 complex. SMARCA3 was immunoprecipitated from HEK 293 cells that were transfected with either control AcGFP1 or PASIP-AcGFP1 construct. The immune complexes were subjected to immunoblot analysis using AcGFP1-, SMARCA3-, AnxA2- and p11 antibodies. **d** Recombinant AAVs, expressing either control AcGFP1 or the PASIP-AcGFP1, were stereotaxically injected into the dentate gyrus of MC-Cre transgenic mice. **e** Representative immunolabeling images to show mossy cell-specific expression of control AcGFP1 or PASIP-AcGFP1 construct. AcGFP1+ cells (green) were colocalized with most CRT+ cells in the hilus (red) as indicated (solid arrowheads), but not with a few CRT+ immature neurons in the subgranular zone (open arrowheads) to show the cell-type specific gene delivery. Scale bar, 50 μm. **f** Experimental design. Control AcGFP1-injected or PASIP-AcGFP1-injected mice were administered saline (SAL) or fluoxetine (FLX) for 3 weeks. After behavioral testing, all the animals were labeled with BrdU for the last 3 h prior to perfusion. **g** Representative images with α-BrdU immunostaining results. BrdU+ cells in the subgranular zone (SGZ) are as indicated in each image (solid arrowheads). Scale bars, 100 μm. **h** Quantification of BrdU-positive cells in the SGZ. Two-way ANOVA, [AAV × drug interaction $F(1,51) = 4.129$; $p = 0.0498$, AAV factor $F(1,51) = 18.54$; $p < 0.0001$, drug factor $F(1,51) = 8.65$; $p = 0.0049$], followed by the Turkey's post hoc test. **i** Representative images of DCX-positive cells in control-AcGFP and PASIP-AcGFP1 mice. **j** Quantitation of DCX-positive cells in the SGZ and the GCL. Two-way ANOVA, [AAV × drug interaction $F(1,25) = 6.364$; $p = 0.0184$, AAV factor $F(1,25) = 18.54$; $p < 0.0001$, drug factor $F(1,25) = 8.65$; $p = 0.0049$], followed by the Turkey's post hoc test. **k** Novelty suppressed feeding test (NSF). Two-way ANOVA, [AAV × drug interaction $F(1,54) = 4.09$; $p = 0.0481$, AAV factor $F(1,54) = 4.084$; $p = 0.0327$, drug factor $F(1,54) = 4.584$; $p = 0.0368$], followed by the Turkey's post hoc test. **l** Home cage feeding levels. Data are represented as means ± SEM. Pair-wise comparison by post hoc test; $*p < 0.5$, $**p < 0.01$, $***p < 0.001$, ns, nonsignificant. MC, mossy cells; CRT, calretinin; GCL, granule cell layer; IML, inner molecular layer; DCX, double-cortin; Rel. DCX IF, relative double-cortin immune-fluroscence. See also Figure S3, Figure S4, and Figure S5

Effects of cell type-specific inhibition of the p11/AnxA2/SMARCA3 complex on neuronal activity of mossy cells

We first examined the effects of chronic antidepressant administration on neuronal activity of mossy cells. Mossy cells are spontaneous firing neurons in the hilus, whose activity modulates neuronal circuitry and function of the dentate gyrus [46]. To identify mossy cells for electrophysiological recordings, we labeled mossy cells with a fluorescence protein, AcGFP1, by injecting Cre-dependent AcGFP1 AAVs into mossy cell-specific Cre (MC-Cre) mice (Fig. 3a). AcGFP1 was shown to be restrictively expressed in mossy cells in the dentate gyrus (Fig. 3b). Mossy cells were further confirmed by their morphology, tonic firing pattern and membrane capacity (50–100 pF). Electrophysiological recordings showed that chronic fluoxetine treatment significantly enhanced the spontaneous firing rate of mossy cells (Fig. 3c, d), but acute treatment of the drug had no effect on mossy cell activity (Fig. 3e, f).

Previous studies have shown that acute restraint stress decreases c-fos immunoreactivity in hilar mossy cells of the hippocampus [47]. We further investigated whether neuronal activity of mossy cells was altered by chronic stress exposure and the following antidepressant administration. After using the chronic unpredictable mild stress (CUMS) paradigm, which is a rodent model of chronic stress-induced depression [48] (Figure S6a), we counted the active mossy cells in the hilus by immunostaining c-Fos, a neural activation marker [47, 49]. c-Fos immunoreactivity in mossy cells was significantly reduced by chronic exposure to unpredictable stress (Figures S6b and S6c). In constrast, chronic fluoxetine administration to the stressed animals resulted in a remarkable increase in c-Fos immunoreactivity in mossy cells (Figures S6d, S6e and S6f), which was consistent with our electrophysiological recording shown above (Fig. 3c, d). These results revealed that the neuronal activity of mossy cells was suppressed by chronic stress, and the effect was reversible by chronic fluoxetine administration.

We next examined the regulation by p11 and SMARCA3 of mossy cell activity. We found that the spontaneous firing rate of mossy cells was greatly reduced in *p11* cKO mice (Fig. 3g, h) and *Smarca3* cKO mice (Fig. 3i, j) compared to control mice. In addition, we investigated the effects of inhibition of the p11/AnxA2/SMARCA3 complex on neuronal activity of mossy cells. Previous studies have shown that the p11/AnxA2/SMARCA3 complex is induced in the mossy cells by chronic fluoxetine administration and is further targeted to the nuclear matrix to regulate gene transcription [50, 51]. Thus, we additionally generated a PASIP-AcGFP1-NLS construct that was specifically targeted to the nucleus of mossy cells due to triple nuclear localization signals (3× NLS) fused at the C-terminus (Fig. 3k). We generated a series of Cre-dependent viral vectors, including control AcGFP1, PASIP-AcGFP1, and PASIP-AcGFP1-NLS (Figure S7a). By co-transfection into cultured HEK 293 cells or stereoinjection into the dentate gyrus of mossy cell-specific Cre (MC-Cre) transgenic mice, we verified the nuclear-specific expression of the PASIP-AcGFP1-NLS construct (Figure S7b) and also the cell-type specific expression of the PASIP-AcGFP1 constructs in the dentate mossy cells (Fig. 3l). Of note, the firing rate of mossy cells was significantly decreased by PASIP-AcGFP1 or PASIP-AcGFP1-NLS expression compared to control AcGFP1 expression (Fig. 3m, n). We further observed that



c-Fos immunoreactivity was markedly reduced by PASIP-AcGFP1 expression in mossy cells, but not by control AcGFP1 expression (Figures S8a–S8c), verifying a significant role of the p11/AnxA2/SMARCA3 complex in the regulation of mossy cell excitability.

Collectively, these results show that the neural activity of mossy cells is decreased during chronic stress-induced depression and this decrease is reversed by chronic antidepressant administration, possibly through the p11/AnxA2/SMARCA3, an inducible protein complex.

Effects of selective stimulation of dentate mossy cells on adult neurogenesis in the hippocampus

Next we examined whether acute chemogenetic stimulation of mossy cells using the Gq-DREADD system was able to mimic the chronic effects of fluoxetine. To modulate the dentate mossy cells, we delivered viral vectors expressing control mCherry (Control) or hM3D-Gq-mCherry (Gq-DREADD), into hilus regions of MC-Cre transgenic mice (Fig. 4a, Figures S9a-S9c). By using immunostaining with c-Fos, we

◀ **Fig. 3** Neuronal activity of mossy cells is enhanced by chronic SSRI treatment via p11/AnxA2/SMARCA3 complex. **a** Schematic of AcGFP1 AAV injection into the hippocampus of MC-Cre mice. Ventral hippocampal slices were used for electrophysiological recordings. **b** Representative fluorescence images of AcGFP1-labeled mossy cells in a hippocampal section of the AAV-injected mice. Magnifications: 5× or 40× objective lens. **c**, **d** Representative traces (**c**) and Whisker box plot (**d**) showing the spontaneous firing rate of mossy cells by chronic FLX administration on 8-week-old mice (oral administration, 18 days, $n = 9$ cells/3 mice per group). Scale bar: 1 s, 50 mV. Two-tailed, unpaired *T*-test; $p = 0.044$. **e**, **f** Representative traces (**e**) and Whisker box plot (**f**) showing the spontaneous firing rate of mossy cells from 8-week-old mice injected with a single dose of saline or fluoxetine (10 mg/kg). $n = 9$ cells/3 mice per group. Scale bar: 1 s, 50 mV. Two-tailed, unpaired *T*-test; $p = 0.9401$. **g**, **h** Reduced neuronal activity of mossy cells in *p11* cKO mice. Mossy cells were labeled by injecting Cre-dependent AcGFP1 AAV into *p11* wild-type (+/+, MC-Cre)(Control) and *p11* conditional KO ($p11^{(f/f);MC\text{-}Cre}$) (*p11* cKO). Representative traces (**g**) and Whisker box plot (**h**) showing the spontaneous firing rate of mossy cells in the dentate gyrus of control and *p11* cKO mice at 16 weeks of age. $n = 10$ cells/3 mice for control; $n = 6$ cells/3 mice for *p11* cKO. Scale bar: 300 ms, 50 mV. Two-tailed, unpaired *T*-test; $p = 0.0255$. **I**, **j** Reduced electrophysiological activity of mossy cells in *Smarca3* cKO mice. Mossy cells were labeled by injecting the Cre-dependent AcGFP1 AAV into *Smarca3* wild-type (+/+, MC-Cre) (Control) and *Smarca3* conditional KO ($Smarca3^{(f/f);MC\text{-}Cre}$) (*Smarca3* cKO). Representative traces (**i**) and Whisker box plot (**j**) showing the spontaneous firing rate of mossy cells in the dentate gyrus of control and *Smarca3* cKO mice at 16 weeks of age. $n = 9$ cells/3 mice per group. Scale bar: 300 ms, 50 mV. Two-tailed, unpaired *T*-test; $p = 0.0073$. **k** Schematic design for a series of PASIP-fusion constructs, including control AcGFP1, whole cell inhibitor construct (PASIP-AcGFP1) and nucleus-targeted inhibitor construct (PASIP-AcGFP1-NLS). **l** Immunofluorescence images showing mossy cell-specific expression in the dentate gyrus of AAV-injected mice. AcGFP1-positive MC was indicated (filled arrowheads). Scale bar, 100 μm. **m**, **n** Disrupted p11/AnxA2/SMARCA3 complex reduces firing rate of mossy cells. Representative traces (**m**) and Whisker box plot (**n**) showing the spontaneous firing rate of mossy cells expressing each construct: control AcGFP1 ($n = 12$ cells/4 mice), PASIP-AcGFP1 ($n = 8$ cells/3 mice), PASIP-AcGFP1-NLS ($n = 8$ cells/3 mice) in 16-week-old mice. Scale bar: 200 ms, 50 mV. One-way ANOVA [$F(2,21) = 10.08$; $p = 0.0009$], followed by Bonferroni's multiple comparison test. Pair-wise comparison; $^{*}p < 0.01$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, non-significant; GCL, granule cell layer; IML, inner molecular layer; NLS, nuclear localization signal; MC, mossy cell; SAL, saline; FLX, fluoxetine. See also Figure S6, Figure S7, and Figure S8

verified that mossy cells were stimulated by clozapine-N-oxide (CNO) administration in Gq-DREADD mice, when compared to control mice (Fig. 4b, Figures S9d and S9e).

We assessed adult neurogenesis activities using BrdU-labeling of neural progenitor cells and doublecortin (DCX)-immunolabeling of post-mitotic immature neurons, in the presence or the absence of CNO/Gq-DREADD activation. Interestingly, Gq-DREADD stimulation of mossy cells significantly increased the number of BrdU+ proliferating cells and DCX immunostaining intensity in the SGZ of the dentate gyrus compared to control mice (Fig. 4c–g). Mossy cell-dependent regulation of neurogenesis activities was consistent throughout the rostro-caudal axis of the dentate gyrus. (Figures S10a-S10d). Next, we investigated the effects of direct stimulation of mossy cells on basal locomotion, anxiety, and depression-related behaviors (Figures S11a-g). Chemogenetic-stimulation of mossy cells alone increased the duration in the open arms of the EPM test (Figure S11d), but failed to induce significant differences in other types of anxiety-related behaviors (Figures S11a-S11c). Furthermore, acute stimulation of mossy cells with CNO injection did not result in any significant change in the depression-like behaviors including the NSF test, and the TST (Figures S11e–S11g). Collectively, these results suggest that acute stimulation of mossy cells is sufficient to trigger adult neurogenesis, but that is unlikely sufficient to achieve full improvement in anxiety and depressive-like behaviors.

Effect of selective inhibition of dentate mossy cells on antidepressant actions in the hippocampus

Next we examined whether inhibition of the mossy cells influences neurogenic and behavioral effects of chronic antidepressant treatment. With cre-dependent AAV injections, control mCherry (control) and hM4D-Gi-mCherry (Gi-DREADD) were specifically expressed in the mossy cells along the dorso-ventral axis of the dentate gyrus (Fig. 5a, b, Figures S12a–S12c). By using electrophysiological recordings, we verified that the activity of mossy cells was silenced by CNO only in mossy cells expressing Gi-DREADD (Fig. 5c, d).

Using the Gi-DREADD system, we examined the role of mossy cells on chronic fluoxetine-induced neurogenesis and behavioral changes (Fig. 5e). We measured adult neurogenesis activities in control and Gi-DREADD mice. Chronic treatment with SSRI significantly increased the number of BrdU+ proliferating cells in the SGZ of the dentate gyrus of control mice, but the fluoxetine effect was significantly reduced in the Gi-DREADD mice (Fig. 5f, g, Figures S13a and S13b). We next analyzed the expression level of doublecortin (DCX), a marker for postmitotic immature neurons. DCX immunostaining intensity was increased by chronic fluoxetine treatment in control mice, but to a much lower extent in Gi-DREADD mice (Fig. 5h, i, Figures S13c and S13d). Chronic fluoxetine administration promotes the newborn neural progenitors to survive and differentiate into mature neurons [52]. Inhibition of mossy cell activity results in significant reduction in survival and/or differentiation of post-mitotic newborn cells (Fig. 5h, i). These results indicate that mossy cells may modulate chronic fluoxetine-induced proliferation of neural progenitors, differentiation and maturation into new-born granule cells. This phenotype is similar to that observed with genetic KO or inhibition of the p11/AnxA2/SMARCA3 complex in mossy cells.

We further investigated the functional significance of mossy cell activity by profiling general locomotor activity

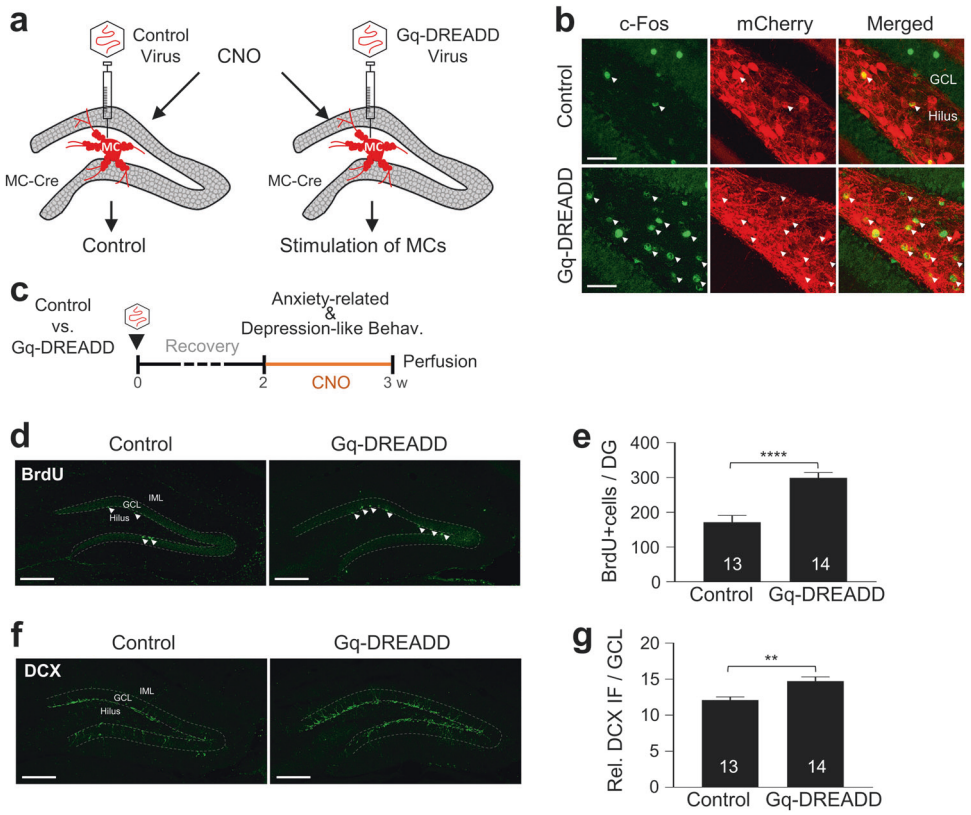
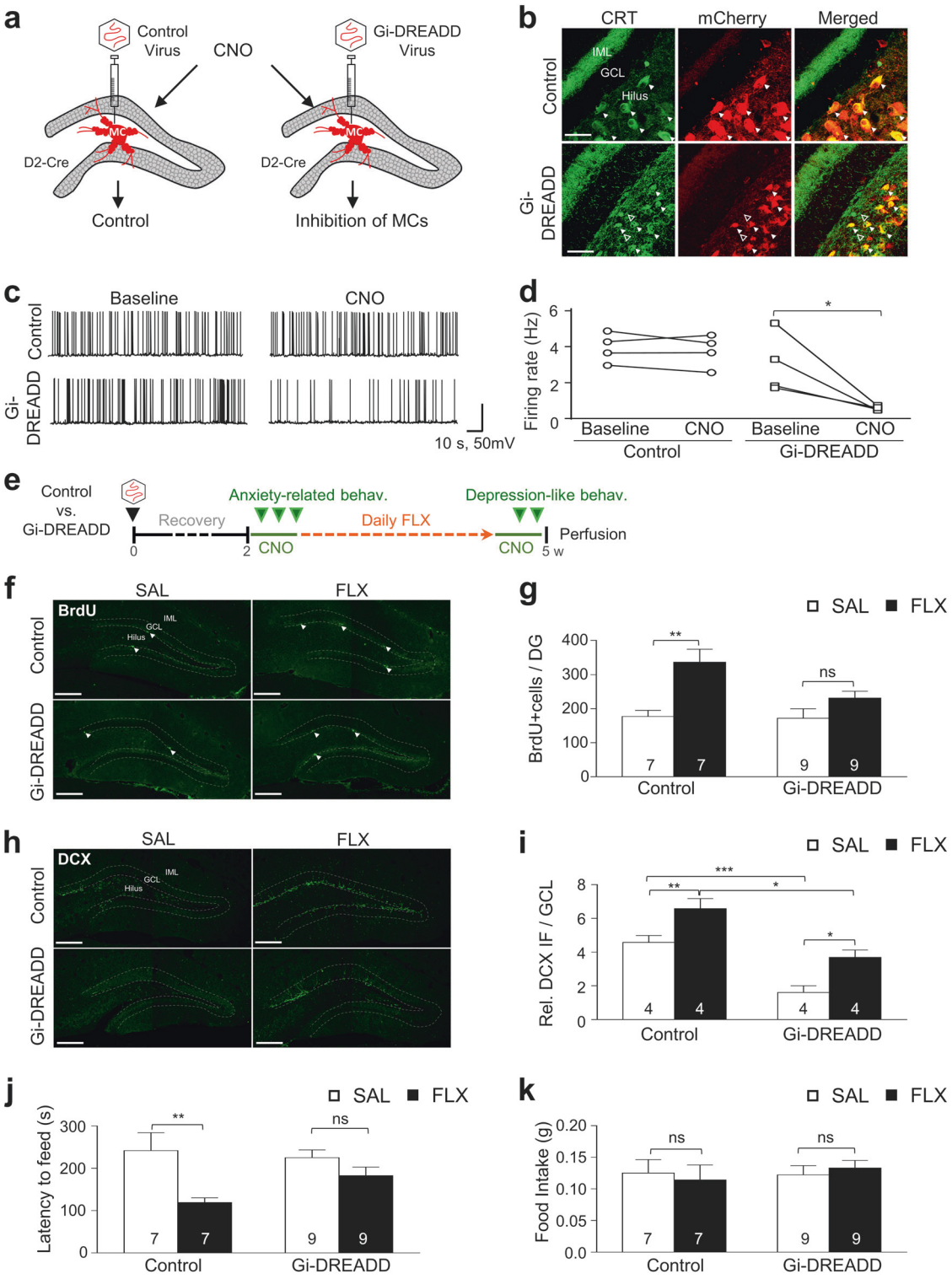


Fig. 4 Acute chemogenetic stimulation of mossy cells increases the adult neurogenesis activities in the dentate gyrus. **a** Schematic illustration of chemogenetic stimulation of mossy cells with Gq-DREADD system. **b** Representative images of c-Fos positive cells (Green) in the dentate hilus of the control or Gq-DREADD mice, 2 h after CNO injection. Scale bar, 50 µm. **c** Schematic of experimental design. Control and Gq-DREADD mice were tested for anxiety-related or depression-like behaviors with CNO injection. One day after the last behavioral testing, all mice were labeled with BrdU for 3 h and then sacrificed with transcardinal perfusion. **d** Representative images of BrdU immunostaining in the dorsal hippocampal sections from the control or Gq-DREADD mice. **e** Quantitation of BrdU-positive cells in the SGZ. Two-tailed, unpaired *T*-test; $p < 0.0001$. **f** Representative images of DCX-immunostaining in the dorsal hippocampal sections from the control and Gq-DREADD mice. **g** Quantitation of relative intensity of DCX immunofluroscence in the SGZ and the GCL of the dentate gyrus. Data are represented as means ± SEM. Two-tailed, unpaired *T*-test; $p = 0.0021$. CNO, clozapine-N-oxide; SGZ, subgranular zone; GCL, granule cell layer; IML, inner molecular layer. See also Figure S9, Figure S10 and Figure S11

and affective behaviors. Control and Gi-DREADD mice did not display a baseline difference in locomotor activity (open field test [OF], Figure S14a), anxiety-related behaviors (thigmotaxis, Figure S14b; light/dark box test [LDB], Figure S14c; elevated plus maze test [EPM], Figure S14d). The novelty-suppressed feeding [NSF] test is based on depressive-like and anxiety behavior and has been widely used to assess the chronic effect of fluoxetine [53]. We examined the possible effect of mossy cell inhibition on behavioral changes after chronic antidepressant treatment, using the NSF test and the tail suspension test [TST], a classical depression test. We found that behavioral responses to chronic fluoxetine treatment were affected by CNO-induced silencing of mossy cell activity. In the NSF test, the reduced latency to feed after chronic fluoxetine treatment was abolished in the Gi-DREADD mice (Fig. 5j). In contrast, the home cage feeding level was unaffected, which suggests that this behavioral change is unlikely due to different hunger levels between comparison groups (Fig. 5k). In the TST, the effect of fluoxetine on immobility was significantly reduced in control mice, but not in Gi-DREADD mice (Figure S14e). Collectively, our results demonstrate that modulation of mossy cell activity in the dentate gyrus is critical for both neurogenic and behavioral responses to chronic fluoxetine administration.

Discussion

In the present study, we have demonstrated a role for hippocampal mossy cells in mediating the behavioral and neurogenic responses to chronic antidepressant treatment. Furthermore, we have shown that p11 and SMARCA3 play crucial roles in modulating mossy cell activity (Figure S15).



Role of mossy cells in the behavioral response to chronic SSRI medication

In addition to established roles in cognitive processes, the hippocampus has been associated with emotional control by taking a prominent position in the limbic circuitry. There is a well-established link between hippocampal dysfunction and the pathogenesis of mood-related disorders. In addition, antidepressive medication has been shown to enhance neuronal plasticity in the hippocampus [54–56]. It thus has been regarded as an important target area for the development of advanced antidepressant therapeutics [3, 57].

◀ **Fig. 5** Chemogenetic silencing of mossy cells attenuates neurogenic and behavioral responses to chronic SSRIs. **a** Schematic illustration of chemogenetic silencing of mossy cells with Gi-DREADD system. **b** Representative images for mossy cell-specific delivery of Gi-DREADD system. Hippocampal sections from AAV-injected mice were co-labeled with a mossy cell marker, CRT. Scale bar, 50 μm. **c**, **d** Representative traces (**c**) and dot plot (**d**) showing the spontaneous firing of mossy cells expressing either control mCherry (Control) or Gi-DREADD-mCherry (Gi-DREADD) before and after bath application of CNO (1 μM). $n = 4$ cells per group. Scale bar: 10 s, 50 mV. Two-way ANOVA with repeated measurement, [AAV x CNO interaction $F(1,6) = 7.261$; $p = 0.0395$, CNO factor $F(1,6) = 6.473$; $p = 0.0438$, CNO factor $F(1,6) = 9.709$; $p = 0.0321$], followed by the Sidak's multiple comparison post hoc test. Control (CNO effect); $p = 0.9355$, Gi-DREADD (CNO effect); $^{*}p = 0.0343$. **e** Schematic of experimental design. Control and Gi-DREADD mice were administrated saline (SAL) or fluoxetine (FLX) for 3 weeks and labeled with BrdU for the last 3 h prior to perfusion. **f** Representative images to visualize proliferating neural stem cells with α-BrdU immunostaining (BrdU, Green, filled arrowheads). **g** BrdU+ cells were quantified along the subgranular zone. Two-way ANOVA, [AAV × drug interaction $F(1,28) = 3.548$; $p = 0.0700$, AAV $F(1,28) = 1.707$; $p = 0.2020$, drug $F(1,28) = 13.55$; $p = 0.001$], followed by the Turkey's post hoc test. **h** Postmitotic immature neurons were immunostained with α-doublecortin antibody (DCX, green). Scale bar, 100 μm. **i** Quantification of relaltive DCX immunofluorescence intensity (Rel. DCX IF) were quantified in the sub-granular and granular zone using Image J software. Pair-wise comparison with two-tailed, unpaired *T*-test. **j** Effect of Gi-DREADD-dependent inhibition of mossy cells on the novelty-suppressed feeding test after chronic fluoxetine treatment. Two-way ANOVA, [AAV x drug interaction $F(1,28) = 4.212$; $p = 0.0496$, AAV factor $F(1,28) = 1.39$; $p = 0.2484$, drug factor $F(1,28) = 15.11$; $p = 0.0006$], followed by the Turkey's post hoc test. **k** Home cage feeding test. Data are represented as means ± SEM. Pair-wise comparison; $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns, nonsignificant; CRT, calretinin; CNO, clozapine-N-oxide; GCL, granule cell layer; IML, inner molecular layer. See also Figure S12, Figure S13, and Figure S14

Mossy cells are very active as reflected by spontaneous action potentials and high-frequency spontaneous excitatory postsynaptic currents (EPSCs) [10, 58]. Here, we found that chronic exposure to unpredictable stress suppresses mossy cell activity as measured by c-fos expression, a neuronal excitation marker, which is reversed by chronic SSRI administration. In addition, our electrophysiological recordings showed that the tonic firing rate of mossy cells is enhanced by chronic, but not acute, treatment with an SSRI, fluoxetine, which is consistent with the induction pattern of p11 and its ternary complex p11/AnxA2/SMARCA3 in the hippocampus [34]. In contrast, mossy cell-specific knockout of *p11* or of *Smarca3* or a disruption of the p11/AnxA2/SMARCA3 complex reduces the tonic firing and c-fos expression of mossy cells, suggesting a significant role of the ternary complex in regulating the neuronal activity of mossy cells. These results suggest that mossy cells are labile neurons that undergo considerable neuroplastic changes in response to external stress or chronic antidepressant administration.

Despite the potential involvement of mossy cells in neurological and neuropsychiatric disorders, our understanding of their functions and pathological implications remains limited. To date, few studies have been performed to examine animal behaviors by manipulating mossy cells. A previous study deleted mossy cells by using a cytotoxic toxin expression and found transient elevation of anxiety and impaired pattern separation in rodents [23]. A recent study adopted an optogenetic approach and showed that mossy cells control spontaneous convulsive seizures and spatial memory [59]. Here we demonstrate that behavioral effects of chronic fluoxetine treatment are significantly diminished when mossy cell activity is suppressed. Furthermore, chemogenetic inhibition of mossy cell activity impairs the SSRI-induced changes in affective behaviors. Collectively, our findings provide strong evidence that mossy cells play a significant role in antidepressant actions, in addition to their reported role in cognitive behaviors. However, acute chemogenetic stimulation of mossy cells is unlikely to be sufficient to achieve full behavioral improvement as seen by chronic SSRI administration. Behavioral benefit after chronic SSRI administration may require cooperative neuroplastic changes in multiple neural cell types that include hippocampal mossy cells.

Role of mossy cells in the neurogenic response to chronic SSRI medication

It has been unknown whether mossy cells are involved in adult neurogenesis, although a previous study suggested that mossy cells provide the first excitatory input to adult-born granule cells [60]. The current study is the first to show that mossy cells influence SSRI-induced neurogenesis. We report here that mossy cells, regulated by the p11/AnxA2/SMARCA3 complex, mediate the neurogenic responses to chronic antidepressant administration. Consistent with our previous reports using *p11* or *Smarca3* conventional KO mice [29, 34], disruption of the p11/AnxA2/SMARCA3 complex in mossy cells impairs adult neurogenesis in response to chronic fluoxetine treatment. Consistently, chemogenetic inhibition of mossy cells results in a decrease in SSRI-induced adult neurogenesis in the hippocampus. Furthermore, acute chemogenetic stimulation of mossy cells is able to trigger early neurogenic activities including the proliferation of the neural stem cells, survival, and differentiation into new-born granule cells. Thus, these results suggest crucial roles of mossy cells in multiple stages of SSRI-induced neurogenesis in the hippocampus.

Despite extensive studies performed over the past 20 years, the molecular mechanisms underlying SSRI-induced neurogenesis remain unclear. Several genetic and pharmacological approaches have demonstrated that 5-HT1A and 5-HT4 receptors, enriched in dentate granule cells, are crucial for SSRI-induced neurogenesis [61–63]. In contrast, the basal maintenance level of hippocampal neurogenesis is

not altered in either 5-HT1A receptor-deficient or 5-HT4 receptor-deficient mice, which indicates the specific involvement of these serotonin pathways in SSRI-induced neurogenesis. Here we observed that the p11/AnxA2/SMARCA3 complex in mossy cells regulates SSRI-induced neurogenesis, but not the basal level. Given that mossy cells and granule cells make extensive reciprocal connections to construct the neural circuitry of the dentate gyrus, it is plausible that serotonergic regulation of both granule cells and mossy cells may cooperate to induce the neurogenesis and further behavioral response to chronic SSRI administration.

In conclusion, we have demonstrated that mossy cells regulate affective behaviors as well as neurogenesis in response to SSRIs, in addition to their established role in cognitive behaviors. The present study contributes to a better understanding of the neural mechanisms mediating the beneficial effects of SSRI medication and should contribute to the development of novel antidepressant therapeutics to target specific neural cell types in the mood-regulatory circuits.

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Author contributions SO, JC, PG, and YSO designed the experiments. SO and JA performed and analyzed the behavior test. JC performed and analyzed electrophysiology experiments. SO performed and analyzed immunofluorescence data. SO, JC, PG, and YSO wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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