Post-natal treatment by a blood-brain-barrier permeable calpain inhibitor, SNJ1945 rescued defective function in lissencephaly

Shiori Toba1*, Yasuhisa Tamura2*, Kanako Kumamoto1*, Masami Yamada1, Keizo Takao3,4, Satoshi Hatton1,5, Tsuyoshi Miyakawa3,4,5, Yosky Kataoka6, Mitsuyoshi Azuma6, Kiyoshi Hayasaka7, Masano Amamoto6, Keiko Tominaga8, Anthony Wynshaw-Boris10, Hideki Waniuchi11, Yuichiro Oka12,13,14, Makoto Sato12,13,14, Mitsuhiro Kato7 & Shinji Hirotsune1

1Department of Genetic Disease Research, Osaka City University Graduate School of Medicine, Asahi-machi 1-4-3 Abeno, Osaka 545-8586, Japan, 2Cellular Function Imaging Laboratory, RIKEN Center for Molecular Imaging Science, Minatojima minamimachi, Chuo-ku, Kobe, Hyogo, Japan, 3Japan Science and Technology Agency, CREST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan, 4Section of Behavior Patterns, Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, 38 Nishigakou Myodaiji, Okazaki, Aichi, 444-8585, Japan, 5Division of Systems Medical Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan, 6Senjyu Laboratory of Ocular Sciences, Senju Pharmaceutical Co., Ltd., 1-5-4, Murotani, Nishiki, Kobe, Hyogo 651-2241, Japan, 7Department of Pediatrics, Yamagata University Faculty of Medicine, Tada-nishi 2-2-2, Yamagata 990-9585, Japan, 8Pediatric Emergency Center, Kitakyusu City Hospital. Nishimotomachi 4-18-1, Yatatodaiji Kitakyusu City, Japan, 9Department of Internal Medicine, Tokyo Metropolitan Fuchu Medical Center for the Disabled, Musashidai, Fuchu-shi, Tokyo, Japan, 10Department of Pediatrics and Institute for Human Genetics, University of California, San Francisco, School of Medicine, San Francisco, CA 94143-0794, 11Department of Pathology, Osaka City University Graduate School of Medicine, Asahi-machi 1-4-3 Abeno, Osaka 545-8586, Japan, 12Division of Cell Biology and Neuroscience, Department of Morphological and Physiological Sciences, Faculty of Medical Sciences, University of Fukui, 13Research Center for Child Mental Development, University of Fukui, 14Research and Education Program for Life Science, University of Fukui.

Toward a therapeutic intervention of lissencephaly, we applied a novel calpain inhibitor, SNJ1945. Peri-natal or post-natal treatment with SNJ1945 rescued defective neuronal migration in Lis1+/− mice, impaired behavioral performance and improvement of 18F-FDG uptake. Furthermore, SNJ1945 improved the neural circuit formation and retrograde transport of NFG in Lis1+/− mice. Thus, SNJ1945 is a potential drug for the treatment of human lissencephaly patients.

Results

SNJ1945 rescued defective distribution of cytoplasmic dynein and membranous components in the cell and defective migration in Lis1+/− neurons. In vitro administration of SNJ1945 protected LIS1 from proteolysis, resulting in the augmentation of LIS1 levels in Lis1+/− mouse embryonic fibroblast (MEF) cells and leading to rescue of the aberrant distribution of cytoplasmic dynein and intracellular components including mitochondria and β-COP positive vesicles (Supplementary Fig. 1a–e). SNJ1945 also rescued defective distribution of cytoplasmic dynein and membranous components in human fibroblasts from an isolated lissencephaly sequence (ILS) patient, suggesting that SNJ1945 will be similarly effective in the human (Supplementary Fig.
In addition, SNJ1945 improved neuronal migration of Lis1<sup>−/−</sup> cerebellar granular neurons (Supplementary Fig. 3). Notably, administration of even large doses did not result in obvious adverse effects on granular neurons (Supplementary Fig. 4). Oral administration of SNJ1945 to pregnant dams resulted in substantial increases of LIS1 levels in the brain of fetuses, as did oral administration directly to perinatal offspring or adults (Fig. 1). Importantly, LIS1 levels increased in the brain three weeks after birth (Fig. 1c, f), indicating that indeed SNJ1945 passed through the BBB and inhibited proteolytic degradation of LIS1. Quantitative determination of drug concentrations in tissue homogenates via liquid chromatography-tandem mass spectrometry (LC-MS/MS) is commonly conducted using the standards. We measured the concentration of SNJ1945 in the brain using LC-MS/MS (Supplementary table 1). LC-MS/MS analysis indicated the brain distribution of SNJ 1945.

To demonstrate whether there was therapeutic benefit in vivo, we designed four different administration approaches (Fig. 2a): (1) intra-peritoneal injection starting at E9.5 (100 µg/g) followed by oral
Figure 2 | Rescue of defective corticogenesis in Lis1\(^{+/−}\) mice by SNJ1945. (a) Administration of SNJ1945 via four protocols (see methods). (b) Apoptotic cell death was examined by TUNEL staining at E15.5. One representative data set is shown (b-a, b, c, d, e). Histogram plots of the relative frequency of TUNEL positive cells to the total number of cells are shown at the bottom (b-f). The staining patterns are representative of different experiments as indicated at the bottom of the panel. \(n\) is the number of examined brains. Statistical examination was performed by unpaired Student’s \(t\)-test. Error bars in graphs were expressed as mean ± SEM. (c) Neu-N staining of mid-sagittal sections of the hippocampus is shown. Severe cell dispersion and splitting of CA3 region were observed in the Lis1\(^{+/−}\) mouse hippocampus (c-c), which improved by the administration of SNJ1945 (c-d, e, f, g). Other examples are shown in Supplementary Figure 6. (d) Cortical phenotypes were examined using a layer specific maker, Brn-1 (layer 2 and 3). The distribution of Brn-1 positive cells is indicated at the right side of each panel (d-a, b, c, d, e, f, g). Brn-1 positive cells were more dispersed in the Lis1\(^{+/−}\) mouse (d-c). Quantitation of the thickness of Brn-1 positive cells is summarized at the bottom (d-h). The staining patterns are representative of different experiments as indicated at the bottom of the panel. Statistical examination was performed by unpaired Student’s \(t\)-test. Error bars in graphs were expressed as mean ± SEM. (e) BrdU birthdating analysis revealed neuronal migration defects in the Lis1\(^{+/−}\) mouse. Quantitation of the thickness of BrdU positive cells is summarized at the bottom (e-o). The distribution of BrdU labeled cells in each bin that was equally divided the cortex from ML to SP. The staining patterns are representative of five different experiments. The shift downward toward the ventricular side as Lis1 was disrupted. Treatment of SNJ1945 rescued neuronal migration to more superficial (later born and further migrating) areas.
administration after birth (200 μg/g), (2) oral administration starting at E9.5 (200 μg/g) followed by oral administration after birth (200 μg/g), (3) oral administration starting in the peri-natal period (200 μg/g) and (4) oral administration starting ten days after birth (200 μg/g). We previously reported a mild reduction of the density of cells in the neocortex of the Lis1+/− mice. At E15.5 when later migrating neurons are generated, a significant acceleration of apoptotic cell death in the ventricular zone was observed. These results prompted us to investigate apoptotic cell death during corticogenesis by TUNEL staining at E15.5 (Fig. 2b). In Lis1−/− mice, apoptotic cell death was clearly increased. In contrast, administration of SNJ1945 suppressed apoptotic cell death in Lis1−/− mice (Fig. 2b). We also examined whether administration of SNJ1945 had any effects on mitosis, since LIS1 is essential for mitotic cell division and neuroepithelial stem cell proliferation. At E13.5, we performed BrdU pulse labeling and found that BrdU incorporation was not significantly different among the five groups (Supplementary Fig. 5), indicating that there was no measureable effect of SNJ1945 on proliferation of neuroepithelial stem cells. We next examined the effect of SNJ1945 on the cortical and hippocampal layering of neurons. Lis1+/− mice exhibited laminar splitting and discontinuities of pyramidal cells in the CA3 and CA2 region of the hippocampus (Fig. 2c), as we previously demonstrated. After administration of SNJ1945 in utero, Lis1−/− mice also displayed splitting and discontinuities in the pyramidal cell layer of the hippocampus, but these defects were markedly improved compared with untreated mice (Fig. 2c and Supplementary Fig. 6a–c). To examine cortical lamination, we analyzed Brn-1 immunoreactivity, to label neurons of layer 2 and 3. In Lis1+/− mice, Brn-1 positive cells (which migrate at later stages) exhibited a broader distribution compared to Lis1+/− mice. Administration of SNJ1945 resulted in more tightly packed layer 2/3 neurons in Lis1−/− mice (Fig. 2d), suggesting that neuronal migration in the cortex was also improved by the inhibition of LIS1 degradation. In both the hippocampus and cortex, oral administration starting postnatally was also partially effective but less effective than when treatment started in utero (Fig. 2c, d and Supplementary Fig. 6a–c). To confirm that the morphological defects observed in Lis1−/− mice were improved by SNJ1945 treatment, we performed quantitative BrdU birthdating analysis. In Lis1−/− mice, the distribution of labeled cells was shifted downward toward the ventricular zone in the cortex, and BrdU-labeling was more diffusely localized (Fig. 2e), as we previously demonstrated. These migration defects associated with the disruption of Lis1 were partially rescued in the presence of SNJ1945 (Fig. 2e). Thus, we concluded that oral administration or intra-peritoneal injection of SNJ1945 in utero are effective in rescuing defective neuronal migration. Importantly, oral administration commencing postnatally was also partially effective, resulting in improvement of brains structure including hippocampus and cortex. In contrast, oral administration starting ten days after birth did not result in any obvious benefits, suggesting that some fraction of neurons was still migrating at the time of birth, which was essentially complete by ten days after birth.

SNJ 1945 partially rescued impaired motor behavior. Lis1+/− mice displayed abnormal behavior and impaired spatial learning. Therefore, we examined whether administration of SNJ1945 to Lis1−/− mice is effective in improving motor behavior compared to untreated Lis1+/− mice. Body weight and temperature were not significantly different among groups, (Supplementary Table 1). As shown in our previous report, Lis1+/− mice exhibited shorter wire-hang latency compared with wild-type mice, and this decreased latency was rescued by SNJ1945 treatment (Fig. 3a). This improvement was most significant in the group in which treatment was started from fetal times (Lis1+/− E9.5). Next, we examined rotarod performance. The latency to fall for the SNJ1945-minus group was significantly less than wild type mice, as we previously reported. Impaired performance on the rotarod test was significantly improved in the SNJ1945-treated groups (Fig. 3b, Supplementary Fig. 7). This functional improvement was also observed not only in the Lis1+/− E9.5 group, but also in the Lis1−/− P0 group, in which treatment was started perinatally. In our previous report, we demonstrated that in utero treatment of a calpain inhibitor from fetal stages improved gait performance. Consistent with this finding, administration of SNJ1945 significantly improved the percent of stride in swing and swing to stance ratios in the Lis1+/− E9.5 group (Fig. 3c). Most importantly, these improvements were also observed in the Lis1+/− P0 and Lis1−/− P10 groups. The Pursolt forced swim test is the most commonly used test for assessment of depression-like behavior in animal models. Mice were placed in an inescapable cylinder half-filled with water to induce the behavioral state of despair. We measured periods of immobility, in which longer immobile times indicated higher degrees of despair (Fig. 3d). We found that Lis1−/− mice displayed significantly increased immobile times compared with controls. SNJ1945 treated Lis1−/− groups displayed immobile times intermediate between Lis1−/− mice and Lis1+/− mice. In particular, improvement close to normal levels was observed in the Lis1−/− E9.5 group. Our findings suggest that SNJ1945 treatment can rescue depression-like status in Lis1−/− mice. It is possible that the rescue of this behavioral abnormalities in Lis1−/− mice by SNJ1945 treatment results from the improved coordination of motor function by SNJ1945. Another possibility is that treatment of SNJ1945 rescued depression-like status in Lis1+/− mice, which led to improvement of behavioral performance.

Small-animal positron emission tomography (PET) was used for evaluation of brain functional improvement by SNJ1945 treatment. We further addressed whether the rescue of defective behaviors in Lis1+/− mice by SNJ1945 treatment was associated with the improvement of brain function. Small-animal imaging studies using PET have been increasingly applied in murine models for drug discovery. We here employed PET imaging with 2-deoxy-2-[18F]-fluoro-D-glucose (FDG) to evaluate brain neural activity based on glucose metabolism. We found aberrations in FDG uptake patterns in Lis1+/− mice that responded to SNJ1945 treatment. PET scans with FDG in human lissencephaly patients demonstrated two layers in the cerebral cortex that could be differentiated based on metabolic activity, corresponding to the inner cellular layer of the lissencephalic cortex and the molecular, outer cellular, and cell-sparse layers, respectively. In our PET imaging, we were not able to observe such a layered pattern in Lis1+/− mice due to the limitation of spatial resolution. Lis1−/− mice displayed similar glucose utilization in the cortex, hippocampus and cerebellum compared to wild type mice (Supplementary Fig. 8). Interestingly, we found significantly reduced glucose utilization in the basal forebrain, hypothalamus and amygdala (Fig. 4a, b). Most importantly, these reductions in FDG uptake were reversed by the treatment with SNJ1945 (Fig. 4a, b). The recovery of glucose metabolism is consistent with the improvement of behavioral performance by treatment with SNJ1945.

SNJ1945 improved neural network formation and facilitated clustering of GABA receptors in amygdala. Because behaviors and brain metabolism are highly linked to the interactive dynamics of neural circuits and synaptic formation in the brain, it is possible that SNJ1945 also rescued defective network formation and synapto genesis in Lis1+/− mice, which might account for the functional improvement of behavioral performance and FDG uptake even without migratory rescue (such as after treatment commencing at P10). To address this question, we injected TdTomato into embryos to visualize neural networks at E16.5. In particular, we have focused on neural networks in amygdala (Fig. 5a). While there were no notable differences in the size of the soma, Lis1+/− mice displayed a striking simplification of dendrites compared to Lis1+/− mice.
Branching and length of each branch were decreased to approximately 29% and 27%, respectively. Importantly, the poor development of dendritic arbors and decreased each length in Lis1\(^{1/2}\) mice were rescued by SNJ1945 treatment (Fig. 5b, c, d, Supplementary Fig. 9), suggesting that neurons in the amygdala partially maintain plasticity, and proper network formation was facilitated by the treatment after P10.

The amygdala has an important role in processing emotional information\(^{21–23}\). Inappropriate processing within the amygdala is thought to induce anxiety disorders. Benzodiazepines, which act specifically on GABA\(_A\) receptors, are commonly used as anxiolytics. This implies that GABAergic synapses within the amygdala may have an important role in inducing or compensating for these disorders. GABA\(_A\) receptors are clustered at synaptic sites to achieve a...
high density of postsynaptic receptors opposite the input axonal terminals. This allows for an efficient propagation of GABA mediated signals, which mostly result in neuronal inhibition. A key organizer for GABAA receptors is gephyrin that forms oligomeric superstructures beneath the synaptic area. In addition, gephyrin plays a crucial role in synaptic dynamics and is a platform for multiple protein-protein interactions and bringing receptors. Thus, we examined the distribution of gephyrin as an indicator for functional GABAA receptors in amygdala. In Lis1+/+ mice, somata and proximal dendrites of amygdala neurons exhibited a variety of gephyrin clusters from very small round puncta to large and bright clusters (Fig. 5b). While Lis1+/- mice displayed similar pattern of gephyrin clusters with Lis1+/+ mice, they were significantly decreased (Fig. 5b, e). Decreased gephyrin clusters in Lis1+/- mice were rescued by SNJ1945 treatment commencing at P10 (Fig. 5b, e). Thus, we concluded that post-natal treatment of SNJ1945 was effective for recovery of defective network formation and decreased receptor distribution in amygdala. SNJ1945 augmented retrograde transport of nerve growth factor (NGF) in dorsal root ganglia (DRG) neurons. Neural growth
factors are crucially important for activity-dependent plastic changes in synaptic strength and network refinement. We assumed that the SNJ1945 dependent rescue of neural network formation and receptor distribution might be attributable to the recovery of retrograde transport of neural growth factors. Retrograde axonal transport of nerve growth factor (NGF) signals is critical for the survival, differentiation, and maintenance of peripheral sympathetic and sensory neurons and basal forebrain cholinergic neurons. To examine retrograde transport of NGF, we used quantum dots (Qd-NGF) to track retrograde transport of NGF in cultures of mouse DRG neurons. Using pseudoTIRF microscopy, we tracked the movement of Qd-NGF in live DRG neurons in real time. We applied non-compartmentalized cultures of DRG neurons, which displayed both directional movements of Qd-NGFs. Live-cell
Figure 6 | Retrograde transport of NGF in DRG neurons. To show that the Qd-NGF complex can be internalized at axon terminals and transported in a retrograde fashion to neuron cell bodies, quantum dots were conjugated to NGF (Qd-NGF) and incubated with DRG neurons followed by pseudoTIRF microscopy examination. (a) Qd-NGF were internalized and transported to the cell body. White dotted line indicates the contour of DRG neurons. DRG neurons from \( Lis1^{+/+} \) mice (a-a), \( Lis1^{+/−} \) mice (a-b) and \( Lis1^{−/−} \) mice with P10 treatment (a-c) are shown. Note: in \( Lis1^{−/−} \) mice, Qd-NGF dots were internalized, but aberrantly accumulated at the tips of DRG neurons (red arrowhead). (b) Each percentage of abnormal accumulation was presented as mean ± SEM. \( n \) indicates the number of examined DRG neurons. Aberrant accumulation was exclusively observed in \( Lis1^{−/−} \) mice, which was rescued by SNJ1945 treatment. (c) Transport dynamics of Qd-NGF containing endosomes. Time-lapse video images of endosomes are shown. Lapsed time is shown at the bottom of panel. Retrograde transport of Qd-NGF containing endosomes in \( Lis1^{−/−} \) mice was decreased (c-b). (d) Trajectories of endosomes from \( Lis1^{+/+} \) mice (d-a), \( Lis1^{+/−} \) mice (d-b) and \( Lis1^{−/−} \) mice with P10 treatment (d-c) are shown. (e) Number of transported endosomes per DRG neuron was presented as mean ± SEM. \( n \) indicates the number of examined DRG neurons. Note: We found that significant reduction of the frequency in \( Lis1^{+/−} \) mice, which was rescued by SNJ1945 treatment. (f) Histograms of velocities of retrograde-directed endosomes. (g) Mean velocities are 0.62 μm/s in \( Lis1^{+/+} \) mice, 0.60 μm/s in \( Lis1^{+/−} \) mice and 0.65 μm/s in \( Lis1^{−/−} \) mice with P10 treatment. There was no significant difference in each group.
imaging revealed that Qd-NGFs were internalized at axon terminals and transported in a retrograde fashion to cell bodies in DRG neurons from \textit{Lis1\textsuperscript{-/-}} mice (Supplementary movie 1). Importantly, Qd-NGFs were internalized, but accumulate aberrantly at tips of DRG neurons from \textit{Lis1\textsuperscript{-/-}} mice (Fig. 6a, b). This aberrant accumulation was rescued by treatment of SNJ1945 (Fig. 6a, b). Our observations were similar with transport defects peroxisomes and endosomes in the genetic absence of \textit{Lis1\textsuperscript{null}} of filamentous fungus \textit{Aspergillus nidulans}\textsuperscript{26}. Next, we characterized the transport of Qd-NGF containing endosomes. Kymographs from time-lapse videos of Qd-NGFs indicated that the retrograde transport of Qd-NGF containing endosomes moved in a stop-and-go manner (Fig. 6c, d, Supplementary movie 1). Strikingly, we found that the frequency of retrograde transport of Qd-NGF containing endosomes was significantly decreased in DRG neurons from \textit{Lis1\textsuperscript{-/-}} mice (Fig. 6c, d, e, Supplementary movie 2), whereas the frequency of anterograde transport of Qd-NGF containing endosomes was not affected (Supplementary Fig. 9). In clear contrast, the velocity of retrograde transport of Qd-NGF containing endosomes was intact in DRG neurons from \textit{Lis1\textsuperscript{-/-}} mice (Fig. 6f, g), which was also consistent with \textit{Aspergillus nidulans}\textsuperscript{26}. We next examined the effect of SNJ1945 in the decreased frequency of the retrograde transport. Importantly, the treatment of SNJ1945 clearly recovered the defective retrograde transport of Qd-NGF containing endosomes (Fig. 6c, d, e, Supplementary movie 3). Presumably, the protection of L1IS1 degradation by SNJ1945 restored proper dynenin distribution, resulting in the recovery of retrograde transport of Qd-NGF containing endosomes, which may stimulate network formation and receptor distribution.

**Discussion**

SNJ1945 is permeable to the BBB, and was effective in rescuing defects in \textit{Lis1\textsuperscript{-/-}} mice after treatment commenced perinatally. These findings suggest that SNJ1945 may be considered for the treatment of lissencephaly patients postnatally. In support of this, we demonstrated that SNJ1945 improved behavioral performances and brain glucose metabolism after treatment ten days after birth without histological rescue of brain disorganization. We also demonstrated that SNJ1945 stimulated network formation and receptor distribution, explaining functional rescue by SNJ1945. These functional rescues are partially attributable to restoration of growth signal, characterized by the recovery of retrograde transport of Qd-NGF containing endosomes. These findings support a potential therapeutic approach with a novel calpain inhibitor, SNJ1945, in the human ILS patient that has a \textit{LIS1} mutation.

**Methods**

Bu\textsuperscript{d}U birthdating and proliferation studies. All mouse experiments were performed under the approval from the experimental animal committee of Osaka City University Graduate School of Medicine or the approval of the experimental animal committee of Osaka City University Graduate School of Medicine, National Institute for Physiological Sciences and Fujita Health University.

For bromodeoxyuridine (Br\textsuperscript{d}U) experiments, pregnant dams (E15.5) were injected with Br\textsuperscript{d}U (50 \textmu g/g, i.p.), and the distribution of Br\textsuperscript{d}U-positive cells was determined at P5. For pulse labeling to examine proliferation of neuroepithelial stem cell, pregnant dams (E13.5) were injected with Br\textsuperscript{d}U (150 \textmu g/g, i.p.). Subsequently, the distribution of Br\textsuperscript{d}U-positive cells was determined one hour after injection. The incorporation of Br\textsuperscript{d}U in cells was detected with a mouse anti-Br\textsuperscript{d}U monoclonal primary antibody (Roche) followed by an alkaline phosphatase–conjugated secondary antibody (Boehringer Mannheim). We analyzed three independent mice for each genotype.

**Histological examination and immunohistochemistry.** After perfusion with 4\% PFA fixative, tissues from wild type and mutant mice were embedded in paraffin and sectioned at 5 \textmu m thickness. After deparaffinization, endogenous peroxidase activity was blocked by incubating the sections in 1.5\% peroxide in methanol for 20 min. The sections were then boiled in 0.01 M citrate buffer, pH 6.0, for 20 min and cooled slowly. Before staining, the sections were blocked with rodent block (LabVision) for 60 min. The sections were washed in PBS and incubated with an anti-Bm-1 antibody (Santa Cruz).

Cell culture and immunocytochemistry. Human fibroblasts were grown in D-MEM supplemented with 10\% FBS. To inhibit calpain, cells were incubated with 200 \textmu M SNJ1945 or control DMSO for 2 hrs. Cells were fixed in 4\% PFA in PBS followed by permeabilization with 0.2\% Triton X-100 in PBS. Coverslips were blocked for one hour with Block Ace (Yukijirushi) in PBS supplemented with 5\% BSA, and were incubated for 1 hr in primary antibody, washed, and incubated for 1 hr using Alexa 546-conjugated secondary antibodies (Molecular Probes). Primary antibodies were an anti-JCOP antibody (Sigma) and an anti-DJC1 antibody (Chemicon).

**Behavioral analysis.** \textit{Lis1\textsuperscript{-/-}} mice and \textit{Lis1\textsuperscript{-/-}} mice that were treated with and without SNJ1945 were used for behavioral experiments, as described in the figure legend for Figure 2a. \textit{Lis1\textsuperscript{-/-}} mice had a single \textit{Lis1} mutant allele. In this study mice were used without the genetic background. All behavioral tests were carried out in a small cage that was at least 9 weeks old at the start of testing. Raw data from the behavioral tests, the date on which each experiment was performed, and the age of the mice at the time of the experiment are shown in the mouse phenotype database (http://www.mousephenotype.org/). Mice were group-housed (four mice per cage) in a room with a 12 h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. The room temperature was kept at 23 ± 2 °C. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all apparatus were cleaned with diluted sodium hypochlorite solution to prevent a bias due to olfactory cues. Three independent experiments were performed for behavioral tests. One group consisted of equal numbers of mice. \textit{Lis1\textsuperscript{-/-}} mice without treatment, \textit{Lis1\textsuperscript{-/-}; Lis1\textsuperscript{-/-}} mice with oral administration from E9.5 (200 \textmu g/g) followed by oral administration after birth (200 \textmu g/g), \textit{Lis1\textsuperscript{-/-}; Lis1\textsuperscript{-/-}} mice with oral administration from peri-natal period (200 \textmu g/g) \textit{Lis1\textsuperscript{-/-}; Lis1\textsuperscript{-/-}} mice with oral administration from ten days after birth (200 \textmu g/g). Experiments were done in the following sequences; the first group (12 each): the general health and social interaction test (Supplementary Fig. 4A), rotarod (RR) and gait analysis (GA); the second group (16 each): GHNS, LD, RR and GA; the third group (24 each): GHNS, LD, open field (OF), elevated plus maze (EP), hot plate (HP), one-chamber social interaction test (SI), RR, Crawley’s sociability and preference for social novelty test (CST), startle response/prepulse inhibition test (PPPI), Porsolt forced swim test (PS), fear conditioning test (FC), tail suspension test (TS), and social interaction test in home cage (HC-SI). Behavioral data were obtained automatically by applications based on the public domain Image J program and modified for each test by Tsuyoshi Miyakawa (available through O’HARA & CO., Tokyo, Japan)! Each behavioral test was separated from each other at least by 1 day. Briefly, the rotarod test, using an accelerating rotarod (UGO Basile Accelerating Rotation, rat, mice performed by panning mouse on rotating drum (mouse at drum speed) and measuring the time each animal was able to maintain its balance on the rod. The speed of the rotarod accelerated from 4 to 40 rpm over a 5-min period. Gait analysis was performed using ventral plane videoography as described. Mice were placed on the treadmill belt that moves at a speed of 24.7 cm/s. Digital video images of the underside of mice were collected at 150 frames per second. The paw area indicates the temporal placement of the paw relative to the treadmill belt. The color images were converted to their binary matrix equivalents, and the areas (in pixels) of the approaching or retreating paws relative to the belt and camera were calculated throughout each stride. Plotting the area of each digital paw print (paw contact area) individually in time provides a dynamic gait signal representing the temporal record of paw placement relative to the treadmill belt. For Porsolt forced swimming test, the apparatus consisted of four plastic cylinders (20 cm height × 10 cm diameter). The cylinders were filled with water (23 °C) up to a height of 7.5 cm. Mice were placed into the cylinders, and their behavior recorded over a 10-min test period. Data acquisition and analysis were performed automatically, using Image PC software (see above). All behavioral testing procedures were approved by the Animal Care and Use Committee of National Institute for Physiological Sciences and Fujita Health University.

**MicroPET scan and data analysis.** PET imaging data were obtained in male mice (20–30 g) using a small animal PET camera (microPET Focus-220, Siemens Medical Systems, Heidelberg, Germany) with a transaxial resolution of 1.4 mm in full width at half maximum. Tissue images were acquired in a 128 × 128 × 95 matrix with a pixel of 0.475 mm and a slice thickness of 0.796 mm. Before PET scanning, mice were intravenously injected with \textit{18F-FDG} (approximately 0.5 MBq/g B.W.) through a cannula inserted into the tail vein and were kept in their home cage for 30 min for the \textit{18F-FDG} uptake under freely moving condition. Subsequently, the mice were placed in the small-animal PET scanner under isoflurane anesthesia (4% for induction and 1.5% for maintenance) with O\textsubscript{2} and N\textsubscript{2}O gas. Static acquisitions were performed during 30 min. PET images were reconstructed using a filtered backprojection (FBP) algorithm. The image data acquired from microPET were analyzed by ASIPro VM (ver. 6.0, Concorde Microsystems Inc.) and PMOD Technologies (ver. 3.4, PMOD Technologies Ltd.) software. The PET and magnetic resonance (MR) images were co-registered using a PMOD software. MR images were obtained from \textit{Lis1\textsuperscript{-/-}} mice and \textit{Lis1\textsuperscript{-/-}} mice used for the PET study under isoflurane anesthesia with a 7 tesla MR scanner (BioSpec 70/20, Bruker). Volumetric regions of interest were placed on the several brain regions (cingulate cortex, hippocampus, thalamus, caudate, amygdala, basal forebrain and septum, brain stem, midbrain, superior colliculus) based on the images. Relative \textit{18F-FDG} uptake was determined by normalized count data to those in the whole brain. Each value was presented as mean ± SEM. Statistical analysis was performed using the SPSS Statistics Student software. Data
were analyzed using one-way ANOVA followed by post hoc Tukey’s test for comparison among groups. Significance threshold was assumed at P < 0.05.

In utero transfection. Expression vectors were introduced into fetal brains by an in utero electroporation-mediated gene transfer method. Briefly, pregnant mice were deeply anesthetized on E16.5, and the uterine horns were exposed. Approximately 2 μl of a transfection solution was injected into the lateral ventricle from outside the uteri with a glass micropipette (GD-1.5, Narishige, Tokyo, Japan). Each embryo in the uterus was then placed between the tweezer-type electrodes described above and electronic pulses (45 V; 50 msec duration) were applied five times at intervals of 950 msec (GU-121, Bexco tbl). The uterine horns were then placed back into the abdominal cavity to allow the embryos to continue normal development. Histological examination was performed 5 days after in utero injection (P2–P3). Histological examination was performed 35 days after in utero injection (P30). SNJ1045 as applied from P10 by oral administration.

Examination of retrograde transport of NGF by pseudoTIRF microscopy and live imaging. NGF was conjugated with Q655 via carboxyl group substitution by using the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Pierce Biotech, Rockford, IL). An inverted microscope (Olympus 1 X 71) was modified for pseudoTIRF illumination. The laser beam (488 nm) was focused at the back focal plane of the objective lens (ApoX 60, 1.49 Oil, Olympus). The incident angle was adjusted to be slightly smaller than the critical angle so that the laser beam could penetrate ~1 µm into the aqueous solution. To image transport of Q4-NGF in live neurons, DRG neurons were incubated with Q4-NGF. Fluorescence images were filtered with a Q655/15 emission filter. Time-lapse images were acquired by using an EMCCD camera (ImageM, Hamamatsu photonics) at the speed of 5–10 frames per second.

**LC-MS/MS analysis.** The SNJ1945 concentration in the brain was determined by turbo ion spray on an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems) equipped with the ion spray source using multiple reaction monitoring (MRM). Chromatography was performed on a NANOSPACE SI-2 HPLC system (Shiseido) with Shiseido Capcell pak C18 MG-II column. The extraction of SNJ1945 from the brain and the measuring condition were described in the literature.

1. Beckmann, N. et al. In vivo mouse imaging and spectroscopy in drug discovery. NMR Biomed 20, 154–185 (2007).
2. Reiner, O. et al. Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit repeats. Nature 364, 717–721 (1993).
3. Hattori, M., Adachi, H., Tsujimoto, M., Arai, H. & Inoue, K. Miller-Dieker lissencephaly lensephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase [corrected]. Nature 370, 216–218 (1994).
4. Vallee, R. B., Tait, C. & Faulkner, N. E. LIS1: cellular function of a disease-causing gene. Trends Cell Biol 11, 155–160 (2001).
5. Wynshaw-Boris, A. Lissencephaly and LIS1: insights into the molecular mechanisms of neuronal migration and development. Clin Genet 72, 296–304 (2007).
6. Yamada, M. et al. LIS1 and NDEL1 coordinate the plus-end-directed transport of cytoplasmic dynein. Embo J 27, 2471–2483 (2008).
7. Yamada, M. et al. Inhibition of calpain increases LIS1 expression and partially rescues in vivo phenotypes in a mouse model of lissencephaly. Nat Med 15, 1202–1207 (2009).
8. Koumura, A. et al. A novel calpain inhibitor, (1S)-3-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl-3-methylbutyl) carbamic acid 5-methoxy-3-oxapentyl ester, protects neuronal cells from cerebral ischemia-induced damage in mice. Neuroscience 157, 309–318 (2008).
9. Shirasaki, Y., Yamaguchi, M. & Miyashita, H. Retinal penetration of calpain inhibitors in rats after oral administration. J Ocul Pharmacol Ther 22, 417–424 (2006).
10. Oka, T. et al. Amelioration of retinal degeneration and proteolysis in acute ocular hypertensive rats by calpain inhibitor (1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl-3-methylbutyl)carbamic acid 5-methoxy-3-oxapentyl ester. Biochem Pharm 77, 475–486 (2008).
11. Hirotsune, S. et al. Activation of Aurora-A is Essential for Neuronal Migration via Modulation of Microtubule Organization. J Neurosci 32, 11050–11166 (2012).
12. Davis, M., Walker, D. L. & Myers, K. M. Role of the amygdala in fear extinction measured with potentiated startle. Ann N Y Acad Sci 985, 218–232 (2003).
13. Pare, D., Quirk, G. J. & Ledoux, J. E. New vistas on amygdala networks in conditioned fear. J Neurophysiol 89, 1–2 (2004).
14. Takahashi, H. et al. Selective control of inhibitory synapse development by Slitrk3-PTPdelta trans-synaptic interaction. Nat Neurosci 15, 398–399, 538–382 (2012).
15. van Versendaal, D. E. et al. Elimination of inhibitory synapses is a major component of learning. Neuron 67, 713–723 (2010).
16. Chhatwal, J. P., Myers, K. M., Resler, K. J. & Davis, M. Regulation of gephyrin and GABAA receptor binding within the amygdala after fear acquisition and extinction. J Neurosci 25, 502–506 (2005).
17. Cui, B. et al. At one time, live tracking of NGF axonal transport using quantum dots. Proc Natl Acad Sci U S A 104, 13666–13671 (2007).
18. Egan, M. J., Tan, K. & Keck, M. E. LIS1 is an activator factor for dynexin-driven organelle transport. J Cell Biol 197, 971–982 (2012).
19. Yamasaki, N. et al. Alpha-CalMKL deficiency causes immature dentate gyrus, a novel candidate endophenotype of psychiatric disorders. Mol Brain 1, 6 (2008).

**Acknowledgements**

We thank Azusa Inagaki, Kaori Nakakubo, Yukimi Kira and Yoriko Yabunaka for technical support, Hiromichi Nishimura and Keiji Fujimoto for mouse breeding. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan from the Ministry of Education, Science, Sports and Culture of Japan to Shinji Hirotsune and Knowledge Cluster Initiative (Stage-2) Research Foundation to Shinji Hirotsune. This work was also supported by Takeda Science Foundation and the Sumitomo Foundation to Shinji Hirotsune and NIH grants NS41030 and HD47380 to Anthony Wynshaw-Boris. This work was also supported in part by Grant-in-Aid for Scientific Research(B) of Japan Society for the Promotion of Science (JSPS) (22088056 to Masami Yamada). Accessible and Seamless Technology Transfer Program (A-STEP) through Target-driven R&D, Japan Science and Technology Agency (AS23120224G to Masami Yamada), Grant-in-aid for Scientific Research on Innovative Areas of The Ministry of Education, Culture, Sports, Science and Technology (MEXT) (#11001981 to Masami Yamada) and The Uehara Memorial Foundation (to Masami Yamada). This work was also supported by KAKENHI (Grant-in-Aid for Scientific Research on Young Scientists A (16680015), Scientific Research (B) (21300121, 21390069), Exploratory Research (19653081), Integrative Brain Research (IBR-shin), and Innovative Areas (Comprehensive Brain Science Network) from the Ministry of Education, Science, Sports and Culture of Japan (MEXT) of Japan, grant from Neuroinformatics Japan Center (NIT), grants from CREST of Japan and Technology Agency (IST), and the Funding Program for Next Generation World-Leading Researchers (Next Program). This work was also supported by the Multidisciplinary program for elucidating the brain development from molecules to social behavior (Fukui Brain Project) and the Grants-in-Aid for Scientific Research and Strategic Research Program for Brain Sciences (“integrated research on neuropsychiatric disorders”) by the Ministry of Education, Culture, Sports, Science and Technology of Japan to Makoto Sato. We declare no conflicts of interests.

**Author contributions**

S.H. performed mouse histological examination and mouse behavioral analysis. M.Y. performed mouse histological examination. K.T., S.H. and T.M. performed mouse behavioral analysis (Figure 3). Y.K. and Y.T. performed PET analysis (Figure 4). K.K., Y.O., M.Y. and T.T. performed mouse histological examination and mouse behavioral analyasis (Figure 3). Y.K. and Y.T. performed PET analysis (Figure 4). M.A. provided us SNJ1945. K.H., M.A., K.T. and M.K. provided us a tubeflow cell line from the human lissencephaly patient. A.W.-B. and S.H. organized experiments and wrote a manuscript.

**Supplementary information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**License:** This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/