PX-RICS-deficient mice mimic autism spectrum disorder in Jacobsen syndrome through impaired GABA\(_A\) receptor trafficking

Tsutomu Nakamura\(^1\), Fumiko Arima-Yoshida\(^2\), Fumika Sakaue\(^1\), Yukiko Nasu-Nishimura\(^1\), Yasuko Takeda\(^1\), Ken Matsuura\(^1\), Natacha Akshoomoff\(^3\), Sarah N. Mattson\(^4\), Paul D. Grossfeld\(^5\), Toshiya Manabe\(^2\) & Tetsu Akiyama\(^1\)

Jacobsen syndrome (JBS) is a rare congenital disorder caused by a terminal deletion of the long arm of chromosome 11. A subset of patients exhibit social behavioural problems that meet the diagnostic criteria for autism spectrum disorder (ASD); however, the underlying molecular pathogenesis remains poorly understood. PX-RICS is located in the chromosomal region commonly deleted in JBS patients with autistic-like behaviour. Here we report that PX-RICS-deficient mice exhibit ASD-like social behaviours and ASD-related comorbidities. PX-RICS-deficient neurons show reduced surface \(\gamma\)-aminobutyric acid type A receptor (GABA\(_A\)R) levels and impaired GABA\(_A\)R-mediated synaptic transmission. PX-RICS, GABARAP and 14-3-3z/\(\zeta\)/\(\theta\) form an adaptor complex that interconnects GABA\(_A\)R and dynein/dynactin, thereby facilitating GABA\(_A\)R surface expression. ASD-like behavioural abnormalities in PX-RICS-deficient mice are ameliorated by enhancing inhibitory synaptic transmission with a GABAAR agonist. Our findings demonstrate a critical role of PX-RICS in cognition and suggest a causal link between PX-RICS deletion and ASD-like behaviour in JBS patients.
Autism spectrum disorder (ASD) represents a group of aetio logically different neurodevelopmental conditions characterized by a triad of behavioural deficits: impairment in social interaction, difficulty with verbal and non-verbal communication and repetitive behaviour or restricted interests. ASD is a highly heritable condition, with estimated heritability indices of 80–90% (ref. 3). Several lines of evidence suggest that an imbalance between neuronal excitation and inhibition (E/I) within specific cortical circuits is a shared cellular mechanism for the behavioural and cognitive symptoms in several psychiatric diseases, including ASD. Several known ASD-causative genes encode neuron-associated proteins that are essential for the formation and maintenance of glutamatergic excitatory synapses. Recent studies have also indicated that impaired presynaptic release of y-aminobutyric acid (GABA), and the resulting dysfunction of GABA-mediated inhibitory signalling, has been associated with ASD. These findings suggest that a balance between glutamate and GABA signalling is essential for normal cognitive function.

ASD-like behaviour is also known to appear as one of the comorbid symptoms of several developmental syndromes such as fragile-X syndrome. Studying these syndromes will assist in better understanding the pathogenesis of non-syndromic ASD. Jacobsen syndrome (JBS; MIM 147791), also known as 11q deletion syndrome or partial 11q monosomy syndrome, is a rare congenital disorder caused by a deletion within the distal part of the long arm of chromosome 11 (ref. 14). The incidence of JBS has been estimated to be ~1 of every 100,000 births. JBS patients commonly suffer from a variety of pre- and post-natal developmental anomalies such as intellectual disability, characteristic facial dysmorphism, heart dysplasia and thrombocytopenia. Prospective genotype/phenotype studies have revealed several candidate genes that may be causative for each symptom of JBS. Recently, Akshoomoff et al. have presented evidence that approximately half of JBS patients exhibit behavioural problems that meet the diagnostic criteria for ASD, and identified four annotated genes localized within a tiny (0.24 Mb) interstitial deletion region on 11q24.3 as candidate genes responsible for ASD-like behaviour in JBS. They have concluded that PX-RICS is the most promising candidate gene based on its predominant expression in the brain and its critical roles in dendritic spine morphology and axon elongation.

PX-RICS (ARHGAP32 isoform 1), a longer splicing isoform of RICS (ARHGAP32 isoform 2), is a GTPase-activating protein (GAP) for Cdc42, containing multiple domains for protein–protein interactions. RICS is expressed exclusively in the brain in humans and regulates N-methyl-D-aspartate (NMDA) receptor-mediated signalling at the postsynaptic density and axonal elongation at the growth cone. In contrast, PX-RICS is expressed in relatively wide range of tissues but is predominantly expressed in the brain. PX-RICS has a unique phospholipid-binding domain not shared by RICS: the Phox-homology (PX) domain that mediates its interaction with phosphatidylinositol-4-phosphate (PI4P). Recently, we have shown that PX-RICS interacts with GABA type A receptor (GABAAR)-associated protein (GABARAP) and 14-3-3ε/0 to facilitate trafficking of the N-cadherin/β-catenin cell adhesion complex in non-neuronal cells.

In this study, we investigated the molecular mechanisms underlying the autistic-like behaviour of PX-RICS-deficient mice. Our results demonstrate an essential role for PX-RICS in normal cognitive function and suggest that PX-RICS deficiency contributes to the behavioural deficits in JBS patients.

Results

PX-RICS-deficient mice exhibit ASD-like social behaviour. JBS is a haploinsufficiency disorder in which most cases carry a de novo terminal deletion of one chromosome 11q (ref. 14). We thus included PX-RICS+/− mice in addition to PX-RICS+/+ and PX-RICS−/− mice in all behavioural assays. We first examined voluntary social interaction using the three-chamber test (Supplementary Fig. 1a). In the empty–empty (habituation) session, no significant differences were observed between the time spent exploring both wired cages (Fig. 1a,b) and between the time stay in left and right chambers (Supplementary Fig. 1b,c) in all genotypes. When a stranger mouse was placed in one wired cage (stranger–empty session), every genotype had a similar preference for the stranger-containing cage or chamber (Fig. 1a,b and Supplementary Fig. 1b,c), suggesting that PX-RICS+/− mice have normal voluntary sociability. In the subsequent stranger– stranger session in which a novel stranger mouse was placed in the previously empty wired cage, PX-RICS+/+ mice spent more time with the novel stranger mouse (Fig. 1a,b and Supplementary Fig. 1b,c). In contrast, PX-RICS−/− mice did not show such a preference for social novelty and instead showed a tendency to spend more time with a familiar stranger mouse (Fig. 1a,b and Supplementary Fig. 1b,c). PX-RICS+/+ mice exhibited an intermediate phenotype in the familiar–stranger session. No significant differences in exploration and locomotor activity were observed between genotypes in each session (Supplementary Fig. 1d–f).

We next performed a reciprocal social interaction test in which dyadic pairs of test (every genotype) and stimulus (PX-RICS+/+) mice could freely move and mutually interact in an open arena. We found that PX-RICS−/− mice spent less time on social activities (Fig. 1c) such as nose-to-nose sniffing, anogenital sniffing (Fig. 1d) and close huddling (Fig. 1e). In addition, the rate of successful social interactions of PX-RICS−/− test mice was significantly lower than that of PX-RICS+/+ test mice, regardless of whether the approach to the test mouse was initiated by the stimulus or the test mouse itself (Fig. 1f). Interestingly, PX-RICS−/− test mice frequently showed apathetic or avoidance responses to approach from the stimulus, resulting in an extremely poor success rate (34.5% in −/− versus 61.4% in +/+).
in +/+ of social interactions. This result suggests that PX-RICS−/− mice show normal voluntary social interaction as in the three-chamber test, but show less interest in or avoidance of passive social interaction. PX-RICS+/− mice showed intermediate phenotypes.

The results from both social interaction tests demonstrate that PX-RICS deficiency leads to inappropriate or poor social interaction in mice, as is commonly observed in ASD individuals. To further evaluate social interaction, we investigated mice for social dominance and social hierarchies using the
tube test, a useful paradigm in predicting impairments in social interaction. In the social dominance tube test, PX-RICS\(^{-/-}\) rarely won against unfamiliar PX-RICS\(^{+/+}\) or PX-RICS\(^{+-}\) mice (Fig. 1g), suggesting that PX-RICS\(^{-/-}\) mice are socially subordinate to PX-RICS\(^{+/+}\) and PX-RICS\(^{+-}\) mice, and thus tend to avoid passive social contact with other unfamiliar mice. The test mice displayed no aggressive behaviour; they only pushed their opponent, tried to squeeze themselves into the space between the opponent and the tube wall, or remained still.

Social interaction of nocturnal rodents is primarily driven by olfactory perception. We thus examined responses to olfactory cues using the olfactory habituation/dishabituation test. We found that PX-RICS\(^{+/+}\) and PX-RICS\(^{+-}\) mice show strong habituation/dishabituation responses to all olfactory cues presented (Supplementary Fig. 1g). In contrast, whereas PX-RICS\(^{+-}\) mice showed normal responses to non-social odours, they spent much less time interacting with unfamiliar social odours than PX-RICS\(^{+/+}\) and PX-RICS\(^{+-}\) mice. This result suggests that PX-RICS\(^{-/-}\) mice have decreased interest in unfamiliar social olfactory cues, although their olfactory perception is not impaired.

Mouse pups, when separated from their mother, emit communicative ultrasonic vocalizations (USVs) during the first 2 or 3 postnatal weeks. We thus measured maternal separation-induced USVs to quantify social communication deficits. As shown in Fig. 1h, USVs of PX-RICS\(^{+/+}\) pups reached a maximum on postnatal day (PND) 7 and then decreased gradually to almost zero by PND21. In contrast, PX-RICS\(^{+-}\) pups emitted USVs much less frequently after PND7 and required more time to emit the first call on PND14 (Supplementary Fig. 2a). No significant differences were observed in the mean duration of calls, total calling time, peak frequency and peak amplitude between genotypes (Supplementary Fig. 2b–e). These results suggest atypical postnatal development of mother–pup communication in PX-RICS\(^{-/-}\) pups.

Repetitive behaviour is the most noticeable symptom in ASD individuals. We monitored repetitive self-grooming behaviour and found that PX-RICS\(^{-/-}\) mice spend more than twice as much time on repetitive self-grooming, persisting intermittently for an unusually long (more than 2 min) period (Fig. 1i). The marble-burying test also revealed that PX-RICS\(^{-/-}\) mice have more than two-fold higher activity of repetitive digging behaviour (Fig. 1j,k and Supplementary Fig. 2f). PX-RICS\(^{+-}\) mice exhibited intermediate phenotypes in these assays. These results clearly demonstrate increased repetitive behaviour in PX-RICS\(^{-/-}\) mice.

ASD individuals often exhibit inflexible adherence to routines. To assess the ability of mice to switch flexibly from a previously established habit to a novel habit, we employed acquisition and reversal learning tasks in a water T-maze. Learning performance was represented by the number of trials required to achieve five consecutive correct arm choices (Supplementary Fig. 2b–e). These results suggest that PX-RICS\(^{-/-}\) mice have a tendency to adhere a previously established habit and less ability to adapt their behaviour to a novel situation.

**PX-RICS-deficient mice exhibit ASD-related comorbidities.** In ASD individuals, the triad of core behavioural problems is commonly accompanied by neurodevelopmental comorbidities, including impaired motor coordination and epilepsy. We investigated whether these ASD-related phenotypes are present in PX-RICS\(^{-/-}\) mice. The accelerating rotarod test revealed that PX-RICS\(^{-/-}\) mice can stay on a rotating rod for significantly less time than PX-RICS\(^{+-}\) controls (Fig. 2a), indicating impaired motor coordination. PX-RICS\(^{-/-}\) mice displayed intermediate motor coordination between PX-RICS\(^{+/+}\) and PX-RICS\(^{-/-}\) mice (Fig. 2a).

When suspended by the tail, several mouse models of neuropsychiatric disorders display a limb-clasping phenotype, a dystonic posture characterized by holding the fore- and/or hindlimbs together. This behaviour is a pathological reflex that indicates lesions in the cerebellum, basal ganglia or neocortex. We observed severe limb-clasping behaviour in 68.2% of PX-RICS\(^{-/-}\) mice (Fig. 2b), which is similar to a mouse model of Rett syndrome, one of the constituent conditions of ASD.

The prevalence of epilepsy is much higher in autistic children (~30%) than non-autistic individuals (~1%), which suggests a common molecular basis for cerebral hyperexcitability and impaired cognitive function. To assess the susceptibility to epilepsy, we scored progressive seizures induced by kainic acid, a specific agonist for the kainate subtype of ionotropic glutamate receptors, with Racine’s scale. PX-RICS\(^{-/-}\) mice were more sensitive to kainic acid than their PX-RICS\(^{+-}\) littermates (Fig. 2c). Seizures in the PX-RICS\(^{-/-}\) mice were more severe (Fig. 2d) and progressed more rapidly to each seizure stage (Fig. 2e).

Taken all behavioural data together, we concluded that PX-RICS deficiency in mice leads to behavioural deficits reflecting the core symptoms of ASD and its related comorbidities. We propose that PX-RICS is the gene responsible for ASD-like symptoms in JBS.

**Impaired GABA\(_{A}\)R surface expression in PX-RICS\(^{-/-}\) mice.** Results from the behavioural tests prompted us to investigate the cellular function of PX-RICS underlying ASD-like behaviour. We previously shown that the PX-RICS/GABARAP/14-3-3 complex facilitates dynemin-/dynein-dependent trafficking of the N-cadherin/β-catenin complex in non-neuronal cells. GABARAP was originally identified as a protein that binds to the γ2 subunit of GABA\(_{A}\)R and is known to promote GABA\(_{A}\)R trafficking to the neuronal surface. We thus hypothesized that the PX-RICS/GABARAP/14-3-3 complex facilitates surface expression of GABA\(_{A}\)Rs in neurons and that impairment of this trafficking mechanism causes ASD-like behaviour in PX-RICS-deficient mice.

We first tested the possibility that PX-RICS is involved in GABA\(_{A}\)R trafficking to the neuronal surface. We utilized γ2EP, a γ2 subunit tagged at its N-terminal extracellular domain with ecliptic pHluorin, a pH-sensitive variant of green fluorescent protein that is non-fluorescent in acidic environments (pH < 6). Thus, γ2EP-containing GABA\(_{A}\)Rs are non-fluorescent during trafficking but become readily visualized when expressed on the neuronal surface. When γ2EP was expressed in PX-RICS\(^{-/-}\) cortical neurons and observed in pH 7.4 buffer, robust dot-like fluorescent signals could be detected (Fig. 3a). This fluorescence was almost entirely derived from surface-expressed γ2EP because transient exposure to a pH 6.0 buffer quenched the fluorescent signals. Total γ2EP fluorescence was revealed by subsequent exposure to an NH\(_{4}\)Cl-containing pH 7.4 buffer, which equilibrates the intracellular pH to 7.4. In contrast, in
PX-RICS−/− cortical neurons, surface fluorescence was markedly reduced, but total fluorescence in pH 7.4 NH₄Cl-containing buffer was similar to that in PX-RICS+/+ neurons (Fig. 3a). Quantification of the fluorescence intensities revealed that the amount of surface-expressed γ2EP is markedly decreased in PX-RICS−/− neurons (Fig. 3b). This result was confirmed by the cell surface labelling and surface biotinylation assays (Supplementary Fig. 3a,b). Internalization activity for γ2 was similar between the PX-RICS+/+ and PX-RICS−/− cortical neurons, suggesting that the reduction in surface γ2EP in PX-RICS−/− neurons is due to impaired trafficking to the plasma membrane, not to enhanced endocytic withdrawal of surface γ2EP (Supplementary Fig. 3e–h). The cell surface labelling and surface biotinylation assays revealed that surface-expressed γ2 is markedly reduced in PX-RICS−/− hippocampal neurons and cerebellar granule neurons (CGNs) (Supplementary Fig. 3a,c,d). Taken together, we concluded that surface expression of GABAARs is impaired in PX-RICS−/− neurons.

Figure 2 | PX-RICS−/− mice display impaired motor coordination and a seizure-prone phenotype. (a) Accelerating rotarod test. PX-RICS−/− mice showed poor motor coordination. (b) Limb-clasping behaviour in PX-RICS−/− mice. When picked up by the tail, PX-RICS+/+ mice splayed their limbs outwards away from the abdomen. In contrast, PX-RICS−/− mice continuously held all four limbs together in a bat-like posture. (c–e) Increased susceptibility to kainate-induced epileptic seizures in PX-RICS−/− mice. (c) Representative time-dependent progression of seizure severity in four pairs of PX-RICS+/+ and PX-RICS−/− littermates. (d) A breakdown of the maximum seizure severity in PX-RICS+/+ and PX-RICS−/− mice. (e) Latency for each seizure stage. Significant differences were not determined in latency to score 6 due to the small number of PX-RICS+/+ mice that reached the stage. n = 20 (PX-RICS+/+) and 20 (PX-RICS−/−). Data are represented as means ± s.e.m. NS, not significant; **P < 0.01; ***P < 0.001. Two-way analysis of variance with Bonferroni’s post hoc test (a), χ²-test (d) and unpaired two-tailed Student’s t-test (e).
The efficacy of GABAergic inhibitory transmission is modulated by altering the number of GABAARs expressed on the neuronal surface. We next examined whether reduced surface expression of GABAARs in PX-RICS−/− neurons affects inhibitory synaptic transmission. We measured miniature inhibitory postsynaptic currents (mIPSCs), which reflect postsynaptic responses to GABA released from single synaptic vesicles in the presynaptic terminal, in hippocampal CA1 pyramidal cells using the whole-cell patch-clamp recording technique (Fig. 3c) (ref. 39). Quantitative analysis of mIPSCs revealed no significant difference (P = 0.89) in the mIPSC frequency (evaluated by the interval between mIPSCs) between PX-RICS+/+ (99.4 ± 19.4 ms) and PX-RICS−/− (95.4 ± 21.4 ms) neurons (Fig. 3d), suggesting that presynaptic neurotransmitter release is similar between PX-RICS+/+ and PX-RICS−/− neurons. In contrast, the mIPSC amplitude of PX-RICS−/− neurons (26.8 ± 1.2 pA) was significantly smaller (P = 0.0075) than that of PX-RICS+/+ neurons (32.5 ± 1.6 pA; Fig. 3e). These electrophysiological data indicate that postsynaptic responsiveness to inhibitory inputs is attenuated in PX-RICS−/− mice, which is in line with the reduced surface expression of GABAARs.

**Figure 3 | Decreased surface GABAAR expression and GABA-mediated inhibitory synaptic transmission in PX-RICS−/− neurons.** (a) Sequential fluorescent (pHluorin) and differential interference contrast (DIC) images of PX-RICS+/+ (left) and PX-RICS−/− (right) live cortical neurons expressing γ2EP. The settings of the microscope, lasers and detectors were kept unchanged during image acquisition. Scale bars, 10 μm. (b) Decreased surface expression of γ2 in PX-RICS−/− neurons. Surface expression was calculated as follows: surface expression (%) = 100 × (I7.4 – I6.0) / (I7.4N – I6.0N), where I7.4, I6.0, I7.4N, and I6.0N represent the fluorescent intensities in the imaging buffer at pH 7.4, pH 6.0, pH 7.4 containing NH4Cl and pH 6.0 containing NH4Cl, respectively. n = 20 (PX-RICS+/+) and 20 (PX-RICS−/−) fields. (c) Representative traces of mIPSCs (left) and averaged traces of mIPSCs (right) in hippocampal CA1 pyramidal cells and PX-RICS+/+ and PX-RICS−/− mice. In total, 221 and 330 consecutive events with no overlap with other events were averaged for PX-RICS+/+ and PX-RICS−/− neurons, respectively. (d,e) The median intervals (d) and amplitudes (e) of mIPSCs in PX-RICS+/+ and PX-RICS−/− neurons. n = 18 (PX-RICS+/+) and 20 (PX-RICS−/−) cells. Data are represented as means ± s.e.m. NS, not significant; **P < 0.01; ***P < 0.001 (unpaired two-tailed Student’s t-test).

PX-RICS forms a trafficking complex for GABAARs. We next analysed whether GABARAP, 14-3-3 and dynein/dynactin participate in PX-RICS-mediated GABAAR transport. We found that the γ2 subunit, GABARAP and PX-RICS form a ternary complex in cortical neurons (Fig. 4a) and are colocalized in the perinuclear region of the soma (Fig. 4b–d). Immunofluorescent staining with organelle-specific markers revealed that this region corresponds to the endoplasmic reticulum, endoplasmic reticulum exit sites and endoplasmic reticulum–Golgi intermediate compartments (Supplementary Fig. 4b–l). The introduction of siRNA against 14-3-3, PX-RICS and GABARAP are localized in the dendritic satellite secretory organelle exit site- and trans-Golgi network-specific markers (Supplementary Fig. 4b–l), suggesting that PX-RICS and GABARAP are localized in the dendritic satellite secretory organelle exit site- and endoplasmic reticulum–Golgi intermediate compartment and trans-Golgi network-specific markers (Supplementary Fig. 4b–l), suggesting that PX-RICS and GABARAP are localized in the dendritic satellite secretory organelle exit site- and endoplasmic reticulum–Golgi intermediate compartment and trans-Golgi network-specific markers (Supplementary Figs 5 and 6). This localization is consistent with their involvement in local delivery of GABAARs to the dendritic surface. Further ultracentrifugation analysis revealed that 14-3-3ζ/ζ and dynactin1, as well as γ2 and GABARAP, coimmunoprecipitated with PX-RICS in high-molecular-weight (<440 kDa) fractions (Fig. 4e,f). We confirmed that 14-3-3ζ/ζ and dynactin1 are colocalized with γ2, PX-RICS and GABARAP in the soma and dendrites of cortical neurons (Fig. 4g–i and Supplementary Figs 5 and 6). The introduction of siRNA against any one of them into cortical neurons resulted in a drastic decrease in GABAAR expression.
Figure 4 | PX-RICS is central to a large cargo-adaptor-motor complex in cortical neurons. (a) Co-immunoprecipitation analysis. Note that GABARAP (solid arrowhead) but not GABARAPL1 (open arrowhead) preferentially coprecipitates with γ2 and PX-RICS. (b–d) Colocalization of PX-RICS, GABARAP and γ2 in the perinuclear region of the soma and in specific compartments of the dendrites (white arrowheads). Scale bars, 5 μm. (e) Mouse cortical neurons solubilized with 1% digitonin were fractionated by linear sucrose density gradient (10–40%) ultracentrifugation, and each fraction was subjected to immunoblotting with the indicated antibodies. Arrows at the top indicate the mobilities of the molecular weight markers. (f) Fractions 6, 7 and 8 in e, the highest-molecular-weight fractions that contain all of the indicated proteins, were pooled and subjected to immunoprecipitation with anti-PX-RICS antibody, followed by immunoblotting with the indicated antibodies. Kinesin superfamily protein 5 (KIF5), a microtubule-dependent molecular motor, was used as a negative control for immunoprecipitation: it is present in fractions 6–8 (e), but does not coimmunoprecipitate with PX-RICS in the pooled lysate (f). KIF5 is known to interact with GABARAP to facilitate plus-end-directed retrograde transport of GABA<sub>2</sub>Ars (ref. 65). (g–i) γ2, GABARAP and PX-RICS were colocalized with 14-3-3ζ (g), 14-3-3η (h) and dynactin1 (i) in the perinuclear region of the soma and specific compartments of the dendrites (white arrowheads). Scale bars, 10 μm (soma); 5 μm (dendrite).
Figure 5 | The PX-RICS-dependent trafficking complex is required for surface expression of GABA\(_{\alpha}\)Rs. (a) Mouse cortical neurons (10 DIV) were transfected with the indicated siRNA (10 nM) plus Red Fluorescent Oligo (10 nM). Surface (green) and intracellular (blue) levels of the \(\gamma_2\) subunit in siRNA-introduced neurons (red) were analysed by surface labelling. The settings of the microscope, lasers and detectors were kept constant during image acquisition. Scale bars, 10 \(\mu\)m. (b) Quantitative analysis of surface-expressed and intracellular \(\gamma_2\). \(n = 10\) (images in each siRNA). (c) PX-RICS \(^{-/-}\) cortical neurons (14 DIV) were transfected with plasmids expressing FLAG-tagged wild-type (WT) or mutant PX-RICS as indicated. \(\Delta\)RSKSDP, a mutant lacking the 14-3-3-binding motif (amino acids 1,793–1,798); S1796A, a mutant in which Ser-1796, the CaMKII phosphorylation site in the 14-3-3 binding motif, is replaced with Ala; \(\Delta\)GBR, a mutant lacking the GABARAP-binding region (amino acids 562–796). Surface (green) and intracellular (blue) levels of the \(\gamma_2\) subunit in transfected neurons (red) were analysed by surface labelling. The settings of the microscope, lasers and detectors were kept constant during image acquisition. Scale bars, 10 \(\mu\)m. (d–g) Quantitative analyses of surface-expressed \(\gamma_2\) in entire neurons (d) and the representative dendrites (e). The number (f) and averaged size (g) of surface-expressed \(\gamma_2\) puncta within 40-\(\mu\)m portion of the representative dendrites are also quantified using ImageJ. \(n = 10\) (images in each construct). Data are represented as means ± s.e.m. NS, not significant; ***P < 0.001 (unpaired two-tailed Student’s t-test, compared with siControl (b) or vector control (d–g)).
reduction in surface $\gamma_2$ levels and a simultaneous increase in intracellular $\gamma_2$ levels (Fig. 5a,b).

We further determined whether the PX-RICS/GABARAP/14-3-3 complex is required for surface expression of GABA$_A$Rs. When wild-type PX-RICS was exogenously expressed in PX-RICS$^{-/-}$ neurons, surface expression of the $\gamma_2$ subunit was drastically increased (Fig. 5c–g). In contrast, PX-RICS/ARSKSDP and PX-RICS/S1796A, two mutants lacking the binding ability to 14-3-3 (ref. 23), had no apparent effect on surface expression of $\gamma_2$ (Fig. 5c–g). Similarly, surface $\gamma_2$ levels remained unchanged in PX-RICS$^{-/-}$ neurons expressing PX-RICS-ΔGBR, a mutant defective in GABARAP binding$^{22,23}$ (Fig. 5c–g).

Taken together, these results suggest that $\gamma_2$, GABARAP, PX-RICS, 14-3-3ζ/θ and dynactin1 are assembled into a large cargo-adaptor-motor complex that plays a facilitating role in GABA$_A$R surface expression. On the basis of a similarity to the trafficking mechanism previously proposed for the N-cadherin/β-catenin complex$^{22,23}$, we speculate a possible model for trafficking $\gamma_2$-containing GABA$_A$Rs (Fig. 6).

**A curative effect of clonazepam on autistic-like behaviour.** Cell surface labelling revealed that a small amount of the $\gamma_2$ subunit remains on the surface of PX-RICS$^{-/-}$ neurons (Supplementary Fig. 3a). We speculate that this observation may be due to an auxiliary secretory route for $\gamma_2$-containing GABA$_A$Rs that complements or bypasses the PX-RICS-dependent pathway. We reasoned that ASD-like behaviour in PX-RICS$^{-/-}$ mice may be treatable by potentiating synaptic inhibition through the GABA$_A$Rs remaining on the neuronal surface. Before testing this idea, we first determined an optimum injected dose of clonazepam (CZP), a benzodiazepine agonist for GABA$_A$R, to avoid its sedative and anxiolytic effects that could potentially affect our behavioural experiments. Open-field and elevated plus-maze tests revealed that intraperitoneal administration of CZP in a dose up to 0.03 mg kg$^{-1}$ causes no significant sedative or anxiolytic effects in both PX-RICS$^{+/+}$ and PX-RICS$^{-/-}$ mice (Supplementary Fig. 7a–d).

We then conducted behavioural tests of CZP-treated PX-RICS$^{-/-}$ mice. In the three-chamber social interaction test, we found that vehicle-treated PX-RICS$^{-/-}$ mice show impaired preference for social novelty, whereas preference for social novelty in CZP-treated PX-RICS$^{-/-}$ mice is almost completely normalized (Fig. 7a–d). No significant differences were observed in exploration and locomotor activity in each session (Supplementary Fig. 7e–g). Similarly, the water T-maze test revealed that learning performance of CZP-treated PX-RICS$^{-/-}$ mice in the reversal learning session is remarkably improved compared with vehicle-treated PX-RICS$^{-/-}$ mice (Fig. 7e,f and Supplementary Fig. 7h), indicating that persistence to a previously established habit is significantly alleviated by CZP administration. In each test, intraperitoneal injection of 0.03 mg kg$^{-1}$ CZP had no effect on the social behaviour of PX-RICS$^{+/+}$ mice. These results demonstrate that ASD-like behaviour in PX-RICS$^{-/-}$ mice is caused by impaired postsynaptic GABA signalling and that GABA$_A$R agonists have the potential to treat ASD-like behaviour in JBS patients and possibly non-syndromic ASD individuals.

**Discussion**

JBS is a contiguous gene disorder caused by large terminal deletion of chromosome 11q (ref. 14). Simultaneous loss of multiple genes results in a wide variety of congenital defects, including intellectual disability$^{14}$. Recently, Akshoomoff et al.$^{18}$ have conducted cognitive and behavioural assessments of JBS patients using standardized diagnostic procedures and shown that more than half of JBS patients meet the criteria for ASD diagnosis. Furthermore, they defined the 0.24-Mb autism critical region in distal 11q and identified four annotated genes: KCNJ1, KCNJ5, TP53AIP1 and RICS/PX-RICS (Supplementary Fig. 8) (ref. 18). KCNJ1 encodes the renal outer medullary potassium...
Figure 7 | CZP shows an ameliorating effect on impaired social interaction and inflexible behavioural change in PX-RICS−/− mice.

(a–d) Three-chamber social interaction test of vehicle- or CZP-treated mice. Time spent near each wired cage (a,b) and time spent in each chamber (c,d) are shown. Voluntary sociability and social preference are represented as a preference index (b,d), as in Fig. 1. In the familiar-stranger session (novel/familiar), CZP-treated PX-RICS−/− mice showed a positive preference index comparable to PX-RICS+/+ mice, indicating that impaired preference for social novelty can be corrected by improving postsynaptic GABA signalling. E, empty; FS, familiar stranger; M, middle chamber; NS, novel stranger; S, stranger. (e,f) Water T-maze test of vehicle- or CZP-treated mice. CZP-treated PX-RICS−/− mice showed reversal learning performance comparable to PX-RICS+/+ mice in both the number of correct arm choices (e) and latency to find an escape platform (f). Data are represented as means ± s.e.m. NS, not significant; **P<0.01; ***P<0.001. One-way analysis of variance (ANOVA) with Tukey’s post hoc test (a–d) and two-way ANOVA with Bonferroni’s post hoc test (e,f).

channel (ROMK), a member of the inward-rectifying K⁺ channel (Kᵣ) family that plays important roles in K⁺ secretion into the tubular lumen in the nephron. Mutations of KCNJ1 cause antenatal Bartter syndrome type 2, a renal tubular disorder characterized by hypokalaemia, metabolic alkalosis and hyperreninemic hyperaldosteronism with normal blood pressure. KCNJ5 encodes the G-protein-activated inward rectifier K⁺ channel that regulates aldosterone secretion. Mutations of KCNJ5 are implicated in primary hyperaldosteronism and cardiac long QT syndrome type 13 (ref. 44). TP53AIP, p53-regulated apoptosis-inducing protein 1, is a transcriptional target gene for p53 tumour suppressor protein and is specifically expressed in the...
The encoded protein is localized in the mitochondrion and mediates p53-dependent apoptosis. Given their reported localizations and functions, it is unlikely that KCNJ1, KCNJ5, and TP53AIP1 are involved in cognitive function and cause the ASD-like behaviour observed in JBS. In contrast, PX-RICS is the only candidate that is expressed predominantly in the brain and known to play functional roles in neurons. The present study has shown that loss-of-function of PX-RICS in mice results in various ASD-like behaviour associated with deficits in PX-RICS-dependent GABAAR surface expression (Supplementary Fig. 8). Our findings strongly support the notion that PX-RICS is the gene responsible for ASD-like symptoms in JBS patients and also provide evidence that a gene encoding a trafficking factor is implicated in ASD-like behaviour (Supplementary Fig. 8). We therefore propose that JBS-related ASD (and possibly a subset of ASD) is a trafficking disease.

Our findings also demonstrate that PX-RICS-mediated GABAAR trafficking is one of the biological systems that support normal cognitive function. Recently, dysfunctional GABA signalling has been increasingly reported to be associated with ASD. GABA-releasing neuron-specific deletion of the gene for methyl-CpG-binding protein 2 (Mecp2), a chromatin-associated transcription factor, in mice leads to a variety of Rett syndrome-like behavioural features. Mice lacking the contactin-associated protein-like 2 gene (Cntnap2) encoding a neuronal transmembrane protein of the neuropxin superfamily display a reduced number of GABA-releasing interneurons and ASD-related phenotypes. Forebrain interneuron-specific deletion of the Scnla gene, which encodes the α subunit of the sodium channel neuronal type I, in mice results in decreased firing activity of GABA-releasing interneurons and ASD-related behaviour. These findings demonstrate that dysfunction of the presynaptic mechanisms for releasing GABA contributes to ASD-like behaviour. In contrast, the present study has shown that dysfunction of the postsynaptic mechanism for trafficking GABAARs in GABA-receiving neurons is also associated with ASD-like behaviour. Collectively, GABA signalling is essential for normal cognitive function and seriously affects social behaviour if impaired in either GABA-releasing or GABA-receiving neurons.

Another important finding is that the ASD-like behavioural phenotypes of PX-RICS-deficient mice can be successfully rescued by treatment with a low dose of CZP, which suggests that compensatory potentiation of postsynaptic GABAergic signalling could be an effective therapeutic strategy for controlling ASD-like behavioural problems. Despite the increasing number of children diagnosed with ASD and the high prevalence of ASD of ~1% of the population, currently available pharmacological therapies remain supportive. A recent whole-exome-sequencing study has identified a rare missense single-nucleotide variation in the PX-RICS gene in a non-syndromic ASD patient, suggesting that PX-RICS deficiency can be a pathogenic factor for this type of ASD as well. We believe that our PX-RICS-deficient mice can be a unique disease model that properly recapitulates the pathogenic process of ASD-like behaviour in JBS patients and a valuable tool for the discovery of permanent cure drugs, as well as for a better understanding of non-syndromic ASD and related neurodevelopmental disorders.

Our previous reports revealed that PX-RICS is a GAP that selectively targets Cdc42 and that PX-RICS deficiency leads to an increase in neuronal Cdc42 activity. We also found that the Cdc42GAP activity is required for PX-RICS-mediated transport of the N-cadherin/β-catenin complex. How is Cdc42 activity connected with PX-RICS-mediated GABAAR trafficking? In this context, it is notable that collobystin, a brain-specific GDP/GTP exchange factor for Cdc42, is present in the scaffold protein complex for synaptic GABAARs and regulates gephyrin-dependent GABAAR anchoring and clustering. Collybistin binds to phosphatidylinositol (PIs), with a preference for PI3P and PI4P, through its pleckstrin homology domain, as PX-RICS does. Thus, the balance between the activities of the Cdc42 GDP/GTP exchange factor and GAP (that is, collybistin and PX-RICS) might control the synaptic expression and localization of GABAARs.

In summary, we have found that surface expression of GABAARs is impaired in PX-RICS−/− mice. More importantly, stimulation of GABA signalling with a GABAAR agonist improved some autistic-like phenotypes of PX-RICS−/− mice. Consequently, we conclude that dysfunctional PX-RICS-mediated GABAAR trafficking in postsynaptic neurons is a plausible cause for the observed behavioural and molecular deficits. It is possible that alternative binding partners of PX-RICS are also relevant for GABAAR-mediated signalling; PX-RICS is known to interact with more than 60 proteins other than GABARAP and 14–3–3, many of which have potential roles in important neuronal processes such as neurite extension, regulation of rho GTPases pathway and transcriptional regulation. Clearly, further investigation is needed to test the involvement of other putative PX-RICS interactors in GABA signalling, as well as other proteins implicated in complementary or compensatory pathways.

**Methods**

**Mice.** All animal experiments were reviewed and approved by the University of Tokyo Institutional Animal Care and Use Committee, and were conducted according to the University of Tokyo Guidelines for Care and Use of Laboratory Animals. Mice were housed in clean plastic cages (CLEA Japan) lined with paper bedding (Japan SLC) at a constant temperature of 23 °C in a 12-h light/dark cycle (lights off at 21:00), with food and water available ad libitum. PX-RICS-deficient mice were generated as described elsewhere. Mutant mice were backcrossed to C57BL/6N (CLEA Japan) background to the F10 generation. The mice used in behavioural and electrophysiological studies were generated by breedings of heterozygous mutant males and females in the C57BL/6N background. Young adult male mice (8–10 weeks old) were used in all behavioural tests except for USV analysis and the foot-clasping test, in which both male and female pups of PND5−21 and PND21 were used, respectively. Unless otherwise noted, all tests were conducted with a naive cohort. Six- to eight-week-old male mice were used for electrophysiological analysis. The embryos and newborns for primary neuronal cultures were generated by crossing wild-type C57BL/6N males and females, or homozygous mutant males and females in the C57BL/6N background. Embryonic day (E) 16–18 embryos were used for primary culture of cortical and hippocampal neurons, and PND7 pups for CGNs.

**Behavioural tests.** Behavioural assays of mice were designed and performed in accordance with the standard procedures described previously. Detailed procedures are described in the following sections. Full statistical data for the behavioural tests are presented in Supplementary Table 1.

**Three-chamber social interaction test.** Mice were tested for voluntary social interaction as previously described. The three-chamber apparatus (O’HARA & CO., LTD.) was a non-transparent acrylic open arena (61 × 40 × 22 cm) with a removable floor placed in a soundproof chamber (74 × 50 × 83 cm). The arena was divided into left, middle and right chambers (20 × 40 × 22 cm) by two semi-transparent partitions with a small rectangular opening (5 × 3 cm) in the middle chamber. The test mouse could travel through which. Finally, after a novel stranger mouse was placed in another wired cage that had served as the empty cage in the previous sessions, the test mouse was again allowed to freely explore for 10 min (familiar−stranger

**Three-chamber open field test.** Three-chamber open field test was performed as described previously. Mice were tested in the open field apparatus 1 h before beginning the test. For 3 days before testing, age-matched wild-type males unfamiliar to the test mice (stranger mice) were left inside the wired cages for 20 min per day for habituation. A test mouse was first placed in the centre of the middle chamber and left to habituate for 10 min in the absence of stranger mice in the wired cages (empty−empty session). Subsequently, a stranger mouse was introduced into one of the two wired cages. Then, the test mouse was placed in the centre of the middle chamber and allowed to freely explore all three chambers for 20 min. Finally, after a novel stranger mouse was placed in another wired cage that had served as the empty cage in the previous sessions, the test mouse was again allowed to freely explore for 10 min (familiar−stranger

**Three-chamber social interaction test.** Mice were tested for voluntary social interaction as previously described. The three-chamber apparatus (O’HARA & CO., LTD.) was a non-transparent acrylic open arena (61 × 40 × 22 cm) with a removable floor placed in a soundproof chamber (74 × 50 × 83 cm). The arena was divided into left, middle and right chambers (20 × 40 × 22 cm) by two semi-transparent partitions with a small rectangular opening (5 × 3 cm) in the middle chamber. The test mouse could travel through which. Finally, after a novel stranger mouse was placed in another wired cage that had served as the empty cage in the previous sessions, the test mouse was again allowed to freely explore for 10 min (familiar−stranger
session). Movement of the test mouse was recorded by a charge-coupled device camera and analysed online with Time CSI video tracking software (O’HARA & CO., LTD). The open arena, partitions, wired cages and weights were cleaned with 70% ethanol and wiped with paper towels before the next trial. Time spent in close interaction near each wired cage and time spent in each chamber were converted into a preference index, which was calculated as 100 × (time with a right empty cage)/[(time with a right empty cage) + (time with a left empty cage) − 50 (in the empty-empty session)] = 100 × (time with a stranger mouse)/[(time with a stranger mouse + time with an empty cage) − 50 (in the stranger-empty session)] or as 100 × (time with a novel stranger mouse)/[(time with a novel stranger mouse + time with a familiar stranger mouse) − 50 (in the familiar-stranger session)].

**Ultrasonic vocalizations.** Maternal separation-induced USVs were monitored as described previously19. Male mouse 8–10 weeks old was placed in a clean empty cage, leaving the home cage empty for 5 min, and then returned to its home cage. Ultrasonic vocalizations were recorded by the UltraSoundGate system (Avisoft Bioacoustics) for recording and SASLab Pro software (Avisoft Bioacoustics) for quantitative analysis. The cylinder was cleaned with 70% ethanol and wiped with paper towels between recordings.

**Repetitive behaviour.** Self-grooming behaviour was examined as described previously28. A male mouse 8–10 weeks old was placed in a clean empty cage, leaving the home cage empty for 5 min, and then returned to its home cage. Cumulative time spent in full-body grooming was measured with a stopwatch.

Digging behaviour was evaluated using the marble-burying test as described previously28. Twenty black glass marbles (15 mm diameter) were gently placed evenly spaced apart in a 4 × 5 arrangement on 5-mm-thick fresh paper bedding in a clean cage (27.5 × 17 × 13 cm). After taking a still image of marbles from straight above the cage, a male mouse 8–10 weeks old was gently placed in the centre of the cage and left for free exploration for 30 min. Then, the mouse was removed and a still image of the marbles was taken again from straight above the cage. Digging behaviour was quantified by evaluating pre- and post-test images with ImageJ software (National Institutes of Health). Strong colour contrast between the black marbles and the white bedding enabled us to easily define the marble area. The marble was judged as ‘buried’ if more than 50% of the marble area was covered by the paper bedding. The number and area of marbles buried were evaluated.

**Water T-maze test.** Perservative behaviour was assessed using the water T-maze as previously described27,28. The apparatus was a T-shaped transparent acrylic open tank 30 cm deep with a long main arm (96 cm in length and 17.5 cm in width) and two perpendicular side arms (36 cm in length and 17.5 cm in width). A white escape platform (6 × 6 cm, 21.5 cm in height) was designed to fit snugly to the end of the side arms to prevent it from wobbling. The apparatus was filled with tap water at 23 ± 1 °C to a depth of 22.5 cm so that the top of the escape platform was positioned 1 cm below the water surface. The water was coloured white with poster paint (Nicker Colour Co., Ltd.) to have the submerged escape platform invisible in the test tank.

In a habituation swim trial on day 1, a male mouse 8–10 weeks old was placed on the water surface at the starting end of the main arm facing towards the side arms and then gently released. After 60 s of free swimming without the escape platform, the mouse was gently wiped with a towel, placed in an empty cage on a warming plate (38 °C setting) for 5 min, and then returned to its home cage. On the following 3 days (day 2–4), acquisition swim trials were performed in which the escape platform was submerged at the end of the right arm of the T-maze. Fifteen consecutive swim trials were performed every 3 days. In each trial, the latency to reach the escape platform and the result of arm choice were recorded. The mouse was waited from the platform after 20 s. After reaching the escape platform, the mouse was guided onto the escape platform and left for 20 s when it could not find the platform within 35 s. In the last 3 days (day 5–7), reversal learning trials were performed as described above, except that the escape platform was placed at the end of the left arm.

**Accelerating rotarod test.** Motor coordination was assessed using an accelerating rotarod apparatus (O’HARA & CO., LTD) as described previously28. A male mouse 8–10 weeks old was placed on the Kenya rotarod apparatus (O’HARA & CO., LTD) with the vehicle solution for injection in a volume of 10 ml kg⁻¹ body weight at a dose of 30 mg per kg body weight in a total volume of 0.2–0.25 ml. The animal was monitored and video-recorded in a clear cage for 2 h. The video recordings were used to confirm the visual scoring of seizures. Seizure severity was scored according to a modified Racine’s scale23: 0, no response; 1, behavioural arrest with mouth and facial twitches; 2, head nodding; 3, forelimb clonus; 4, seizures clonic characterized by rearing; 5, seizures characterized by rearing and falling; and 6, death from continuous convulsions.

**CZP treatment.** CZP (Wako) was suspended in vehicle solution (0.5% (w/v) methylcellulose 400 (Wako)/phosphate-buffered saline (PBS)) and diluted with the vehicle solution for injection in a volume of 10 ml kg⁻¹ at a dose of 0.03–0.05 mg kg⁻¹. The diluted CZP suspension or vehicle solution was intraperitoneally administered to a male test mouse 8–10 weeks old 30 min before behavioural testing.

The sedative effect of CZP was evaluated as described previously12. The apparatus for the open-field locomotor activity test was a non-transparent acrylic open arena (O’HARA & CO., LTD) without bedding. The CZP-injected mouse was placed in the centre of the arena, and its movement was monitored by a charge-coupled device camera for 10 min. Total distance moved and total immobile time were analysed online with the Time CSI software (O’HARA & CO., LTD). The arena was cleaned with 70% ethanol and wiped with paper towels between trials.

**Footh-clapping test.** To test foot clapping, both male and female mice on PND21 were suspended by the tail for 30 s.
The anxious effect of CZP was evaluated using an elevated plus-maze as described previously. The apparatus (O’HARA & CO., LTD) was a plus-shaped maze fixed at 50 cm in height above the floor comprising two open and two closed arms (25 cm in length and 5 cm in width). The former had small ledges along their edges to prevent a test mouse from losing its footing, whereas the latter were enclosed by semi-transparent acrylic walls (15 cm in height) on three sides. The CZP-injected mouse was placed in the centre of the maze facing towards one of the closed arms and then gently released into the closed arm. Movement of the mice was video-recorded for 10 min. Time spent in the open and the closed arms, and the number of entries into each arm were measured by visual observation of the movies with a stopwatch. The floors and walls of the maze were cleaned with 70% ethanol and wiped with paper towels between tests.

The three-chamber social interaction test and the water T-maze test of the CZP- or vehicle-injected mice were performed as described above.

Reagents. Rabbit polyclonal antibodies (pAbs) to PX-RICS and GABARAP were generated as described previously. Briefly, anti-PX-RICS and anti-GABARAP pAbs were generated by immunizing rabbits with recombinant human PX-RICS (amino acids 53–112) and human GABARAP (full length) fused to glutathione S-transferase, respectively. The antibodies were purified by affinity chromatography by using columns to which the antigens used for immunization had been linked.

Cell culture and transfection. Cortical and hippocampal neurons were isolated from E16–18 mouse embryos and plated into 6- or 12-well tissue culture plates or 35-mm glass-bottom dishes precoated with 1 mg ml⁻¹ poly-1-lysine (SIGMA) as described previously. Cells were cultured in Neurobasal medium (Invitrogen) supplemented with B-27 supplement (Invitrogen) and 0.5 mM l-glutamine (Invitrogen). A unit of 10 μM cytosine β-D-arabinofuranoside (Ara-C; SIGMA) was added to the culture medium for the first 3 days. Half of the medium was changed every 3 days. CGNs were isolated from PND7 pups in the same way except that Neurobasal-A medium (Invitrogen) containing 25 mM KCl was used. Transfection of primary cultured cortical neurons was performed at 14 days in vitro (DIV) using FuGENE 6 (Roche) for plasmid constructs and at 10 DIV using Lipofectamine RNAiMAX (Invitrogen) for siRNA. Three hours after transfection, the media were replaced with conditioned media.

Fluorescent imaging of pHluorin-tagged γ/2L in live neurons. pHluorin is a pH-sensitive green fluorescent protein variant that exhibits minimal fluorescence at acidic pH (pH < 6.5) and a robust fluorescent signal at neutral pH. The tagged receptors are thus non-fluorescent during trafficking when the N terminus of the γ2 subunit resides in the acidic lumen of transport vesicles but become readily visualized when exposed to the extracellular neutral pH environment at the cell surface. A mammalian expression construct for the γ2L isoform tagged with eclipser pHluorin (γ2EP) was a kind gift from Dr Moss (Tufts University). Unlike the γ2S isoform, surface expression of γ2L requires the γ and β subunits; thus, this method monitors surface expression only of the correctly assembled receptor complex. NA1A (pcDNA3.1-RNAiMAX for RNAiMAX or Invitrogen) for siRNA. Three hours after transfection, the media were replaced with conditioned media. Forty-eight hours after transfection, fluorescent and differential interference contrast images were obtained with an LSM510META laser scanning confocal microscope (Zeiss). Just before image acquisition, culture media were replaced with imaging media in the following order. IM-7.4 (standard buffer): 10 mM HEPES (pH 7.4), 125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂ and 10 mM d-glucose; IM-6.5 (low-pH obtained with an LSM510META laser scanning confocal microscope (Zeiss). The settings of the microscope, lasers and detectors were kept unchanged during image acquisition.

Surface labelling. Surface labelling was performed as described. The rabbit pAb to γ2 (Synaptic Systems) used for surface labelling recognizes the N-terminal extracellular region of the subunit. Primary and secondary antibodies used for surface labelling before fixation were diluted in conditioned medium from 14 DIV cortical or hippocampal neurons or from 10 DIV CGNs. The cell images were obtained with an LSM510META laser scanning confocal microscope (Zeiss). The settings of the microscope, lasers and detectors were kept constant during image acquisition. Supplementary Fig. 3a: PX-RICS γ/ +/ and PX-RICS γ/ −/− cortical or hippocampal neurons (14 DIV) were surface-labelled with rabbit pAb to γ2 as described above. After being rinsed in PBS, the neurons were incubated with Alexa Fluor 594 labelled anti-rabbit IgG and Alexa Fluor 549 labelled anti-chicken IgY for 30 min at RT and then with Alexa Fluor 488 labelled-anti-rabbit IgG and Alexa Fluor 549 labelled-anti-chicken IgY for 60 min at RT. Supplementary Fig. 4b–k: mouse cortical neurons (14 DIV) were surface-labelled with rabbit pAb to γ2 for 30 min at RT and rinsed in PBS. After being fixed with 2% paraformaldehyde in PBS for 15 min at RT and then blocked with 0.1% Triton X-100 in PBS for 5 min, the neurons were incubated with unconjugated anti-rabbit IgG (50 μg ml⁻¹; SIGMA) for 1 h at RT to block the unlabelled anti-γ2 antibody on the surface. The neurons were then permeabilized with 0.2% Triton X-100 in PBS for 5 min and stained with the indicated combinations of the primary antibodies for 60 min at RT and then with Alexa Fluor 488, Alexa Fluor 594- and Alexa Fluor 647-labeled secondary antibodies for 60 min at RT. Antibodies to organellar-specific markers were as follows: chicken pAb to calreticulin (1:800; Abcam); goat pAb to Sec23 (1:800; Santa Cruz Biotechnology); goat pAb to EGRIC3 (1:50; Santa Cruz Biotechnology); mouse monoclonal antibody (mAb) to GM130 (1:200; Transduction Laboratories); and mouse mAb to syntaxin 1 (1:1000; Transduction Laboratories).

Surface biotinylation assay. Biotinylation of neuronal surface proteins was performed as described previously. Mouse cortical neurons (14 DIV) or CGNs (10 DIV) were washed three times with ice-cold PBS and incubated with 0.25 mg ml⁻¹ EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS for 20 min at 4 °C with gentle agitation. After an immediate rinse with ice-cold quenching buffer (50 mM glycine in PBS), the neurons were further washed three times in ice-cold quenching buffer for 5 min each. The cells were lysed in lysis buffer T (10 mM Tris-HCl (pH 6.8), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail and phosphatase inhibitor cocktail) and cleared lysates (0.5 mg of protein) were incubated with 60 μl of 50% slurry of streptavidin–agarose beads (Thermo Scientific) for 2 h at 4 °C. Beads were washed five times in lysis buffer T and then once with PBS. Bound proteins were analysed by immunoblotsing with rabbit pAb to γ2 (1:1,000; Synaptic Systems), mouse mAb to transferrin receptor (1:500, Zymed) and mouse mAb to α-tubulin (1:500; Calbiochem). Band intensities were quantified using ImageJ software.

Electrophysiological analysis. To measure IPSCs, hippocampal slices were prepared, and whole-cell patch clamp recordings were performed as previously described. Briefly, PX-RICS γ/ +/ and PX-RICS γ/ −/− mice 6–8 weeks old were anaesthetized deeply with halothane and decapitated, and then, the brains were removed. Transverse hippocampal slices (400-μm thick) were cut with a tissue
slicer (Vibratome 1500 or Vibratome 3000) in Krebs–Ringer solution (119.0 mM NaCl, 2.5 mM KCl, 1.0 mM NaH₂PO₄, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 26.2 mM NaHCO₃ saturated with 11.0% O₂ and 5% CO₂). The slices were incubated for at least 1 h at RT in an interface-type holding chamber filled with Krebs–Ringer solution, and then, a slice was transferred to a submersion-type recording chamber, where the solution was perfused at 1.5–2.0 ml min⁻¹ at 25°C. Whole-cell patch–clamp recordings were made from pyramidal cells in the CA1 region. The seal resistance of the recording was maintained at about 70 mV. A recording patch pipette (3–6 MΩ) was filled with the internal solution (140 mM NaCl, 10.0 mM HEPES, 0.2 mM EGTA, 2.0 mM MgATP, 0.3 mM Na₂GTP, 8.0 mM NaCl and 5.0 mM QX314 (pH 7.2, 295–305 mosM)). mIPSCs were recorded in the presence of the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (10 mM), the NMDA receptor antagonist (−)−2-amino-5-phosphono pentanoic acid (AP5: 50 μM) and the voltage-gated sodium channel blocker tetrodotoxin (1 μM; all from Tocris Bioscience). mIPSCs were analysed using the Mini Analysis (6.0.7) software (Synaptosoft). All of the events detected, only the events that did not overlap with other events were accepted for mIPSC amplitude analysis, whereas all the events were included in the interval analysis. Any significant difference of the median of mIPSC amplitudes and intervals between the genotypes was evaluated with the unpaired two-tailed Student’s t-test.

Immunoprecipitation and immunoblotting. Mouse cortical neurons (14 DIV) were lysed in lysis buffer T. The lysates were preincubated with protein A-Sepharose (GE Healthcare) for 1 h at 4°C. Preincubated lysates (100–500 μg of protein) were incubated with 5 μl of the indicated antibody for 3 h at 4°C, and then, the immunocomplexes were adsorbed to protein A-Sepharose for 1 h at 4°C. After washing extensively with lysis buffer T, the immunoprecipitates were resolved by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The blots were probed with primary antibodies as indicated and visualized with alkaline phosphatase-conjugated secondary antibodies (Promega). Images have been cropped for presentation (Fig. 4 and Supplementary Fig. 3). Full-size images are presented in Supplementary Figs 9 and 10.

Immunofluorescence. Mouse cortical neurons (14 DIV) were fixed with cold methanol for 20 min at −20°C and permeabilized with 0.2% Triton X-100 in Tris-buffered saline for 5 min. The neurons were stained with the indicated combination of primary antibodies for 60 min at RT. Staining patterns were visualized by incubating with the Alexa Fluor 488-, Alexa Fluor 594- or Alexa Fluor 647-conjugated secondary antibodies (1:500; Invitrogen) for 60 min at RT. The cell images were obtained with an LSM510META laser scanning confocal microscope (Zeiss). For immunofluorescence of 14-3-3ζ and dynactin1, the following antibodies were used: rabbit pAb to 14-3-3ζ (1:50; Santa Cruz Biotechnology); rabbit pAb to 14-3-3ζ (1:50; Santa Cruz Biotechnology); and mouse mAb to dynactin1 (1:250; Transduction Laboratories). For double immunofluorescence staining of 14-3-3ζ and early endosome antigen 1 in Supplementary Fig. 3g, mouse mAb to 14-3-3ζ (1:50; Santa Cruz Biotechnology) and rabbit pAb to early endosome antigen 1 (1:100; Cell Signaling Technology) were used. Cell nuclei were visualized with TO-PRO-3 iodide (1:10,000; Invitrogen). Quantitative analyses of the colocalization were performed using ImageJ, based on the previously reported method42.

Sucrose density gradient ultracentrifugation. Density gradient ultracentrifugation was performed as described elsewhere50. Mouse cortical neurons were washed twice with 5 μl of the indicated antibody buffer for 3 h at 4°C, and then, the cells were lysed in 1 ml of lysis buffer D (25 mM HEPES–KOH (pH 6.8), 150 mM NaCl and 0.4% digitonin). After centrifugation for 15 h at 35,000 r.p.m. in a Beckman SW40 rotor, 12 fractions each containing 1 ml were collected from the top of the tube and subjected to immunoblotting or immunoprecipitation. The antibodies used in immunoblotting were as follows: rabbit pAb to αtubulin (1:1,000; Synaptic Systems) and rabbit pAb to αtubulin (1:1,000; Synaptic Systems). Mouse mAbs to dynactin1 and dynactin2 were used. Cell nuclei were visualized with TO-PRO-3 iodide (1:10,000; Invitrogen). Quantitative analyses of the colocalization were performed using ImageJ, based on the previously reported method42.

References

1. Lord, C. Unweaving the autism spectrum. Cell 147, 24–25 (2011).
2. Jeste, S. S. & Geschwind, D. H. Disentangling the heterogeneity of autism spectrum disorder through genetic findings. Nat. Rev. Neuro. 10, 74–81 (2014).
3. Schaaf, C. P. & Zoghbi, H. Y. Solving the autism puzzle a few pieces at a time. Neuron 70, 806–808 (2011).
4. Rubenstein, J. L. & Merzenich, M. M. Model of autism: increased ratio of excitation/inhibition in key neural systems. Genes Brain Behav. 2, 255–267 (2003).
5. Ramamoorthi, K. & Lin, Y. The contribution of GABAergic dysfunction to neurodevelopmental disorders. Trends Mol. Med. 17, 452–462 (2011).
6. Yizhar, O. et al. Neocortical excitation/inhibition balance in information processing and social dysfunction. Nature 477, 171–178 (2011).
7. Ecker, C., Spooren, W. & Murphy, D. G. Translational approaches to the biology of autism: false dawn or a new era? Mol. Psychiatry 18, 435–442 (2013).
8. Delorme, R. et al. Progress toward treatments for synaptic deficits in autism. Nat. Med. 19, 685–694 (2013).
9. Ebert, D. H. & Greenberg, M. E. Activity-dependent neuronal signalling and autism spectrum disorder. Nature 493, 327–337 (2013).
10. Chao, H. T. et al. Dysfunction in GABA signalling mediates autism-spectrum-like stereotypies and Rett syndrome phenotypes. Nature 468, 263–269 (2010).
11. Penagarikano, O. et al. Absence of CNTPAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. Cell 147, 235–246 (2011).
12. Han, S. et al. Autistic-like behaviour in Scn1a<sup>−/−</sup> mice and rescue by enhanced GABA-mediated neurotransmission. Nature 489, 385–390 (2012).
13. Lerman, J. S. The neurology of autism spectrum disorders. Curr. Opin. Neuro. 24, 132–139 (2011).
14. Mattina, T., Perrotta, C. S. & Grossfeld, P. Jacobsen syndrome. Orphanet J. Rare Dis. 4, 9 (2009).
15. Coldren, C. D. et al. Chromosomal microarray mapping suggests a role for Bsx and Neurokinin in neurogenetic and behavioral deficits in the 1q11 deletion disorder (Jacobsen Syndrome). Neurogenetics 10, 89–95 (2009).
16. Ye, M. et al. Deletion of JAM-C, a candidate gene for heart defects in Jacobsen syndrome, results in a normal cardiac phenotype in mice. Am. J. Med. Genet. A 149A, 1438–1443 (2009).
17. Ye, M. et al. Deletion of ETS-1, a gene in the Jacobsen syndrome critical region, causes ventricular septal defects and abnormal ventricular morphology in mice. Hum. Mol. Genet. 19, 648–656 (2010).
18. Akselkoonoff, N., Mattson, S. N. & Grossfeld, P. D. Evidence for autism spectrum disorder in Jacobsen syndrome: identification of a candidate gene in distal 11q. Genet. Med. 17, 143–148 (2014).
19. Okabe, T. et al. RICS, a novel GTPase-activating protein for Cdc42 and Rac1, is involved in the β-catenin-N-cadherin and N-methyl-D-aspartate receptor signaling. *J. Biol. Chem.* **278**, 9920–9927 (2003).

20. Hayashi, T. et al. PX-RICS, a novel splicing variant of RICS, is a main isoform expressed during neural development. *Genes Cells* **12**, 929–939 (2007).

21. Naus-Nishimura, Y. et al. Role of the Rho GTPase-activating protein RICS in neurite outgrowth. *Genes Cells* **11**, 607–614 (2006).

22. Nakamura, T. et al. PX-RICS mediates ER-to-Golgi transport of the N-cadherin/β-catenin complex. *Genes Dev.* **22**, 1244–1256 (2008).

23. Nakamura, T. et al. The PX-RICS-14–3–3 ε complex couples N-cadherin-β-catenin with dynemin-dynactin to mediate its export from the endoplasmic reticulum. *J. Biol. Chem.* **285**, 16415–16154 (2010).

24. Nadler, J. J. et al. Automated apparatus for quantification of social approach behaviors in mice. *Genes Brain Behav.* **3**, 303–314 (2004).

25. Stockhorst, U. & Pietrowsky, R. Olfactory perception, communication, and the nose-to-brain pathway. *Physiol. Behav.* **83**, 3–11 (2004).

26. Scattoni, M. L., Crawley, J. & Ricceri, L. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci. Biobehav. Rev.* **33**, 508–515 (2009).

27. Bednar, I. et al. Selective nicotinic receptor consequences in APPswh transgenic mice. *Mol. Cell. Neurosci.* **20**, 354–365 (2002).

28. Tsai, P. T. et al. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tac1 mutant mice. *Nature* **488**, 647–651 (2012).

29. Lalonde, R. & Strazielle, C. Brain regions and genes affecting limb-clasping responses. *Brain Res. Rev.* **67**, 252–259 (2011).

30. Racine, R. J. Modification of seizure activity by electrical stimulation. II. Motor seizures. *Clin. Neurophysiol.* **52**, 281–294 (1972).

31. Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J. & Olsen, R. W. GABA-A receptor-associated protein links GABA-A receptors and the cytoskeleton. *Nature*** **397**, 69–72 (1999).

32. Leil, T. A., Chen, Z. W., Chang, C. S. & Olsen, R. W. GABA-A receptor-associated protein traffic GABA-A receptors to the plasma membrane in neurons. *J. Neurosci.* **24**, 11429–11438 (2004); **9**, 1437–1445 (2008).

33. Jacob, T. C. et al. Gephyrin regulates the cell surface dynamics of synaptic GABA-A receptors. *J. Neurosci.* **25**, 10469–10478 (2005).

34. Kang, J. Q., Shen, W. Z. & Macdonald, R. L. The GABRG2 mutation, Q351X, associated with generalized epilepsy with febrile seizures plus, has both loss of function and dominant-negative suppression. *J. Neurosci.* **29**, 2845–2856 (2009).

35. Miesenbock, G., De Angelis, D. A. & Rothman, J. E. Visualizing secretion and membrane cargo in neuronal dendrites. *Science* **302**, 865–869 (2003).

36. Rudolph, U. & Knoflach, F. Beyond classical benzodiazepines: novel therapeutic potential of GABA-A receptor subtypes. *Nat. Rev. Drug Discov.* **10**, 685–697 (2011).

37. Nakatani, J. et al. Abnormal behavior in a chromosome-engineered mouse model for human 15q11-13 duplication seen in autism. *Cell*** **137**, 1235–1246 (2009).

38. McFarlane, H. G. et al. Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav.* **7**, 152–163 (2008).

39. Thomas, A. et al. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berlin)* **204**, 361–373 (2009).

40. Boileau, A. J., Pearce, R. A. & Czajkowsk, C. W. The short splice variant of the γ2 subunit acts as an external modulator of GABA-A receptor function. *J. Neurosci.* **30**, 4895–4903 (2010).

41. Rudolph, U. & Knoflach, F. Beyond classical benzodiazepines: novel therapeutic potential of GABA-A receptor subtypes. *Nat. Rev. Drug Discov.* **10**, 685–697 (2011).

42. Racine, R. J. Modification of seizure activity by electrical stimulation. II. Motor seizures. *Clin. Neurophysiol.* **52**, 281–294 (1972).

43. Watanabe, A. et al. P53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell 85*, 899–906 (2001).

44. Matsuda, K. et al. P53AIP1 regulates the mitochondrial apoptotic pathway. *Cancer Res.* **62**, 2883–2889 (2002).

45. Han, S., Tai, C., Jones, C. J., Scheuer, T. & Catterall, W. A. Enhancement of inhibitory neurotransmission by GABA-A receptors having h3-β2 subunits ameliorates behavioral deficits in a mouse model of autism. *Neuron* **81**, 1282–1289 (2014).

46. De Bubeis, S. et al. Synaptic, transcriptional and chromatin genes disrupted in autism. *Nature** **515**, 209–215 (2014).

47. Kins, S., Betz, H. & Kirsch, J. Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat. Neurosci.* **3**, 22–29 (2000).

48. Ludoold, M. et al. Specificity of collybistin-phosphoinositide interactions: Impact of the individual protein domains. *J. Biol. Chem.* **291**, 244–254 (2016).

49. Crawford, J. N. Designing mouse behavioral tasks relevant to autistic-like behaviors. *Mental Retard. Dev. Disabil. Res. Rev.* **10**, 248–258 (2004).

50. Crawley, J. N. Behavioral phenotyping strategies for mutant mice. *Neuron* **57**, 809–818 (2008).

51. Silverman, J. L., Yang, M., Lord, C. & Crawley, J. N. Behavioural phenotyping assays for mouse models of autism. *Nat. Rev. Neurosci.* **11**, 490–502 (2010).

52. Kardon, J. R. & Vale, R. D. Regulators of the cytoplasmic dynein motor. *Nat. Rev. Mol. Cell Biol.* **10**, 854–865 (2009).

53. Nakajima, K. et al. Molecular motor KIF5A is essential for GABA-A receptor transport, and KIF5A deletion causes epilepsy. *Neuron* **76**, 945–961 (2012).

54. Coyle, J. E., Qamar, S., Rashanskan, K. R. & Nikolov, D. B. Structure of GABARAP in two conformations: implications for GABA-A receptor localization and tubulin binding. *Neuron* **33**, 63–74 (2002).

55. Green, F., O’Hare, T., Blackwell, A. & Enns, C. A. Association of human transferrin receptor with GABARAP. *FEBS Lett.* **518**, 101–106 (2002).

56. Chen, C. et al. GEC1 interacts with the κ opioid receptor and enhances expression of the receptor. *J. Biol. Chem.* **281**, 7983–7993 (2006).

57. Cook, J. L. et al. The trafficking protein GABARAP binds to and enhances plasma membrane expression and function of the angiotensin II type 1 receptor. *Circ. Res.* **102**, 1539–1547 (2008).

58. Lainez, S. et al. GABA-A receptor associated protein (GABARAP) modulates TRPV1 expression and channel function and desensitization. *FASEB J.* **24**, 1958–1970 (2010).

Acknowledgements
We thank S. J. Moss and J. Q. Kang for expression plasmids and A. M. Watabe for her participation in this research at the earliest stage. This work was supported by Grants-in-Aid for Scientific Research, the Takeda Science Foundation, the Uehara Memorial Foundation and in part by the Global COE Program (Integrative Life Science Based on the Study of Biosignalling Mechanisms), the Global COE Program for the Comprehensive Centre of Education and Research for Chemical Biology of the Diseases) and the Strategic Research Program for Brain Sciences, MEXT, Japan.

Author contributions
T.N. designed and performed most of the experiments, analysed the data and wrote the manuscript; F.A.-Y. and T.M. designed and performed the electrophysiological experiments and analysed the data; F.S., Y.N.-N., Y.T. and K.M. bred mice and advised on animal experiments; N.A., S.N.M. and P.D.G. provided information on the deletion mapping of JBS; T.A. supervised this study and wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.
How to cite this article: Nakamura, T. et al. PX-RICS-deficient mice mimic autism spectrum disorder in Jacobsen syndrome through impaired GABA\textsubscript{A} receptor trafficking. Nat. Commun. 7:10861 doi: 10.1038/ncomms10861 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/