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ORIGINAL ARTICLE

Reversal of reduced parvalbumin neurons in hippocampus and amygdala of Angelman syndrome model mice by chronic treatment of fluoxetine

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Abstract

Angelman syndrome (AS) is a neuropsychiatric disorder characterized by autism, intellectual disability and motor disturbances. The disease is primarily caused by the loss of function of maternally inherited *UBE3A*. *Ube3a* maternal-deficient mice recapitulates many essential feature of AS. These AS mice have been shown to be under chronic stress and exhibits anxiety-like behaviour because of defective glucocorticoid receptor signalling. Here, we demonstrate that chronic stress in these mice could lead to down-regulation of parvalbumin-positive interneurons in the hippocampus and basolateral amygdala from early post-natal days. Down-regulation of parvalbumin-positive interneurons number could be because of decrease in the expression of parvalbumin in these neurons. We also find that treatment with fluoxetine, a selective serotonin reuptake inhibitor, results in restoration of impaired glucocorticoid signalling, elevated serum corticosterone level, parvalbumin-positive interneurons and anxiety-like behaviours. Our findings suggest that impaired glucocorticod signalling in hippocampus and amygdala of AS mice is critical for the decrease in parvalbumin interneurons number, emergence of anxiety and other behavioural deficits and highlights the importance of fluoxetine in the recovery of these abnormalities.

Keywords: angelman syndrome, anxiety, chronic stress, fluoxetine, parvalbumin.

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Angelman syndrome (AS) is a neurodevelopmental disorder characterized by severe intellectual disability, ataxia, epilepsy and autistic features (Williams *et al.* 2010; Jana 2012). In most cases, AS is caused by the deletion of the maternal copy of a locus in chromosome 15 (15q11-13), which includes the *UBE3A* gene (Williams *et al.* 2010). Mutations in the *UBE3A* gene are also reported in a subset of AS patients (Kishino *et al.* 1997). The expression of *UBE3A* is imprinted in the brain, with preferential maternal-specific expression (Albrecht *et al.* 1997; Yamasaki *et al.* 2003). *Ube3a* (also known as E6-associated protein) not only function as an ubiquitin ligase but also acts as a transcriptional coactivator for steroid hormone receptors (Huibregtse *et al.* 1995; Nawaz *et al.* 1999). Maternal *Ube3a* knockout mice (AS mice) recapitulates many features of AS including learning and memory impairment and motor dysfunction (Jiang *et al.* 1998; Heck *et al.* 2008). *Ube3a* has been demonstrated to play a crucial role in regulating synaptic function and loss of function of *Ube3a* in AS mice leads to defect in experience-dependent synaptic plasticity (Greer *et al.* 2010; Jana 2012). AS mice also show elevated stress and anxiety-like behaviour, which has been linked to disrupted glucocorticoid receptor (GR) signalling (Godavarthi *et al.* 2012). Anxiety-like behavioural features have also been documented in AS patients during their adulthood (Smith 2001).

Stress-induced changes are not only seen in the principal neurons but also in the GABAergic system. Chronic stress and chronic glucocorticoid treatment modulate expression of GAD67 and GABA-A receptor, in stress-sensitive brain structures (Orchinik *et al.* 2001). Chronic stress and resulting anxiety are known to reduce the number of parvalbumin (PV)-immunoreactive interneurons, a subtype of GABAergic neurons (Czeh *et al.* 2005). In our previous study we

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Abbreviation used: AS, angelman syndrome; BLA, basolateral amygdala; CeA, central amygdala; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; PV, parvalbumin.

reported reduced PV-positive interneurons in the hippocampus of adult AS mice (Godavarthi *et al.* 2012). PV-positive interneurons are essential for hippocampal long-term-potentiation and contextual fear conditioning, deficits that have been reported in AS mice model (Jiang *et al.* 1998; Chen *et al.* 2010). PV interneurons not only provide perisomatic inhibition to the pyramidal cell and modulate their output timing, but have also been shown to play an important role in the sensitive period during development (Hensch 2004). This prompted us to examine whether the changes in PV-positive interneurons and GR expression in AS mice are developmental in nature. In addition, we examined if pharmacological intervention of serotonergic system by fluoxetine (a selective serotonin reuptake inhibitor, SSRI) could attenuate the molecular and behavioural deficit displayed by AS mice. Our study show that the loss of *Ube3a* causes down-regulation in the expression of PV and GR from early post-natal days in stress-sensitive brain structures of AS mice. Chronic treatment of fluoxetine partially reverse the defect in GR signalling and reduced number PV-positive interneurons in the hippocampus and basolateral amygdala (BLA) along with recovery of anxiety-like behaviour in AS mice.

Materials and methods

Materials

TRIzol reagent, fluoxetine hydrochloride, mouse monoclonal anti-parvalbumin, anti-calbindin and anti-β-actin antibodies were purchased from Sigma (Bangalore, India). Rabbit polyclonal anti-*Ube3a*, anti-GR and anti-brain derived neurotrophic factor (BDNF) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse monoclonal anti-glutamic acid decarboxylase (GAD) was procured from Millipore (Bangalore, India) and rabbit polyclonal anti-SGK1 was from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti-FK506 binding protein 5 (FKBP5) was purchased from Cell Signaling Technology. Anti-rabbit immunoglobulin G fluorescein isothiocyanate, AP-conjugated anti-rabbit and anti-mouse IgG, biotinylated anti-mouse IgG, horseradish peroxidase-conjugated anti-mouse IgG, Vectastain Elite ABC kit (Burlingame, CA, USA) reagent and Novared staining kit were purchased from Vector Laboratories (Barlingame, CA, USA). Alexa Fluor 594 was purchased from Molecular Probes. One-step Superscript III Reverse Transcriptase kit was procured from Invitrogen (Carlsbad, CA, USA). iQ SYBR Green Super Mix for quantitative real-time PCR was purchased from Bio-Rad Laboratories, Hercules, CA, USA.

Animals, experiment design and injection

Ube3a heterozygous mice were obtained from Jackson Laboratory (Jackson code: 129-*Ube3a*tm1Alb/J) and maintained in C57BL/6 background. Animals were maintained on a 12-h light/dark cycle, with food pellets and water *ad libitum*. All animal experiments were conducted in accordance with the approval of the Institutional Animal Ethics Committee of National Brain Research Centre. Genotyping was carried out using PCR as described previously (Jiang *et al.* 1998). Crosses of mice were set up to get *Ube3a*-maternal (AS mice) or wild-type mice as shown earlier (Jiang *et al.* 1998). Male mice belonging to age groups P5, P10, P20 and P120 were used for experiments.

Male wild-type and AS mice of 90 days old (eight animals per genotype) were injected fluoxetine subcutaneously (10 μg/gm body weight) for 24 days. Control male mice received subcutaneous saline injections. Animals were subjected to behavioural analysis after 21 days of injection. Following completion of behavioural tests, mice were either killed by cervical dislocation and brain parts were stored at −80°C or anesthetized and transcardially perfused with phosphate buffered saline containing 4% paraformaldehyde (w/v).

Immunoblot analysis

Cortex and hippocampi from both hemispheres were removed into ice-cold phosphate buffered saline and immediately snap frozen into liquid nitrogen. Frozen samples were homogenized in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and complete protease inhibitor cocktail) and were kept on ice for 30 min. Lysed protein samples were centrifuged at 15 000 *g* for 10 min and supernatant was used for immunoblotting after protein estimation as described previously (Rao *et al.* 2010a). The primary antibodies and their dilutions used for the study were as follows: *Ube3a*, 1 : 1000; GR, 1 : 500; SGK1, 1 : 500; BDNF, 1 : 500; Fkbp5, 1 : 1000; GAD, 1 : 5000 and β-actin, 1 : 10 000.

Immunohistochemistry and cell counting

Paraformaldehyde-fixed brains were processed for cryosectioning to obtain 20-μm-thick sections. Sections were then processed for diaminobenzidine (DAB) immunohistochemical staining using reagents from Vector Laboratories as described earlier (Rao *et al.* 2010b). PV, GAD and calbindin antibodies were used at 1 : 5000, 1 : 1000 and 1 : 5000 dilutions respectively. Biotinylated secondary antibody was used at a dilution of 1 : 500 and signal was enhanced using ABC kit and developed using ImmPACT Novared peroxidase substrate (Barlingame, CA, USA). Stained sections were imaged using bright-field microscopy on a Leica DM RXA2 microscope (Wetzlan, Germany). For immunofluorescence staining, secondary antibodies conjugated either with AF-594 or AF-488 was used at 1 : 2000 dilutions and sections were counter-stained by 4′,6-diamidino-2-phenylindole (DAPI) and observed using a fluorescence microscope (Apotome, Zeiss, Jena, Germany). Primary antibodies against *Ube3a*, serum and glucocorticoid inducible kinase (SGK) and GR were used at 1 : 1000, 1 : 500 and 1 : 200 dilutions respectively.

Quantification of PV- and calbindin-positive cells was conducted from one in seven series of diaminobenzidine-stained sections using Neurolucida program (MicroBrightField version 7.0; MBF Bioscience, Williston, VT, USA). Detailed procedure is described in the Supporting Information.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent. Quantitative real-time PCR was carried out using iQ SYBR Green Super Mix after cDNA synthesis from total RNA using an ABI Prism 7500 system (Foster city, CA, USA) as described in Supporting Information.

Behavioural tests

Light/dark box test, open-field test and novel object recognition test were performed for assessing anxiety as described earlier (Godavarthi *et al.* 2012). Details of the test are described in the Supporting Information.

Corticosterone assay

Morning basal corticosterone levels (collected between 10 and 11 am) were measured in wild-type and AS mice that were left undisturbed overnight. Mice were killed by decapitation, trunk blood was collected and serum was separated and stored at −80°C. Serum corticosterone level was measured using enzyme-linked immunoassay kit (Cayman, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using the SigmaStat software (version 2.03; Systat Software Inc., San Jose, CA, USA). Values were expressed as mean ± SD. Two-tailed Student's *t*-test was used for intergroup comparison. One-way ANOVA was used for analysing data with more than two groups. For fluoxetine study, intergroup comparisons were performed by using two-way ANOVA. Student–Newman–Keuls test was used as the *post hoc* test. $p < 0.05$ was considered statistically significant.

Results

Spatial and temporal characterization of PV-immunoreactive neurons in AS mice

We have earlier reported that AS mice are under chronic stress and exhibit heightened anxiety. These adult mice also show decreased number of PV-positive interneurons in their hippocampus (Godavarthi *et al.* 2012). We now examined hippocampal PV expression in AS mice along with wild-type controls at P10, P20 and P120 (Fig. 1a). PV expression can be detected from post-natal day 8 (P8) onwards in the rodent hippocampus (Kawaguchi *et al.* 1987). Stereological analysis revealed a significant reduction in the number of PV-immunoreactive interneurons in hippocampal CA3 and dentate gyrus subfields of AS mice compared to control at P10. AS mice exhibited significant reduction in PV-positive interneurons across all area of hippocampus at P20 and P120 (Fig. 1).

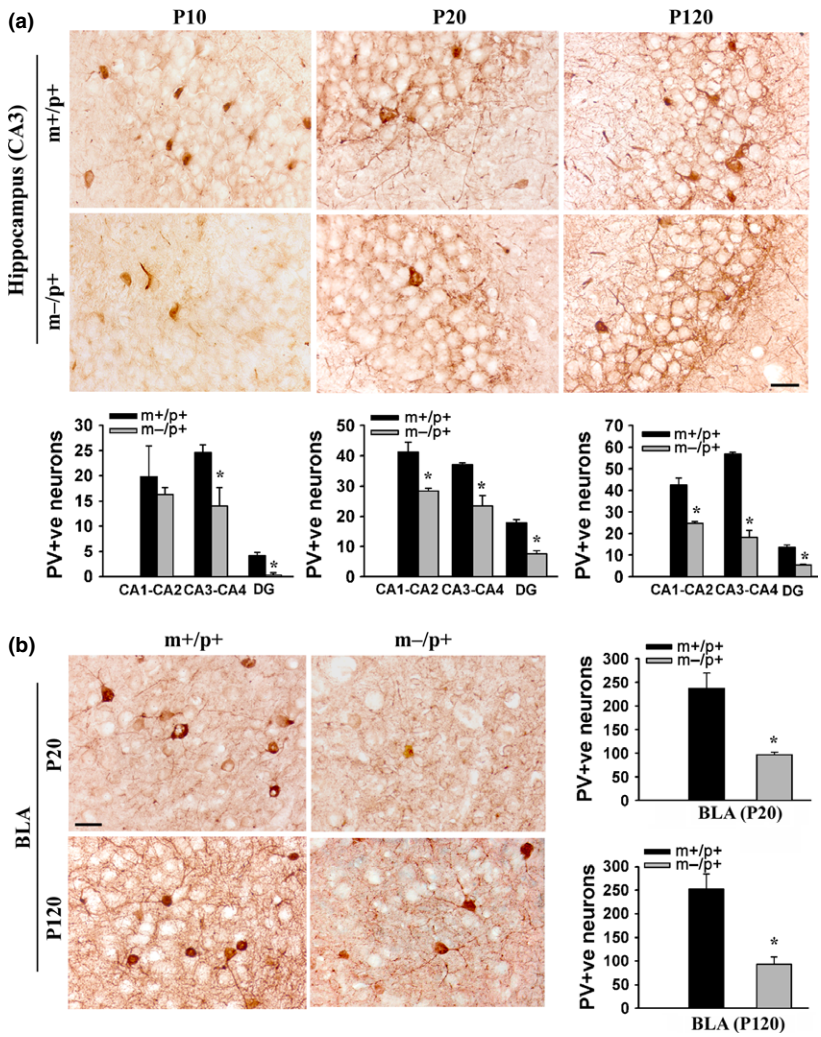

Fig. 1 Down-regulation of parvalbumin (PV)-immunoreactive interneurons number in the hippocampus and basolateral amygdala (BLA) of Angelman syndrome (AS) mice. (a) Comparison of immunohistochemical staining of (PV) in CA3 region of hippocampus of male wild-type (m+/p+) and AS (m−/p+) mice across age group (P10, P20 and P120). Brain sections of 20 μm thickness collected from wild-type and AS mice of different age were placed on the same slide and processed for staining. Bottom panel, bar diagram summarizing numbers of PV-positive cells across CA1-CA2, CA3-CA4 and dentate gyrus regions of the hippocampus at P10, P20 and P120. PV-positive neurons were counted stereologically and expressed as numbers/mm^3 as described in the methods. (b) Comparison of immunohistochemical staining of PV in the BLA of wild-type and AS mice. To the right, PV-stained cell bodies in BLA were stereologically counted and plotted as counts/mm^3. Values are Mean ± SD; $n = 3$ for P10 and P20 and $n = 6$ for P120. *$p < 0.003$ in comparison with wild-type mice. Scale bar, 30 μm.

Amygdala is another important limbic structure involved in modulating stress and anxiety and PV-positive interneurons expression is detected in rodent amygdala from P17 onwards (Berdel and Morys 2000). Therefore, PV immunostaining was performed in the BLA of wild-type and AS mice at P20 and P120. (Fig. 1b). Stereological analysis revealed a significant reduction in number of PV-positive interneurons in the BLA at both P20 and P120 of AS mice with respect to wild-type control (Fig. 1b). We also assessed PV-positive interneurons number in two cortical areas, piriform and perirhinal cortices, which receive input from and project to the limbic regions of the brain. Figure 2a and 2b showed the PV immunostaining in the perirhinal and piriform cortices of wild-type and AS mice respectively. We did not find any

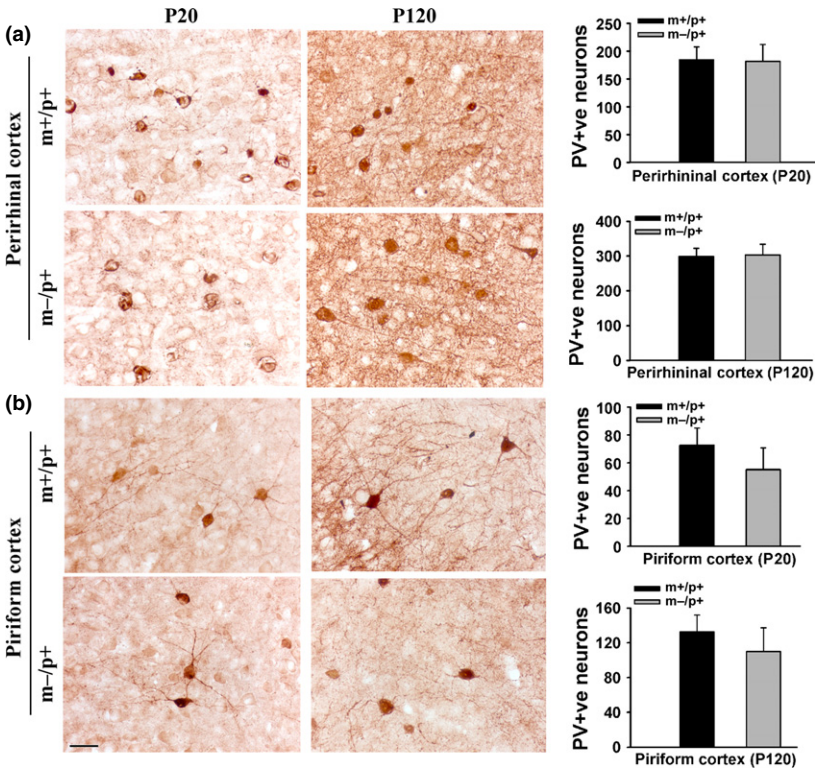

Fig. 2 The number of parvalbumin (PV)-positive interneurons is not altered in perirhinal and piriform cortex of Angelman syndrome (AS) mice. (a, b) Representative immunostaining of PV in perirhinal (a) and piriform cortex (b) of male wild-type (m+/p+) and AS (m−/p+) mice at P20 and P120. To the right, bar graph summarizing the number of PV-positive cells (counted stereologically and expressed as cells/mm^3) in perirhinal (a) and piriform cortex (b) at P20 and P120. Values are Mean ± SD; $n = 3$ for P20 and $n = 6$ for P120. Scale bar, 30 μm.

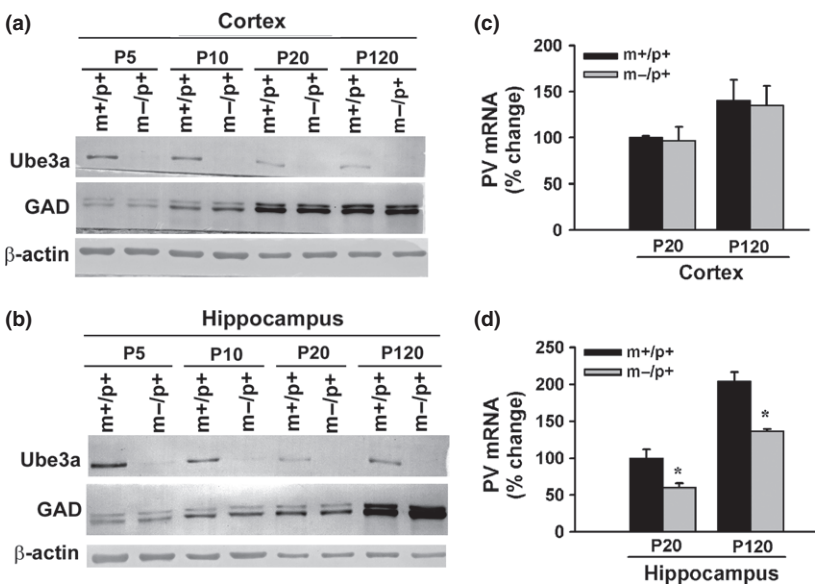

Fig. 3 Hippocampus of Angelman syndrome (AS) mice exhibits decreased expression of parvalbumin (PV) with unaltered GAD level. (a, b) Representative immunoblots comparing age-dependent expression pattern of *Ube3a* and GAD in the cortex (a) and hippocampus (b) of wild-type and AS mice. (c, d) Quantitative real-time PCR for PV mRNA in the cortex and hippocampus of wild-type and AS mice at P20 and P120. Values are Mean ± SD; $n = 3$. *$p < 0.001$ in comparison with wild-type mice.

difference in PV-positive interneurons number in either of the cortical regions between the wild-type and AS mice at either P20 or P120. The expression of calbindin, another class of inhibitory interneurons was unaffected in both hippocampus and BLA of AS mice in compared with wild-type controls across age groups (Figure S1).

Reduced expression of PV in the hippocampus of AS mice

Next, we attempted to check whether there is reduction in number of GABAergic neurons in general, or a specific phenotype of the GABAergic interneurons altered in AS mice. To address this problem, we first checked GAD expression (a general marker for GABAergic interneurons) between

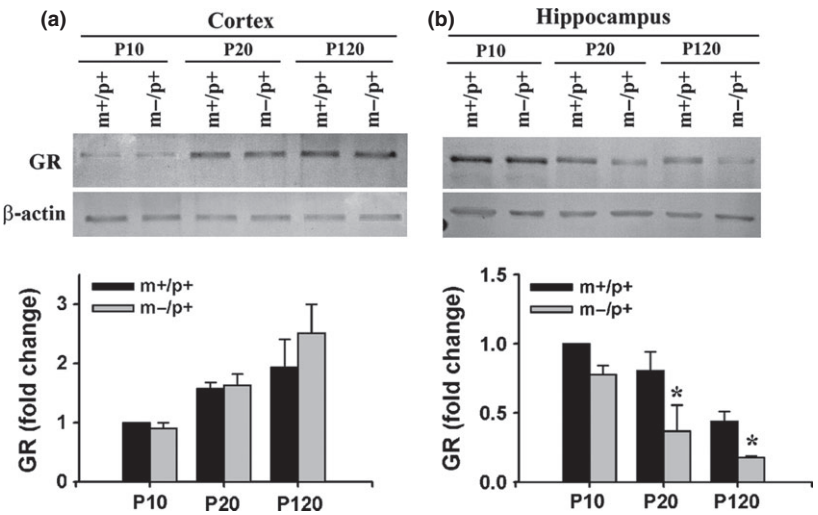

Fig. 4 Down-regulation of glucocorticoid receptor (GR) level in the hippocampus of Angelman syndrome (AS) mice from postnatal day 20. (a, b) Representative immunoblots comparing GR expression in cortex (a) and hippocampus (b) between wild-type and AS mice at P10, P20 and P120. Band intensities of GR in cortex (a, bottom panel) and hippocampus (b, bottom panel) were quantified using NIH Image analysis software and normalized to β-actin. Values are shown as Mean ± SD. $n = 3$ in each group. $^{*}p < 0.05$ in comparison with wild-type mice.

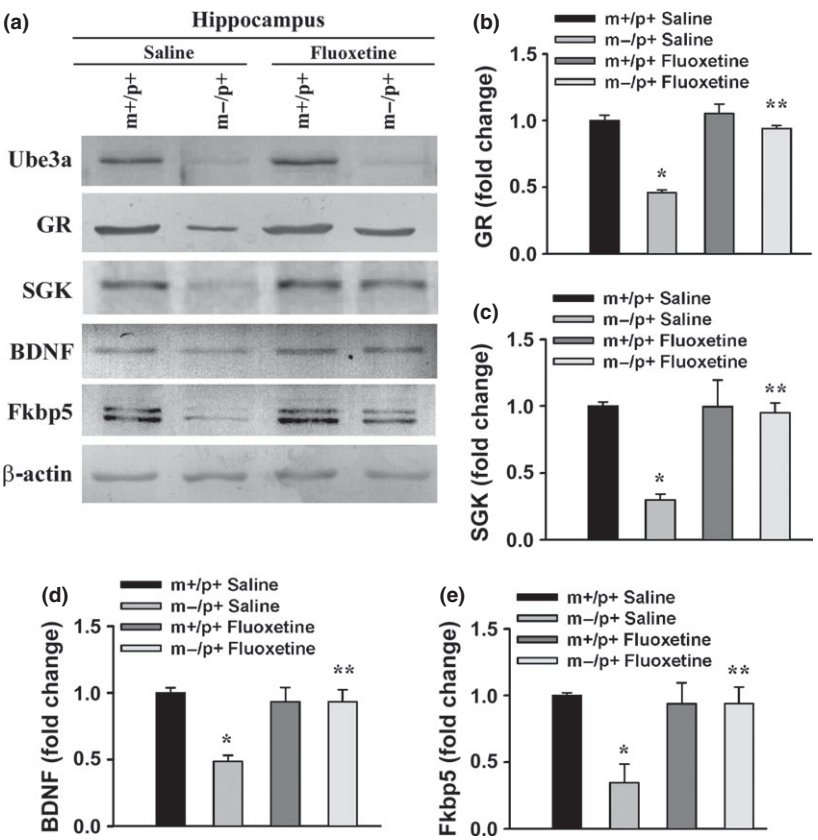

Fig. 5 Chronic treatment with fluoxetine restores the level of glucocorticoid receptor (GR) and its effectors in the hippocampus of Angelman syndrome (AS) mice. (a) Representative immunoblots of *Ube3a*, GR, SGK1, BDNF and FKBP5 in cortex and hippocampus of saline and fluoxetine treated wild-type (m+/p+) and AS (m−/p+) mice. Four-month-old male mice were used for the experiment and fluoxetine injection was given for 24 days. Band intensities of GR, SGK1, BDNF and FKBP5 were quantified using NIH Image analysis software, normalized to β-actin and shown as Mean ± SD; $n = 4$. $^{*}p < 0.001$ in comparison with wild-type mice, while $^{**}p < 0.001$ in comparison with saline-treated AS mice.

wild-type and AS mice. In the cortex, there was no difference in GAD expression across age groups between wild-type and AS mice, as determined by immunoblotting (Fig. 3a). Quantitative RT-PCR showed unaltered PV mRNA levels between wild-type and AS mice cortices at P20 and P120 (Fig. 3c). GAD levels were also unaltered the hippocampus and amygdala between wild-type and AS mice (Fig. 3b and Figure S2). However, PV mRNA level showed ~40% reduction at P20 and ~30% reduction at P120 in AS mice hippocampus in comparison with the wild-type mice (Fig. 3d). This indicates alteration in phenotypic expression of PV among GABAergic interneurons in the hippocampus of AS mice.

Age-dependent changes of GR expression in the hippocampus of AS mice

We have earlier reported impaired expression of GR in the hippocampus of adult AS mice. Having observed age-dependent alteration in PV expression in AS mice, we wanted to examine if change of GR expression in AS mice was also age dependent. In the cortex, wild-type and AS mice showed increasing level of GR from P10 to P120 with least expression at P10. There was no difference in GR level between wild-type and AS mice across age groups (Fig. 4a). In the hippocampus, GR level was highest at P10 and reduced at P20 and P120 in wild-type mice. However, in AS mice, the reduction of GR expression was much more dramatic at P20 and P120 in comparison to wild-type controls (Fig. 4b). This indicates that GR expression is progressively disrupted with age in AS mice beginning at P10.

Fluoxetine treatments partially restore GR signalling and decreased PV-positive interneurons in the hippocampus and BLA of AS mice

We next wanted to test if interventions using anxiolytics can reverse the impaired GR signalling and chronic stress in AS mice. We injected fluoxetine to adult AS mice along with

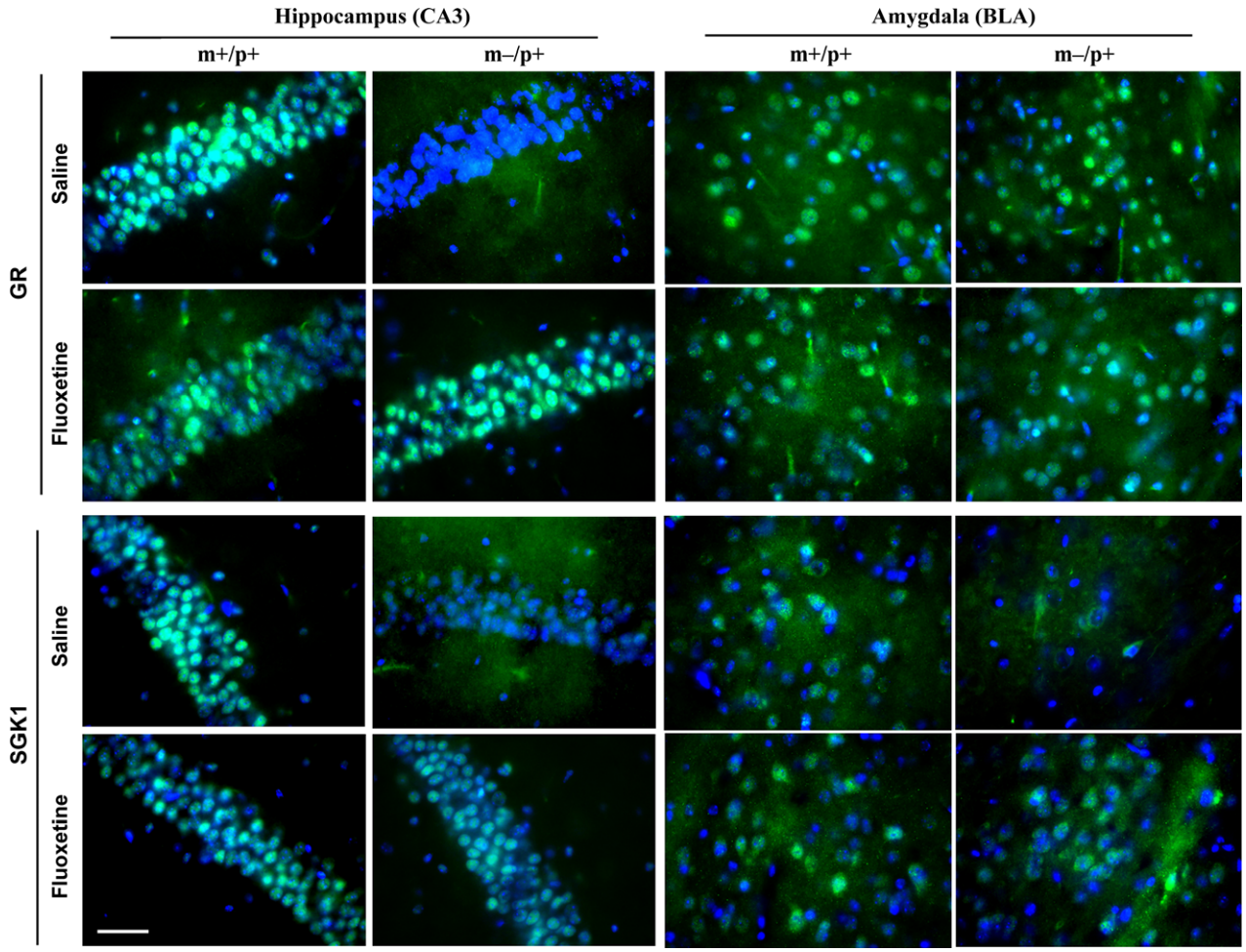

Fig. 6 Fluoxetine treatment reversed the down-regulated glucocorticoid receptor (GR) and its regulatory gene SGK1. Representative immunofluorescence staining of GR and SGK1 in the hippocampus and basolateral amygdala (BLA) of saline and fluoxetine treated wild-type and Angelman syndrome (AS) mice. FITC-conjugated secondary antibody was used to detect GR and SGK1. Nuclei were counter-stained with DAPI. Scale bar, 30 μm.

age-matched wild-type controls for 24 days. Figure 5 showed that chronic treatment of fluoxetine partially restored the impaired expression of GR as well as its downstream target proteins like SGK1, BDNF and FKBP5 in the hippocampus of AS mice. Partial rescue of GR and SGK1 levels in the hippocampus of AS mice was reconfirmed by immunostaining (Fig. 6 and Figure S3). In the BLA of AS mice, GR level was unaffected, while SGK1 level was reduced and this could be because of loss of coactivator function of *Ube3a*. Interestingly, chronic treatment of fluoxetine up-regulated the level of SGK1 in the BLA of AS mice (Fig. 6). Fluoxetine treatment did not have any effect on the expression of *Ube3a* in AS mice brain. PV immunostaining revealed that fluoxetine treatment restored the decreased number PV-positive neurons in both hippocampus (Fig. 7a and Figure S4) and BLA (Fig. 7b) of AS mice.

Long-term fluoxetine treatment normalizes serum corticosterone level and rescues anxiety-like behaviour in AS mice

Since fluoxetine treatment restored GR signalling in the hippocampus, we further examined its effect on serum corticosterone level in AS mice. As shown in Figure 8, morning basal serum corticosterone level was significantly higher in AS mice compared with wild-type control. Chronic treatment of fluoxetine normalized the elevated serum corticosterone level in AS mice to almost wild-type level (Fig. 8a). Since high corticosterone level for prolonged period leads to cognitive dysfunction and anxiety, we conducted battery of tests mainly related to anxiety. In novel object exploration test, AS mice exhibited significantly lower preference for the novel object compared to wild-type animals. Fluoxetine treatment partially improved the novel object preference of AS mice, but data were not statistically significant (Fig. 8b). In the light-dark box test, AS mice spent significantly more time in the dark compartment compared to wild type, which was significantly reversed by fluoxetine (Fig. 8c). In open-field test, AS mice spent significantly more time in outer zone and showed increased frequency of freezing in comparison with wild-type controls, indicating heightened anxiety in these mice (Fig. 9). This anxiety-like behaviour in open field was corrected upon fluoxetine treatment (Fig. 9). Thus, fluoxetine treatment could potentially reduce anxiety in AS mice by restoring GR signalling as well as PV-positive neurons in hippocampus and BLA.

Discussion

In this study, we first demonstrate that the number of PV interneurons was significantly reduced in the hippocampus and BLA of AS mice from the time PV expression is first seen in these regions. However, we have not found any changes in PV interneurons number in piriform and perirhinal cortices and similar findings was recently reported in the visual cortex of AS mice (Wallace *et al.* 2012). The reduction is also specific to PV as calbindin-positive interneurons number was unchanged in these brain regions of AS mice. These results indicate that the PV subpopulation of interneurons may be selectively vulnerable to stress in stress-sensitive brain regions. Interestingly, GAD65/67 levels were unaltered in the hippocampus between the wild-type and AS mice. GABAergic interneurons form 11% of the total population of neurons in hippocampus, of which PV-immunoreactive interneurons form 32–38% (Ribak *et al.*

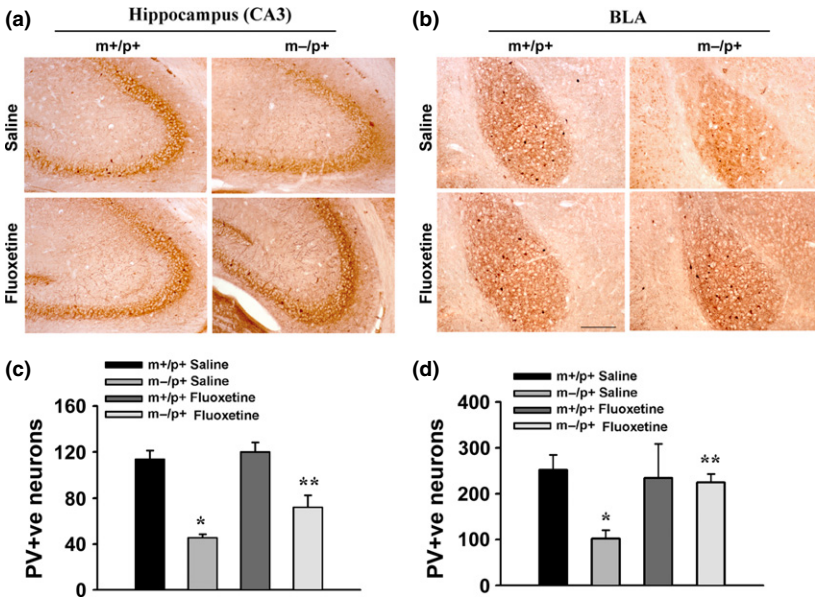

Fig. 7 Rescue of down-regulated parvalbumin (PV)-positive interneurons in the hippocampus and basolateral amygdala (BLA) of Angelman syndrome (AS) mice. Representative immunostaining for PV in CA3 region of hippocampus (a) and BLA (b) of saline- and fluoxetine-treated wild-type and AS mice. Four-month-old male mice were used for the experiment and fluoxetine injection was given for 24 days. Scale bar, 100 μm. Bottom panel shows estimation of PV-positive neurons in hippocampus (c) and BLA (d). PV-positive cell bodies were stereologically counted and expressed as counts/mm^3 as described in the methodology section. Values are Mean ± SD; $n = 4$. *$p < 0.001$ in comparison with wild-type mice, while **$p < 0.01$ in comparison with saline-treated AS mice.

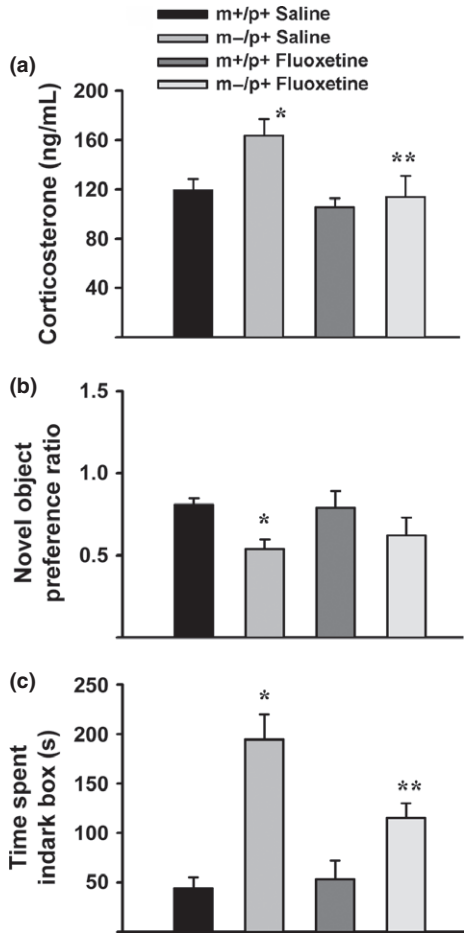

Fig. 8 Normalization of elevated serum corticosterone level and rescue of anxiety-like behaviour in Angelman syndrome (AS) mice upon chronic treatment of fluoxetine. (a) Basal morning blood corticosterone level in the saline and fluoxetine-treated wild-type and AS mice. Blood samples were collected (at 10–11 am) after 21 days of fluoxetine injection. (b) Novel object exploration test. (c) In light/dark box test, fluoxetine-treated AS mice spent less time in dark compartment compared to saline-treated AS mice group. Mice were treated with fluoxetine as described in Figure 5. Tests were conducted on 22 and 23 days of drug treatment. Values are mean ± SD; $n = 8$. *$p < 0.01$ in comparison with wild-type mice, while **$p < 0.01$ in comparison with saline-treated AS mice.

1990). This suggests that decrease in PV immunoreactivity might be because of altered expression of PV. In fact, we have detected a significant decrease in the expression of PV in the hippocampus of AS mice with respect to wild-type controls. PV being a calcium binding protein confers neuroprotective effect (Van Den Bosch *et al.* 2002) and its reduced level or absence could lead to alteration in calcium homeostasis and neuronal death, resulting in fewer PV-positive cell body counts as seen in AS mice.

Chronic stress decreases PV-positive interneuron and stress has been shown to increase hippocampal extracellular GABA levels in rat hippocampus (Czeh *et al.* 2005; de Groote and Linthorst 2007). Also, GR activation has been shown to increase hippocampal inhibitory postsynaptic currents indicating interaction between glucocorticoid signalling and GABAergic transmission (Maggio and Segal 2009). Therefore, a possible mechanism for PV reduction could be because of disrupted GR signalling observed in AS mice. The onset of GR expression precedes PV expression in hippocampus and BLA, however, PV expression precedes GR expression in the cortex (Alcantara *et al.* 1993; Rosenfeld *et al.* 1993; Navailles *et al.* 2010). This indirectly indicates that parvalbumin expression may be partially regulated by GR signalling in hippocampus and amygdala but not in the cortex. Another possible mechanism of PV down-regulation could be via the epidermal growth factor receptor (Erb4) that have been shown to increase GABA release from inhibitory interneurons by down-regulating PV level and its elevated level is shown in the hippocampus of AS mice (Fisahn *et al.* 2009; Kaphzan *et al.* 2012). Elevated GABA level has been reported in AS patients (Ebert *et al.* 1997) and blockade of inhibitory synaptic transmission by Erb inhibitor rescues long term potentiation impairment and contextual fear memory deficits in AS mice (Kaphzan *et al.* 2012). The increased inhibition in the hippocampus of the AS mice can in turn disrupt the hippocampal negative feedback onto hypothalamic–pituitary–adrenal (HPA) axis.

In the BLA, reduced PV expression can result in increased positive input of amygdala to the HPA axis. The intercalated amygdala neurons receive information from BLA and send feed-forward inhibition to the central amygdala (CeA) nucleus (Likhtik *et al.* 2008). Inhibition of the BLA neurons by reduced PV could reduce excitatory input to intercalated neurons, in turn reducing the inhibition on CeA. This would result in enhanced CeA activity and output to HPA axis, inducing fear and anxiety. Our observations are further supported by studies documenting deficits in contextual and cued fear conditioning resulting from the dysfunction of both hippocampus and amygdala in the AS mouse (Jiang *et al.* 1998; Baudry *et al.* 2012; Huang *et al.* 2013). The reduced PV expression in both BLA and hippocampus along with defective GR signalling could result in persistent hyperactivity of HPA axis resulting in increased glucocorticoid level in AS mice.

Because AS mice are under chronic stress and exhibit anxiety, we next attempted to correct these abnormalities by chronically treating the mice with fluoxetine. Fluoxetine and related SSRI are widely used in the treatment of anxiety and depression disorders and therapeutic benefit usually occurs after prolonged use (Fairbanks *et al.* 1997). In fact, acute treatment of various SSRI causes worsening of anxiety symptoms as evident from clinical reports and animals studies (Ravinder *et al.* 2011). We have noted that chronic fluoxetine treatment normalized serum corticosterone level

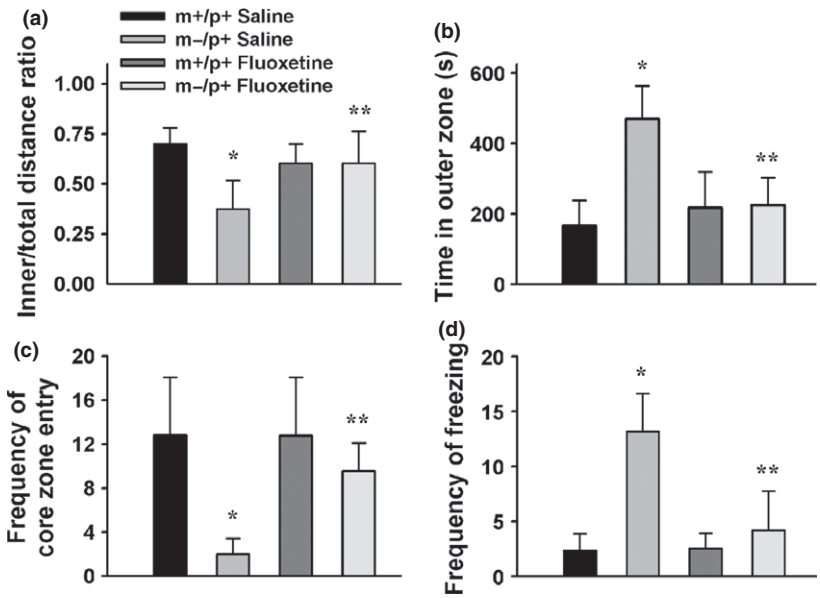

Fig. 9 Chronic treatment of fluoxetine rescues anxiety-like behaviour in Angelman syndrome (AS) mice in the open-field test. The ratio of inner to total distance travelled (a), time spent in outer zone (b), frequency of core zone entry (c) and frequency of freezing (d) were evaluated during 10 min in the open field. Mice were injected with fluoxetine as described in Figure 5. Values are mean ± SD; $n = 8$ in each group. $*p < 0.05$ in comparison with wild-type mice, while $**p < 0.05$ in comparison with saline-treated AS mice.

and corrected anxiety-like behaviour in AS mice. Fluoxetine treatment also partially rescued GR expression and GR signalling in the hippocampus. Most interestingly, fluoxetine treatment also rescued the number of PV-positive neurons in the BLA and hippocampus of AS mice. Prolonged treatment of fluoxetine is shown to alleviate chronic stress and anxiety by multiple ways including increasing GR expression and neurogenesis in the hippocampus (Pariante *et al.* 2003; Yau *et al.* 2004; Surget *et al.* 2011). Fluoxetine could also restore altered serotonin and other monoamines levels reported in fly and mice models of AS (Ferdousy *et al.* 2011; Farook *et al.* 2012). Rescue of GR signalling by fluoxetine might be one of the crucial mechanisms that lead to restoration of HPA axis and the recovery of anxiety-like behaviour in AS mice. Rescue of GR signalling could also reverse the stress-induced impairment in hippocampal long-term potentiation and cognitive deficits in AS mice. In fact, we have noted considerable improvement of short-term memory in novel object recognition test. How fluoxetine increases PV-positive neurons is not clear at present, but again it could be because of restoration of GR signalling or via other factors like BDNF (Patz 2004). Earlier reports have also demonstrated increase in PV-positive neurons in BLA following treatment with anxiolytics or exposure of enriched environment (Hale *et al.* 2010; Urakawa *et al.* 2013). It would be interesting to study how chronic stress or SSRI regulates PV expression in stress-sensitive brain regions.

In conclusion, our study provides evidence that down-regulation of PV in hippocampus and BLA along with altered GR signalling might be involved in the emergence of anxiety-like behaviour in AS mice. Treatment of fluoxetine normalized the GR signalling and PV expression and partially restored anxiety-like behaviour in these mice.

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Representative calbindin immunostaining in hippocampus (CA3), amygdala (BLA) and perirhinal cortex of male wild-type and AS mice.

Figure S2. Immunohistochemical staining of GAD in the cortex, hippocampus and BLA of wild-type and AS mice at P120.

Figure S3. Lower magnification images of GR and SGK immunostaining of entire hippocampus of saline and fluoxetine treated wild-type and AS mice.

Figure S4. Lower magnification images for PV immunostaining showing entire hippocampal region of saline- and fluoxetine-treated wild-type and AS mice.

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