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Upregulation of GSK3β Contributes to Brain Disorders in Elderly REGγ-knockout Mice

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GSK3β regulates some functions of the brain, but the mechanisms involved in the maintenance of GSK3β protein stability remain ambiguous. REGγ, an important proteasome activator for ubiquitin-independent protein degradation, has been shown to degrade certain intact proteins and is involved in the regulation of important biological processes. Here we demonstrate that REGγ promotes the degradation of GSK3β protein *in vitro* and *in vivo*. With increased GSK3β activity, REGγ knockout (REGγ −/−) mice exhibit late-onset sensorimotor gating and cognitive deficiencies including decreased working memory, hyperlocomotion, increased stereotype, defective prepulse inhibition (PPI), and disability in nest building, at the age of 8 months or older. Inhibition of GSK3β rescued the compromised PPI phenotypes and working memory deficiency in the knockout mice. Also, we found an age-dependent decrease in the trypsin-like proteasomal activity in REGγ −/− mice brains, which may be reflective of a lack of degradation of GSK3β. Collectively, our findings reveal a novel regulatory pathway in which the REGγ-proteasome controls the steady-state level of GSK3β protein. Dysfunction in this non-canonical proteasome degradation pathway may contribute to the sensorimotor gating deficiency and cognitive disorders in aging mice.
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INTRODUCTION

The proteasome has important roles in the degradation of proteins involved in neuronal apoptosis and synaptic plasticity (Cline, 2003; Speese *et al*, 2003), and impaired proteasome function is observed not only in aging (Keller *et al*, 2002), Alzheimer's disease (Keller *et al*, 2000), and Parkinson's disease (McNaught *et al*, 2001), but also in schizophrenia (Altar *et al*, 2005; Rubio *et al*, 2013; Watanabe *et al*, 2014). Although most studies focus on ubiquitin-dependent proteasome pathways, the functions of the ubiquitin-independent proteasome in the central nervous system remain unknown.

REG*γ*, also named PA28*γ* or Ki antigen, encoded by *PSME3*, belongs to the REG/11S family of proteasome activators which functions like 'caps' to bind and activate the 20S proteasome (Dubiel *et al*, 1992; Ma *et al*, 1992). The REG*γ* gene is located in 17q21 in humans, a region with high genetic linkage to schizophrenia in Latino populations (Escamilla *et al*, 2009; Lewis *et al*, 2003) and implicated in several other CNS diseases, such as autism (Cantor *et al*, 2005), and bipolar disorder (Ewald *et al*, 2005), which share at least some symptoms with schizophrenia and schizoaffective disorders. Since the discovery of a mammalian target of REG*γ*, SRC3 (Steroid receptor co-activator protein; Li *et al*, 2006), accumulating evidence has revealed diverse functions for the ATP- and ubiquitin-independent REG*γ*–proteasome pathway, including regulation of cell cycle (Kobayashi *et al*, 2013; Li *et al*, 2007), angiogenesis (Liu *et al*, 2014), and hepatic lipid metabolism (Dong *et al*, 2013). REG*γ* is widely expressed in tissues (Yu *et al*, 2010), especially in the brain. Up to now, whether REG*γ* activity is altered in schizophrenia patients or other CNS diseases remains unknown, but the roles of REG*γ* in the central nervous system deserve exploration on the basis of the above evidence.

Our SILAC (stable isotope labeling with amino acids) studies reported here suggest that glycogen synthase kinase-3 beta (GSK3*β*) is potentially regulated by REG*γ*. GSK3*β*, a proline-directed serine/threonine kinase initially identified as a phosphorylating agent of glycogen synthase (Stambolic and Woodgett, 1994), has been well documented as a key functionality in the development of CNS diseases, such as schizophrenia (Agam *et al*, 2006; Freyberg *et al*, 2010; Kozlovsky *et al*, 2002; Lovestone *et al*, 2007). Unlike other

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serine/threonine kinases, GSK3β is constitutively active, regulated by phosphorylation on its serine-9 (Ser9) for inhibition or on tyrosine-216 for enhanced activity (Lochhead *et al*, 2006). Emaniam *et al* found that the phosphorylation level of GSK3β at Ser9 was 73% lower in the prefrontal cortex of schizophrenic individuals, indicating increased activity of GSK3β in schizophrenics (Emamian *et al*, 2004). A number of genes reported to be associated with schizophrenia affect GSK-3 regulatory pathways directly or indirectly. Drugs that induce psychosis, or that are used to treat psychosis, alter GSK-3 signaling. For example, chronic treatment with haloperidol, an anti-schizophrenia drug, can increase the phosphorylation of GSK3β at Ser9 (Emamian *et al*, 2004). However, the molecular mechanism involved in the regulation of the GSK3β protein turnover during schizophrenia-like disorders is poorly understood.

By a series of *in vivo* and *in vitro* analyses, we find that GSK3β can be regulated by REGγ, indicating that the REGγ–proteasome may affect cellular functions in the central nervous system. Unusual behavior phenotypes, including sensorimotor gating and cognitive deficiency reminiscent of schizophrenia-like phenotypes, were observed in elderly REGγ-knockout mice, which appear generally normal when young. Overall, our finding suggests that REGγ deficiency may contribute to late-onset brain disorders via hyperactivation of GSK3β.

MATERIALS AND METHODS

Animals

REG$\gamma-/-$ mice were kindly provided by Dr John J Monaco at the University of Cincinnati (Barton *et al*, 2004). All the mice had a C57BL/6 background. REG$\gamma-/-$ and REGγ+/+ mice were obtained by breeding heterozygous REGγ+/− and genotyping was by standard PCR analysis on tail-snip DNA. The mice were housed (six per cage) at 24 °C and 40–70% humidity on a 12-h light/dark cycle (light on from 0700 to 1900 h) with access to food and water *ad libitum*. If not indicated otherwise, male REGγ+/+ and REG$\gamma-/-$ mice littermates used in this study were 8 months of age in all the experiments. All the experiments were approved by the Animal Ethics Committee at East China Normal University.

Cell Culture, SILAC Labeling and LC MS/MS

A549 cells with REGγ knockdown were grown in EMEM medium (deficient in lysine and arginine; Sigma-Aldrich, St Louis, MO, USA) supplemented with 28 μg/ml $^{12}C_6^{14}N_4$-arginine (Sigma-Aldrich), 73 μg/ml $^{12}C_6^{14}N_2$-lysine (Sigma-Aldrich), 10% FBS, and 1% Pen/Strep (light medium), whereas control A549 cells were grown in EMEM medium supplemented with 28 μg/ml $^{13}C_6^{15}N_4$-arginine (Cambridge Isotope Laboratories, Andover, MA), 73 μg/ml $^{13}C_6^{15}N_2$-lysine (Cambridge Isotope Laboratories), 10% FBS, and 1% Pen/Strep (heavy medium). The cells were grown for more than seven cell doublings in the labeling media to ensure complete labeling.

After cell lysis, equal amounts of nuclear fractions from two populations of cells were mixed, TCA precipitated, digested with LysC/trypsin, and separated by strong cation exchange (SCX) chromatography as previously described. The collected SCX fractions were desalted and subjected to LC MS/MS as described (Kaake *et al*, 2010). MS/MS data were submitted for database searching using a development version of Protein Prospector (v 5.0.0, UC, San Francisco). The Batch-tag program within Protein Prospector (v 5.0.0) was used for database searching against the Swissprot database (2008.06.10). Trypsin was selected as the enzyme and the maximum of missed tryptic cleavages was set as 2. Chemical modifications such as protein N-terminal acetylation, methionine oxidation, N-terminal pyroglutamine, $^{13}C_6^{15}N_4$-labeled Arg, and $^{13}C_6^{15}N_2$-labeled Lys were also chosen as variable modifications. The mass accuracy for parent ions and fragment ions were set as ±20 p.p.m. and 0.6 Da, respectively. Homo sapiens was selected as the restricted species. SILAC ratios were calculated using the Search Compare program in Protein Prospector as described (Wang and Huang, 2008).

Open Field

The open-field activity of mice was measured with TruScan apparatus (Coulbourn Instruments, Allentown, PA, USA). Briefly, the mice were put into a 27 cm × 27 cm × 38 cm chamber illuminated at 50 lux. One hour of free locomotion was tracked by Truscan 2.01 software. Move time and stereotype time in every 5 min were scored.

Prepulse Inhibition (PPI)

The acoustic startle response and the PPI were measured using automated SR Lab startle chambers (San Diego Instruments, San Diego, CA, USA). The tests were performed as described (Gray *et al*, 2009). Briefly, throughout the testing session, mice were exposed to a 65 dB background white noise, during a 5 min habituation period. Each session consisted of 80 trials of which the first and the last six consisted of 'pulse only' startle-inducing stimuli of 120 dB lasting 40 ms. The central 68 trials were a pseudo-randomized program consisting of 10 no-stimulus, 10 startle-120, 10 PPI-73/77/91, and six 73/77/81 alone. In these instances, the prepulse preceded the pulse by 100 ms and lasted for 20 ms. The PPI was expressed as a percentage inhibition of the pulse-alone startle response.

Radial Eight-Arm Maze

During the task, the mice were calorie restricted to keep 80–85% of their normal weight and single housed. For the first 2 days, food was placed at the terminus of every arm of the radial eight-arm maze. Mice were placed in the center of the maze and allowed to seek the food freely for 10 min. For the following 3 days, only one piece of food was put into each terminus and the mice were allowed to find all the food in eight arms until 10 min individually, twice per day. On the test day, the number of arms each mouse entered into was recorded. Every repeated entry was recorded as one error. Error ratios were calculated on the basis of the observations.

Nest-Building

Animals were individually housed in a cage at 1900 h, with one piece of cotton fiber pad as nesting material

(5 cm × 5 cm; Ancare, San Jose, CA, USA). Pictures of the nests were taken by a digital camera on the next morning. The presence and quality of the nests were scored at a five-point scale from 1 to 5 as follows: 1 = nestlet not noticeably touched, 2 = nestlet partially torn up, 3 = mostly shredded but often with no identifiable nest site, 4 = an identifiable, but flat nest and 5 = a near perfect nest.

Medication of Animals

When used in PPI study, 0.5 mg/kg Haloperidol (Sigma-Aldrich) or 5 mg/kg SB216763 (Tocris Bioscience, Bristol, UK) were injected (i.p.) 5 min before the start of PPI experiment. For chronic treatment, SB216763 (1.2 mg/kg, i.p.) were given daily for 1 week.

Primary Neuron Culture and Collection of Brain Samples

For adult primary cortical neurons culture, the cortical neurons from 8-month-old REGγ+/+ and REGγ−/− mice were isolated as previously described (Brewer and Torricelli, 2007). For tissue RNA isolation and protein extraction, animals were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.). The tissue was rapidly dissected on ice and frozen in liquid nitrogen and stored at −80 °C. For immunofluorescence staining and Nissl staining, deeply anesthetized animals were transcardially perfused with PBS (0.01 M phosphate-buffered saline, pH = 7.4) followed by 4% paraformaldehyde. The brains were removed, post-fixed overnight in the fixative, and then dehydrated in 30% sucrose in PBS. Each frozen brain was then sectioned at 10–30 μm in the coronal or sagittal plane using a freezing microtome (CM1850-1-1, Leica, Solms, Germany).

RNA Purification and Real-time PCR

Total RNA was extracted by RNAiso Plus (TAKARA Inc., Dalian, China) according to the manufacturer's instructions. The primers were synthesized by Invitrogen (Carlsbad, CA, USA), and the sequences were as follows: GSK3*β*-f: 5′-TCGAGCCAAGCAGACACTCC-3′; GSK3*β*-r: 5′-ACATTGGGCTCTCCTCGGAC-3′; GAPDH-f: 5′-AGGAGCGAGACCCCACTAACA-3′; GAPDH-r: 5′-GTGATGGCATGGACTGTGGT-3′; c-Fos-f: 5′-CGGGTTTCAACGCCGACTA-3′; c-Fos-r: 5′-TTGGCACTAGAGACGGACAGA-3′; c-Myc-f: 5′-ATGCCCCTCAACGTGAACTTC-3′; c-Myc-r: 5′-CGCAACATAGGATGGAGAGCA-3′. Real-time PCR protocol consisted of 5 min at 95 °C and 40 cycles of 10 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. The amount of target genes was determined by the $2^{-\Delta\Delta Ct}$ method and normalization against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as fold changes.

Plasmid Construction and siRNA

Plasmids Flag-REGγ and HA-REGγ were previously generated (Liu *et al*, 2010). Flag-GSK3*β* was generated by PCR with the primers forward: 5′-CGGAATTCATGGACTACAAGGACGACGATGACAAGATGTCAGGGCGGCCCAG-3′ and reverse: 5′-CCGCTCGAGTCAGGTGGAGTTGGAAGCTG-3′ and was inserted into pcDNA3.1 vector. REGγ siRNA were purchased from GenePharma Company (GenePharma Co., Ltd, Shanghai, China) targeting to the site: 5′-CAGAAGACUUGGUGGCAAA-3′.

Trypsin-like Proteasome Activity Assay

A commercially available indirect enzyme-based luminescent assay was modified (Cat. No. G8631 with substrate for trypsin-like activity (Z-LRR-aminoluciferin), Promega, Madison, WI, USA) to measure the *in vivo* trypsin-like catalytic activity associated with the proteasome in REGγ+/+ and REGγ−/− mice as described before (Strucksberg *et al*, 2010). The resulting luminescence was measured with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) in luminometry mode (Luc 1000). Fold changes in the luminescence values of the tissues from 3-month-old and 8-month-old animals were calculated and displayed.

A detailed description of the Materials and Methods is provided in the Supplementary Text.

Statistical Analysis

Data were analyzed by Student's *t*-test, two-way ANOVA or repeated-measure ANOVA according to the study design. The data for open field and PPI were analyzed by repeated-measure ANOVA and two-way ANOVA, respectively to distinguish genotype effects and phenotypic effects as well as their interactions. For behavior phenotypes with or without drug treatment on the same group of mice, paired-Student's *t*-test was used to analyze the drug effect. For data analysis involved in only single factor, such as protein expression, Student's *t*-test was used. Values in graphs were expressed as mean ± SEM; $P<0.05$ was considered as statistical significance marked by *; $P<0.01$ was considered as highly statistical significance marked by **.

RESULTS

GSK3*β* is Regulated by REGγ *In vitro* and *In vivo*

To identify novel proteins modulated by REGγ deficiency, SILAC (stable isotope labeling by amino acids in cell culture)-based quantitative mass spectrometry was performed in A549 cells with or without REGγ deficiency (Liu *et al*, 2010) to determine changes in relative protein abundance. In this work, 265 proteins were identified to be upregulated with SILAC ratios >2, (see Supplementary Table 1). Among upregulated proteins, GSK3*β* (Glycogen synthase kinase-3 beta) has been further evaluated due to its important roles in the central nervous system as well as in oncogenesis.

Following transient knockdown of REGγ in mouse Neuro-2a (N2a) cells, we found a significant increase of GSK3*β* protein levels (Figure 1a). A similar increase of GSK3*β* with REGγ depletion also was observed in HT-22, a different neuronal cell line (Figure 1b). To test whether GSK3*β* might be regulated by REGγ *in vivo*, we analyzed GSK3*β* expression levels in the prefrontal cortex of REGγ+/+ and REGγ−/− mice at 8 months of age. The results showed that the expression of GSK3*β* (Figure 1c and d), but not GSK3*α* (Glycogen synthase kinase-3 alpha, see Supplementary Figure 1a and b), another isoform of GSK3, was increased

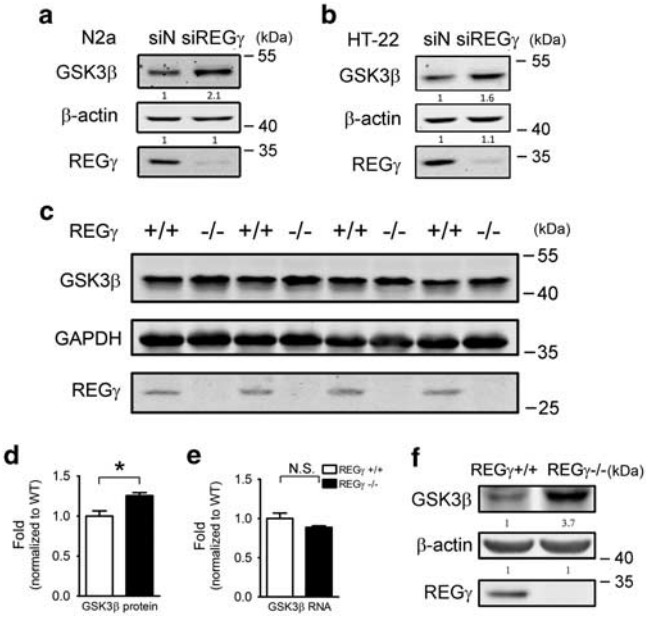


Figure 1 Protein level of GSK3β is increased by deficiency of REGγ *in vitro* and *in vivo*. (a, b) The expression of GSK3β in N2a and HT-22 cell line by knocking down REGγ was shown by western blot, using β-actin as control. (c) GSK3β proteins from prefrontal cortex tissues of four independent pairs of 8-month-old REGγ+/+ and REGγ−/− mice were presented by western blot, using GAPDH as control. (d) Quantification of western blot for (c), REGγ+/+ and REGγ−/− $n=4$ animals each. (e) Quantification of GSK3β mRNA levels from prefrontal cortex tissues in (c), REGγ+/+ and REGγ−/− $n=4$ animals each. (f) Cortical neuron culture of 8-month-old REGγ+/+ and REGγ−/− mice to show the protein levels of GSK3β. All the quantification was presented as mean ± SEM, $^*P<0.05$, NS, not significant.

in the prefrontal cortex of REG$\gamma-/-$ mice compared with wild-type littermates ($P=0.015$). An increased expression of GSK3β also was observed in cultured cortical neuron from 8-month-old REG$\gamma-/-$ mice (see Figure 1f). There were no differences in the mRNA levels for GSK3β between REGγ+/+ and REG$\gamma-/-$ mice by quantitative RT-PCR ($P=0.16$, Figure 1e), indicating that GSK3β is regulated posttranscriptionally. These results suggest that GSK3β is negatively regulated by REGγ *in vitro* and *in vivo*.

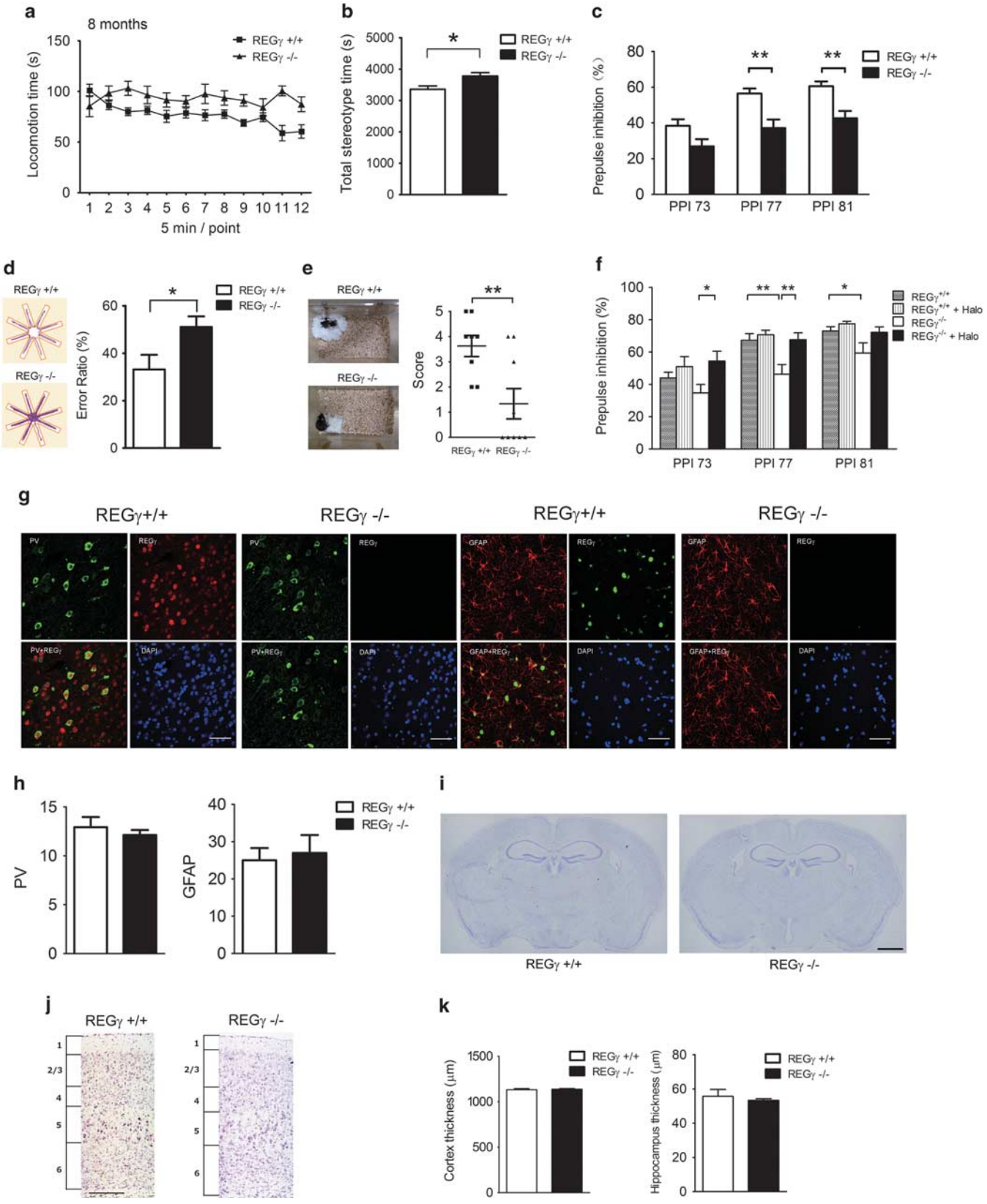
Elderly REG$\gamma-/-$ Mice Exhibit Hyperactivity, Sensorimotor Gating Deficiency, and Aberrant Cognitive Behaviors

Given the important role of GSK3β in psychiatric disease and its increase in elderly REG$\gamma-/-$ mice (8 months), we examined related brain functions in REG$\gamma-/-$ animals. A series of behavioral assays were performed in the REGγ+/+ and REG$\gamma-/-$ mice at 8 months of age. Compared with wild-type littermates, elderly REG$\gamma-/-$ mice (8 months) showed hyperactivity (genotype effect, $F(1,24)=7.737$; $P=0.01$, Figure 2a) and increased stereotype time in 1-h open-field tests ($P=0.012$, Figure 2b). Prepulse inhibition (PPI) of acoustic startle, an index of sensorimotor gating, displayed a significant reduction in elderly adult REG$\gamma-/-$ mice compared with wild-type controls, especially at 77 dB and 81 dB with similar startle amplitudes in each group (see Supplementary Figure 2a), suggesting impaired sensorimotor gating function in elderly REG$\gamma-/-$ mice (interaction effect, $F(2,168)=0.6$, $P=0.55$; genotype effect, $F(1,168)=28.00$, $P<0.0001$; prepulse intensity effect, $F(2,168)=13.82$, $P<0.0001$, Figure 2c). Besides, we found aberrant cognitive ability in elderly REG$\gamma-/-$ mice. The radial eight-arm maze was performed to assess the working memory of REG$\gamma-/-$ mice. The results showed a significant elevation of error ratio, suggesting that the elderly REG$\gamma-/-$ mice had a deficit in working memory ($P=0.027$, Figure 2d). We also noticed that REG$\gamma-/-$ mice had impaired ability in nest-building tests, during which they exhibited significantly lower nesting scores with some cotton pieces untorn, or only scattered nesting materials ($P=0.0081$, Figure 2e). On the other hand, both groups of mice showed similar immobile time in the tail suspension tasks ($P=0.82$, see Supplementary Figure 2b) or sucrose preference tests ($P=0.31$, see Supplementary Figure 2c), both of which are usually applied to measure depression-like emotion in mice. REG$\gamma-/-$ mice also exhibited no significant differences from wild-type mice in the percentage of open-arm entries ($P=0.188$), open-arm time ($P=0.110$) or open-arm distance ($P=0.193$) in elevated plus maze tasks (see Supplementary Figure 2e), reflecting normal anxiety level. The rotarod tests suggested normal motor coordination at 8 months of age ($P=0.93$, see Supplementary Figure 2d). The phenotypes observed in elderly REG$\gamma-/-$ mice are reminiscent of schizophrenia-like behaviors in mouse models (Jones *et al*, 2011). To evaluate this, we tested the effect of haloperidol, a traditional psychotic medicine for schizophrenia (WHO, 2013), on elderly REG$\gamma-/-$ mice. Acute haloperidol treatment could effectively rescue the impaired prepulse inhibition in REG$\gamma-/-$ mice (Figure 2f). Before haloperidol treatment, REG$\gamma-/-$ mice showed decreased PPI, whereas mice treated with haloperidol had no significant differences between the two genotypes ($P>0.05$ for all the three PPI levels). Compared with untreated PPI-73/PPI-77 groups, haloperidol treatment significantly increased prepulse inhibition in elderly REG$\gamma-/-$ mice ($P<0.05$, $P<0.01$, respectively), endorsing the behavior as schizophrenia-like.

REGγ has been reported to be expressed within neurons in the brain (Seo *et al*, 2007). We found by immunostaining that REGγ is expressed in various brain regions, with relatively high levels in the cortex and hippocampus (see Supplementary Figure 3a). Also REGγ is expressed in nearly all NeuN positive cells in the brain and co-localizes with αCAMK2 (see Supplementary Figure 3b), parvalbumin, and GFAP-positive glial cells (Figure 2g). Despite the growth retardation and smaller body size associated with adult REG$\gamma-/-$ mice (Murata *et al*, 1999), results from Nissl staining showed neither striking differences in lamination, nor the cortical/hippocampus thickness, between the REGγ +/+ and REG$\gamma-/-$ mice up to 10 months of age (all $P>0.05$, see Figure 2i–k). NeuN, parvalbumin, αCAMK2, and GFAP were expressed normally in the REG$\gamma-/-$ mouse brain (all $P>0.05$, see Figure 2g and h and Supplementary Figure 3b). These results suggest that the brain structure of REG$\gamma-/-$ mice appears normal and the impact of REGγ deficiency on the brain functions may be mainly at molecular levels.

Increased GSK3*β* Activity in REG*γ* −/− Mice Contributes to the Aberrant Behavioral Testing Results

Owing to behavior abnormalities and increased GSK3*β* protein observed in elderly REG*γ* −/− mice, we analyzed posttranslational modification of GSK3*β* proteins in more details. We found that not only the total GSK3*β* ($P=0.016$), which reflects constitutive activity, but also an activity-enhanced form, p-GSK3*β*-Y216 ($P=0.016$), was increased in



REGγ−/− prefrontal cortex tissues (Figure 3a and b). The inactive form, p-GSK3β-Ser9 remained unchanged ($P=0.24$; Figure 3a and b), resulting in a decreased p-GSK3β-Ser9/GSK3β ($P=0.032$) and increased p-GSK3β-Y216/p-GSK3β-Ser9 ($P=0.029$) ratio (Figure 3b). Our results reflect an increased GSK3β activity in REGγ−/− mice at 8 months of age, which can be inhibited by haloperidol (see Supplementary Figure 4a). Although AKT (Protein kinase B, a serine/threonine kinase) can mediate the inhibition of GSK3β activity through phosphorylation of the Ser9 residue (Dudek *et al*, 1997), expression levels of total AKT 1 and p-AKT 1-S473 were not significantly changed between the two genotypes by western blot ($P>0.05$, Figure 3c and d), indicating AKT is not involved in the regulation of GSK3β. Therefore, we wondered whether GSK3β might be regulated by REGγ, thereby leading to behavioral changes. To determine whether the aberrant behaviors in REGγ−/− mice were caused by elevated GSK3β activity, we treated REGγ−/− mice with GSK3 inhibitor, SB216763, acutely and chronically as described (Chan *et al*, 2012; Datusalia and Sharma, 2014). Cellular analyses of SB216763 treatment in a time-dependent and dose-dependent manner suggest that this inhibitor rapidly and sufficiently regulates downstream genes, such as β-catenin, and so on. (see Supplementary Figure 4b and c). Acute treatment with 5 mg/kg of SB216763 effectively rescued PPI deficiency in REGγ−/− mice ($P<0.05$ for PPI-77/PPI-81), while SB216763 exerted little effect in the control mice ($P>0.05$, Figure 3e). Similarly, chronic treatment with 1.2 mg/kg of SB216763 for 1 week significantly reduced the error ratio for REGγ−/− mice ($P>0.05$, compared with REGγ+/+ mice), but exerted little effect on wild-type mice, in the radial eight-arm maze task (drug × genotype effect, $F(1,33)=4.81$, $P=0.0354$, Figure 3f). Taken together, it might be GSK3β hyperactivity that primarily contributes to the sensorimotor gating and cognitive deficiency in elderly REGγ−/− mice.

REGγ Regulates GSK3β Activity via Triggering Its Degradation

The inverse correlations between GSK3β and REGγ *in vitro* and *in vivo* suggest that REGγ may regulate GSK3β through degradation. Physical interaction between REGγ and GSK3β was observed by co-immunoprecipitation (Figure 4a). To substantiate REGγ-proteasome-dependent degradation of GSK3β, dynamic changes in GSK3β protein levels after inhibition of *de novo* protein synthesis by cycloheximide (CHX) were measured in the REGγ-inducible 293 REGγ wild-type and REGγ N151Y-mut (functional deficiency) cell lines (Li *et al*, 2006; Zhang *et al*, 1998). During the time course of CHX treatment, GSK3β was more stable in REGγ N151Y-mut cells compared with the 293 REGγ wild-type cells (Figure 4b), indicating that a functional REGγ protein is required for the turnover of GSK3β. In support of that notion, when proteasome inhibitor MG132 (20 μM) was added in SH-SY5Y cells, increased GSK3β levels were observed (Figure 4c), suggesting that REGγ-mediated action is proteasome-dependent. As highly expressed GSK3β leads to β-catenin degradation in a ubiquitin-dependent manner (Doble and Woodgett, 2003; Yost *et al*, 1996), we assayed the levels of β-catenin in HeLa shR (a stable cell line with REGγ-knockdown) and shN (a negative control cell line integrated with scrambled shRNA) cells in the presence of CHX. Western blot results showed a faster decrease in β-catenin levels over a time course of CHX treatment in HeLa shR cells (Figure 4d). Similar results showing stabilized GSK3β and faster degradation of β-catenin were observed in REGγ−/− MEF cells (see Supplementary Figure 5a). These experiments suggest that not only GSK3β protein level, but also its activity is elevated in cells depleted of REGγ.

Young REGγ−/− Mice Show Normal Behaviors and Consistently Normal GSK3β Protein Level Owing to 20 S Proteasome Activity

Our observation of no striking differences in locomotor activity in 1 h, stereotype or prepulse inhibition between young REGγ−/− mice (3 months) and wild-type control mice (all $P>0.05$, Figure 5a–c), suggested that the aberrant behaviors in REGγ−/− mice might be 'late-onset'. The GSK3β, p-GSK3β-Ser9, total AKT1, or p-AKT1-S473 expression levels in prefrontal cortex of the young REGγ−/− mice showed no differences compared with wild-type controls by western blot (Figure 5d, Supplementary Figure 6a). Given that the proteasome activator REGγ mainly stimulates the trypsin-like activity of the 20S proteasome (Li *et al*, 2006), we tested the trypsin-like activity in prefrontal cortex tissues from REGγ+/+ and REGγ−/− mice at the age of 3 months or 8 months (Figure 5e). As suspected, the *in vivo* 20S proteasome trypsin-like activity of brain tissues showed no differences between REGγ+/+ and REGγ−/− mice at the age of 3 months ($P=0.43$). However, at 8 months of age, there was a significant decrease of the trypsin-like proteasomal activity in the REGγ−/− brain tissues compared with that in wild-type

Figure 2 Elderly REGγ−/− mice exhibit abnormal behaviors, but normal brain structure. (a) Locomotor activity of REGγ+/+ and REGγ−/− mice was measured by open-field test in 60 min (every 5 min was recorded as 1 point, 12 points in all). The statistical significance between REGγ+/+ and REGγ−/− ($n=12$ animals each group) was described in the text. (b) REGγ−/− mice had more stereotype time compared with REGγ+/+ mice within 60 min in open-field test, REGγ+/+ and REGγ−/− $n=12$ animals each. (c) Prepulse inhibition on 73 dB, 77 dB, and 81 dB of both genotypes were measured, REGγ+/+ and REGγ−/− $n=29$ animals each. (d) Radial eight-arm maze test was used to measure the working memory of both genotypes and the error ratio was presented, REGγ+/+ and REGγ−/− $n=12$ animals each. The left was the representative track of mice of both genotypes in radial eight-arm maze test. (e) The nest-building ability for both genotypes was displayed and the scores were quantified, REGγ+/+ $n=8$, REGγ−/− $n=9$. (f) Prepulse inhibition before and after injection with antipsychotic medicine haloperidol (0.5 mg/kg, i.p., 5 min before the test) for both genotypes was measured, REGγ+/+ $n=10$, REGγ−/− $n=8$, Halo, haloperidol. Alterations of prepulse inhibition of REGγ−/− mice can be observed at 73 and 77 dB. All the mice for behavior studies were 8 months old. (g) The expressions of interneuron marker parvalbumin (PV) and glial cell marker GFAP were shown, Scale bar, 50 μm. (h) Statistical analysis of PV and GFAP positive cell numbers (average for 8–10 views) in both genotypes, REGγ+/+ and REGγ−/− $n=3$ animals each. (i) Nissl staining showed normal appearance of brain anatomy from a REGγ+/+ (wild type, left panel) and REGγ−/− (right panel) mouse. Scale bar, 1 mm. (j) Lamination of cortex from both REGγ+/+ and REGγ−/− mouse by Nissl staining. Scale bar, 100 μm. (k) Statistical analysis of cortex and hippocampus thickness for both genotypes, REGγ+/+ and REGγ−/− $n=3$ animals each. All the quantification above was presented as mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$.

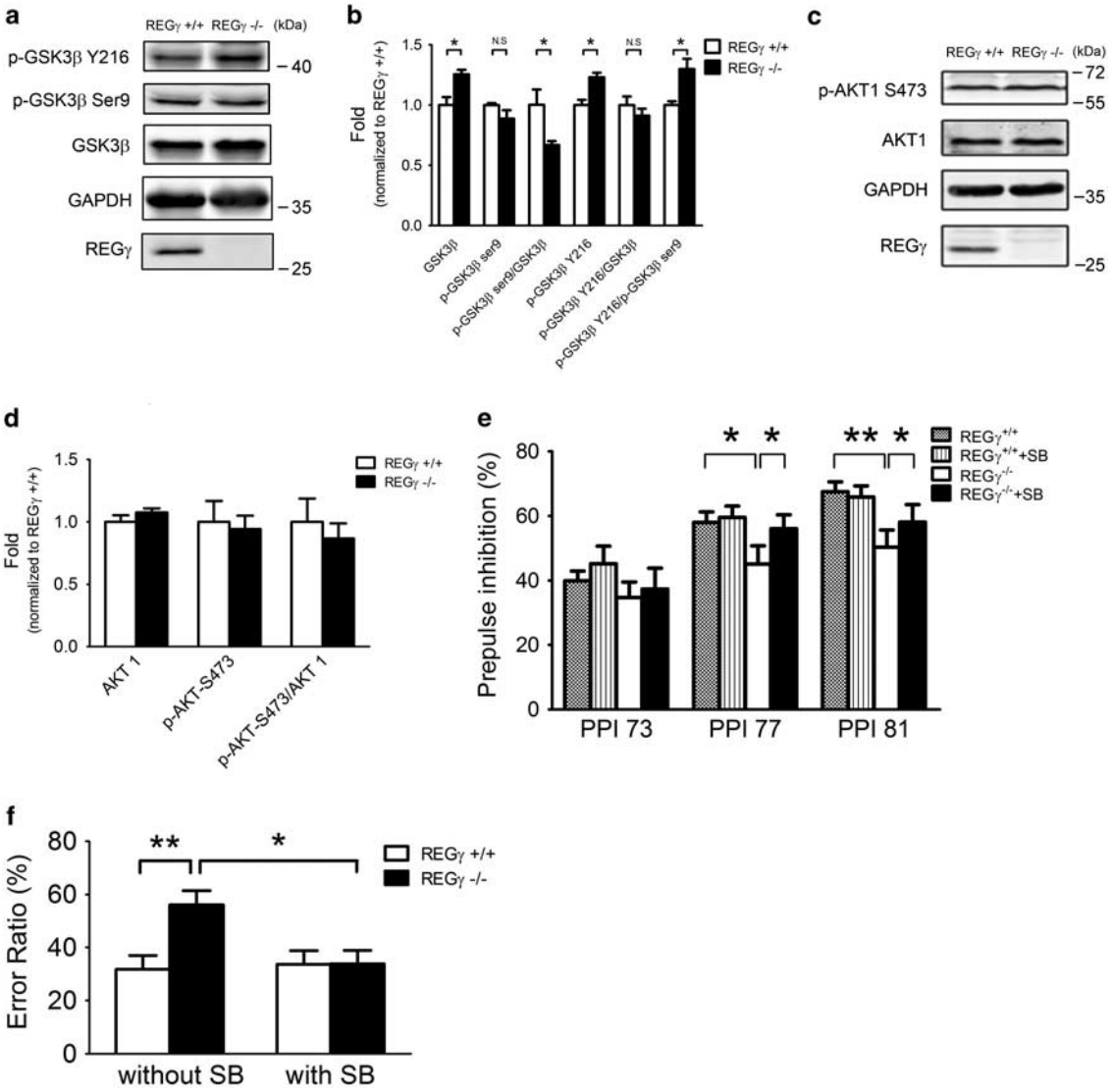


Figure 3 Hyperactivity of GSK3β in REGγ − / − mice contributes to the abnormal behavior. (a) Representative immunoblots of GSK3β and phosphorylated GSK3β in the prefrontal cortex tissues from 8-month-old REGγ+/+ and REGγ − / − mice, using GAPDH as control. (b) Quantification of western blot shown in a, REGγ+/+ and REGγ − / − $n=4$ animals each. (c) Western blot for AKT1 and p-AKT1-S473 expressions in prefrontal cortex tissues of REGγ+/+ and REGγ − / − mice. (d) Quantification of western blot in c, REGγ+/+ and REGγ − / − $n=4$ animals each. (e) Prepulse inhibition of both genotypes of mice before and after injection with GSK3 inhibitor SB216763 (5 mg/kg, i.p., 5 min before the test). Alterations in prepulse inhibition of REGγ − / − mice are significant at 77 dB and 81 dB, REGγ+/+ $n=23$, REGγ − / − $n=14$, SB = SB216763. (f) The eight arm maze was tested with (REGγ+/+ $n=8$, REGγ − / − $n=9$) or without (REGγ+/+ $n=13$, REGγ − / − $n=7$) chronic treatment of SB 216763 (1.2 mg/kg, i.p., once a day, for 1 week). All the quantification was presented as mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, NS, not significant.

controls ($P=0.005$), suggesting an age-dependent decline in ability to degrade endogenous proteins in REGγ − / − mice.

DISCUSSION

Here we have demonstrated that elderly REGγ − / − mice exhibit abnormal stereotypic, including increased spontaneous and stereotypic activities, impaired working memory, deficient prepulse inhibition and disability in nest-building, typical of schizophrenia-related phenotypes in various mouse models, while showing normal anxiety and depression behavior. Certainly, whether REGγ expression level is altered in schizophrenia patients remains unknown but may be worth further analysis.

Given that REGγ is a proteasome activator, loss of REGγ might alter some protein levels in the central nervous system, which could cause behavioral abnormalities. In this study, we show that GSK3β is an important target of REGγ in the prefrontal cortex of brain, and that the hyperactivity of GSK3β induced by REGγ deficiency, at least partially, contributes to the dysfunction in the central nervous system. The function of GSK3β in schizophrenia patients has been evaluated with inconsistent conclusions (Kozlovsky *et al*, 2001). There might be several reasons, including that few brains from schizophrenic patients are available and samples

from postmortem usually have been treated with multiple drugs, including those known to regulate AKT/GSK3*β* and/or Wnt pathways (Freyberg *et al*, 2010). Indeed, results from animal models support the hyperactivity of GSK3*β* in the pathogenesis of schizophrenia. For example, in an infection-based mouse model (also a commonly studied schizophrenia

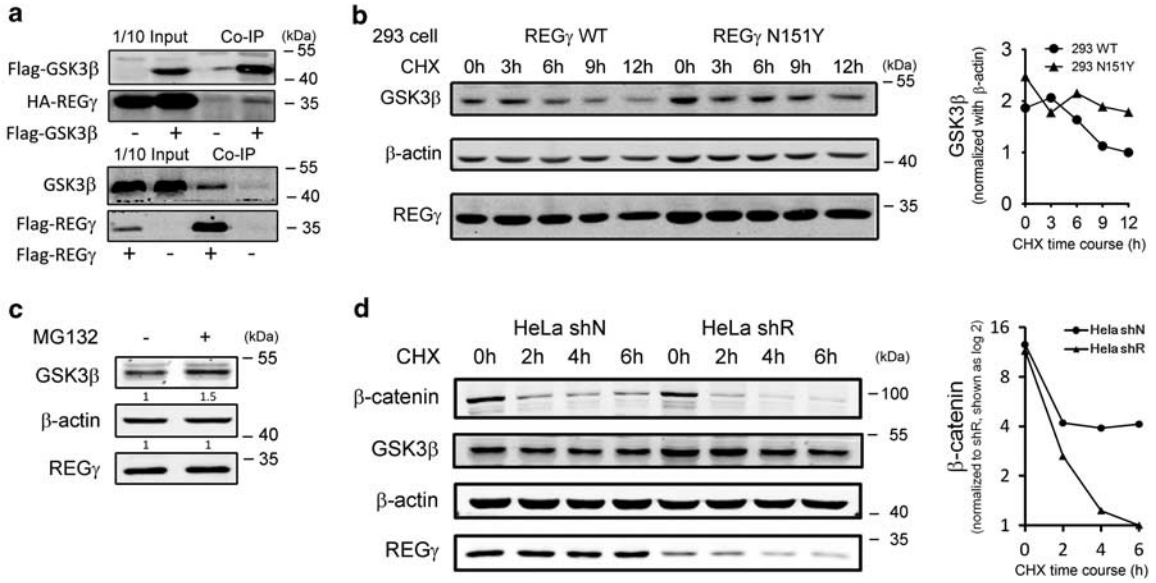


Figure 4 REGγ regulates GSK3β activity via ubiquitin-independent degradation. (a) Co-immunoprecipitation of HA-REGγ by Flag-GSK3β (upper panel) and co-immunoprecipitation of GSK3β by Flag-REGγ (lower panel). (b) Degradation dynamics of GSK3β following a time course CHX treatment in 293 inducible REGγ wild-type and REGγ N151Y-mutant cell lines (CHX, protein synthesis inhibitor, 100 μg/ml). Quantification of GSK3β degradation was normalized with β-actin. REGγ WT, wild-type REGγ; REGγ N151Y, N151Y site-mutant REGγ. (c) GSK3β proteins were stabilized in the presence of MG132 (20 μM), a proteasome inhibitor, in SH-SY5Y cells. (d) Degradation dynamics of GSK3β following a time course CHX treatment in HeLa REGγ shR and shN cell lines (CHX, 100 μg/ml). Quantification of GSK3β degradation was normalized with β-actin and presented as fold changes relative to the expression in HeLa shR cell line in log 2 form.

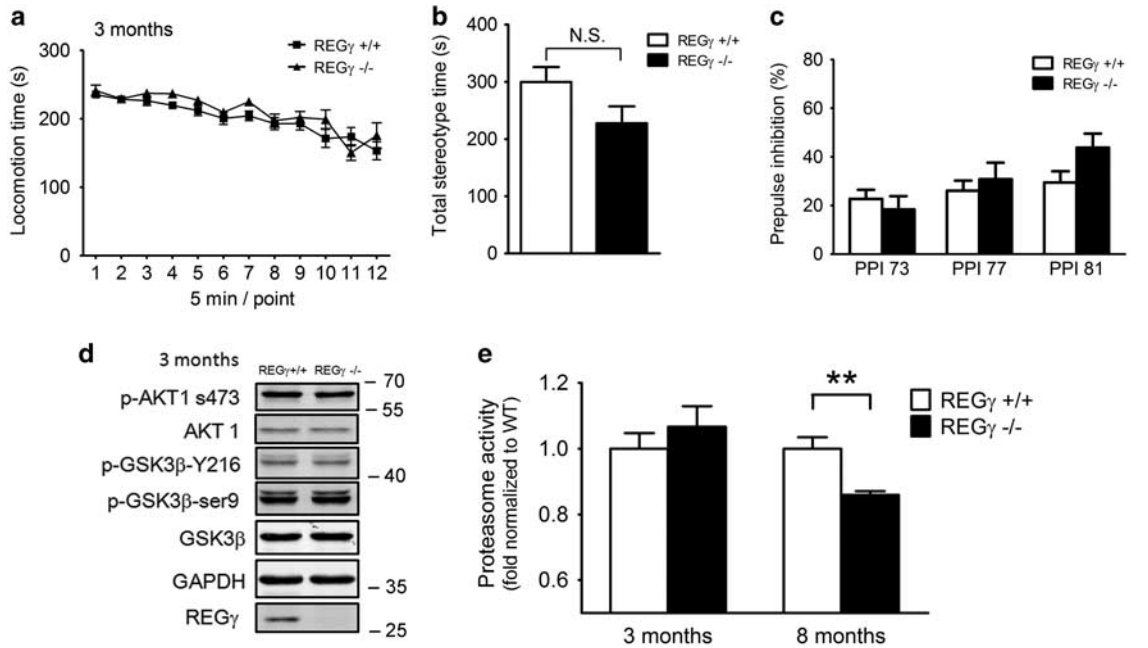


Figure 5 Young REGγ −/− mice show normal behavior and unchanged GSK3β level. (a) Locomotor activity of 3-month-old REGγ+/+ and REGγ −/− mice was measured by open-field test, REGγ+/+ $n=15$, REGγ −/− $n=11$. (b) Stereotype behavior of 3-month-old REGγ+/+ and REGγ −/− mice was measured within 60 min, REGγ+/+ $n=15$, REGγ −/− $n=11$. (c) Prepulse inhibition at 73 dB, 77 dB, and 81 dB of both genotypes in 3 months were measured, REGγ+/+ $n=12$, REGγ −/− $n=11$. (d) Representative immunoblots of indicated proteins in prefrontal cortex tissues from 3-month-old mice. (e) Trypsin-like proteasomal activities in prefrontal cortex tissues from REGγ+/+ and REGγ −/− mice of 3 months and 8 months were measured and presented as fold changes relative to the levels in wild-type mice, 3 months REGγ+/+ and REGγ −/− $n=5$ animals each, 8 months REGγ+/+ and REGγ −/− $n=5$ animals each. All the quantification was presented as mean ± SEM, $^{**}P<0.01$; NS, not significant.

model), an increase in GSK3β protein level and a decreased ratio of p-GSK3β-ser9/GSK3β were found (Willi *et al*, 2013), reminiscent of findings in the REG$\gamma-/-$ mice. Our finding also reveals that the GSK3β protein level could be regulated by the REGγ-mediated proteasome system.

Consistent with the behavioral phenotypes, the GSK3β expression level has no change in the prefrontal cortex from young adult REG$\gamma-/-$ mice. Our work has shown a temporal link between the biochemical and behavioral alterations in REGγ-deficient mice at the ages of 3 and 8 months. The REGγ-dependent activation of trypsin-like activity in the 20S proteasome assay may provide some clues. Despite REGγ depletion, we found no differences in trypsin-like activity between the knockout and the wild-type mice at 3 months of age, suggesting a possible compensation by the ubiquitin-dependent pathway or an age-associated change in REGγ trypsin-like activity. A significantly lower trypsin-like activity occurs in the elderly REG$\gamma-/-$ mice, indicating that an age-dependent decline in proteasome activities correlates with the loss of trypsin-like activity with REGγ depletion. Therefore, when the mice are young, GSK3β activity is maintained at a normal level through compensation by the 20S proteasome system. When the REG$\gamma-/-$ mice are 8 months or older, accumulation of pro-aging factors, environmental stresses, and a decrease in general proteasome activity disrupt the trypsin-like activity of the 20S proteasome, leading to abnormal accumulation of GSK3β protein and a cascade of pathogenesis reflected in altered neuronal function. By this model, active GSK3β accumulates in an age-dependent manner, and leads to a series of aberrant behavioral phenotype in elderly REG$\gamma-/-$ mice.

Taken together, we show that the proteasome activator REGγ regulates GSK3β degradation, and with high GSK3β activity, REG$\gamma-/-$ mice exhibit late-onset hyperactivity, sensorimotor gating deficiency, and working memory disorders. This suggests that the REGγ-mediated proteasome system has an important role in maintaining normal function of the nervous system in adults and REGγ-proteasome dysfunction could contribute to the development of late-onset aberrant brain behaviors.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)