Defective Craniofacial Development and Brain Function in a Mouse Model for Depletion of Intracellular Inositol Synthesis

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myo-Inositol is an essential biomolecule that is synthesized by myo-inositol monophosphatase (IMPase) from inositol monophosphate species. The enzymatic activity of IMPase is inhibited by lithium, a drug used for the treatment of mood swings seen in bipolar disorder. Therefore, myo-inositol is thought to have an important role in the mechanism of bipolar disorder, although the details remain elusive. We screened an ethyl nitrosourea mutant mouse library for IMPase gene (Impa) mutations and identified an Impa1 T95K missense mutation. The mutant protein possessed undetectable enzymatic activity. Homozygotes died perinatally, and E18.5 embryos exhibited striking developmental defects, including hypoplasia of the mandible and asymmetric fusion of ribs to the sternum. Perinatal lethality and morphological defects in homozygotes were rescued by dietary myo-inositol. Rescued homozygotes raised on normal drinking water after weaning exhibited a hyper-locomotive trait and prolonged circadian periods, as reported in rodents treated with lithium. Our mice should be advantageous, compared with those generated by the conventional gene knock-out strategy, especially for understanding the molecular mechanisms underlying the clinical effect of lithium and myo-inositol-mediated skeletal development.

Lithium salts are used as a first-line drug to treat psychiatric illnesses, particularly bipolar (manic depressive) disorder. Evidence indicates that the mood-stabilizing action of lithium is mediated by inhibiting myo-inositol monophosphatase (IMPase, EC 3.1.3.25) activity, thereby inducing intracellular inositol depletion (1–3). IMPase generates myo-inositol, a substrate for the membrane phospholipid phosphatidylinositol, from inositol monophosphate species, which are produced in cells by the multistep dephosphorylation of higher inositol phosphates (“recycling” of inositol) or by the isomerization of D-glucose 6-phosphate (“de novo synthesis” of inositol). Mammalian cells express IMPase 1 and IMPase 2, which are encoded by Impa1/IMPA (4, 5) and Impa2/IMPA2 (6, 7), respectively. Their primary structures are closely related to each other, whereas their three-dimensional structures and enzymatic characteristics vary slightly but significantly (8–10). Importantly, IMPase 1 is more sensitive to lithium inhibition than IMPase 2 in our in vitro assay. This finding strengthens the importance of IMPase 1 as a bona fide target for lithium therapy. However, genetic variations in IMPA2, but not in IMPA1, have been implicated in multiple neuropsychiatric diseases, including schizophrenia (11), bipolar disorder (12, 13), and febrile seizures (14), suggesting a role for the IMPA2 gene in the genetic risk for these illnesses. Although these lines of evidence support critical roles for IMPase and myo-inositol in maintaining normal brain function, it still remains unclear whether and how inositol depletion mediates the therapeutic efficacy of lithium or how intracellular synthesis of myo-inositol impacts the normal development of the brain and other organs. To clarify...
this point, establishing reliable animal models in which the biological effects of inositol depletion are easily detectable as phenotypes is essential.

The potent mutagen, N-ethyl-N-nitrosourea (ENU) primarily causes single base substitutions. We generated a library of ENU-mutated mice and employed a high throughput screening system to detect mutations. We refer to these techniques collectively as RIKEN ENU-based gene-driven mutagenesis system (RGDMS) (15–18). Using RGDMS to generate and analyze mice with ENU-induced mutations, we uncovered unexpected functions for Impa1 in the development of the skull, as well as in mouse behavior.

EXPERIMENTAL PROCEDURES

Mice—All protocols using animals were approved by the Animal Experiment Committee of RIKEN. Mice were housed in groups under constant temperature and humidity with a 12-h light/dark cycle (lights on at 08:00 h). They had ad libitum access to standard lab chow and water. In the rescue experiment, 2% myo-inositol in drinking water was provided to dams until weaning.

Screening of the ENU Mutant Mouse Library—The basic concept of our mutant mouse screening system (RGDMS) is shown in Fig. 1A. The library for Impa mutant mice was screened by PCR using the primer sets, followed by temperature gradient capillary electrophoresis. The information for used primers is available upon request. The mutations identified by temperature gradient capillary electrophoresis were confirmed by Sanger sequencing. Ova from normal mice were fertilized in vitro with sperm stocks harboring one of the identified missense mutations, and thezygotes were implanted in the uteri of female mice to create heterozygous (G2) mice. Founder mice were crossed at least six times with inbred C57BL/6N females (Japan SLC, Shizuoka, Japan) to dilute the original genetic background and irrelevant mutations. Heterozygous males and females were mated to generate homozygotes. The mutant strains are available from the RIKEN BioResource Center under the RBRC numbers shown in Table 1.

Genotyping of Mutant Mice—Genotyping of the Impa1 missense mutants (F81L, T95K, and T96A) was performed as follows: genomic DNA purified from mouse tails was amplified using the primer set forward primer 5'-CTTCATCGCTTGTCTATTATTATCATCCTC-3' and reverse primer: 5'-TTGTC- CTTGGTCCTCCACAGCTTTAGA-3'. Amplicons were sequenced directly, using the forward primer and the BigDye Terminator Version 3.1 cycle Sequencing kit (Invitrogen).

In Vitro Enzyme Assays of Recombinant Proteins—IMPase assays were performed as described (8). In brief, a DNA fragment spanning the open reading frame of mouse Impa1 was amplified from mouse brain Marathon-Ready cDNA (TaKaRa Bio, Ohtsu, Japan), using the primer set mLM1-Fw1, 5'-GTGCGCTCGGC-GAGATAATGGGACAG-3', and mLM1-Rv1, CCCAGGGACAGCA- GCAAGGATGACACTGGA-3', followed by a second PCR assay with the primer set mLM1cds-Fw-EcoRV, 5'-AGTGAGATATCA- ATGGGCAGACCTTTGGCAGAGA-3', and mLM1cds-Rv- Xhol, AGTGACCTGAGCAGCTTTCGTGTCCTCTTGTG-3' (underline sequences denote restriction enzyme recognition sites) to introduce restriction enzyme recognition sites into the PCR product. After digestion with EcoRV and Xhol, the resultant fragment was cloned into the EcoRV/Xhol site of SR-HA, a mammalian expression vector (19) that expresses proteins with N-terminal HA tags, generating SR-HA-mImpa1 WT. Site-directed mutagenesis following the standard DpnI method was performed to produce the three Impa1 mutant constructs as follows: SR-HA-mImpa1 F81L, SR-HA-mImpa1 T95K, and SR-HA-mImpa1 T96A. The nucleotide sequence of each construct was verified. Human kidney-derived HEK293T cells were transfected with one of these three constructs or SR-HA (empty vector), using the calcium phosphate method, and then cultured for 2 days. The HA-tagged recombinant proteins were purified from lysates to near-homogeneity using HA antibody affinity beads. Protein preparations were analyzed using SDS-PAGE, followed by silver staining and Western blotting with an anti-HA antibody.

Bone and Neurofilament Staining—T95K heterozygous male and female mice were intercrossed, and pregnant females were euthanized by cervical dislocation at E10.5, E14.5, or E18.5. Fetuses were removed quickly from uteri, and the tissue samples were harvested. E18.5 and E14.5 fetuses were stained with Alcian blue and alizarin red (20) to visualize bones. E10.5 embryos were subjected to whole-mount immunohistochemistry using an anti-neurofilament antibody (clone 2H3, Developmental Studies Hybridoma Bank, Iowa City, IA) and standard procedures to visualize neural fiber organization. Immune complexes were detected using a combination of anti-mouse IgG labeled with horseradish peroxidase and 3,3'-diaminobenzidine. Western blotting of the tissue extract was performed as described (8). Hematoxylin and eosin staining of brain paraffin sections was performed according to a standard procedure.

Behavioral Tests—Mice were 3–5 months old when tested. Behavioral tests were conducted as described (21, 22), except for circadian rhythm, which was evaluated using a wheel-running apparatus (23) with minor modifications. In brief, individual mice were housed in cages (28 cm wide × 12 cm deep × 15 cm high) equipped with a steel wheel (5.5 cm wide × 15 cm in diameter). For the assessment of circadian rhythm, wheel-running activity was monitored using a computer (O’Hara & Co., Tokyo, Japan) during regular light-dark cycles. Light intensity was set to 150 lux. The ClockLab software (Actimetrics, Wilmette, IL) was used to determine the circadian period.

Statistical Analysis—We used Student’s t test to compare the two groups subjected to behavioral examinations. When data show a biased distribution, the nonparametric (Mann-Whitney U) test was used. When necessary, data were analyzed using two-way repeated measures analysis of variance followed by post hoc Fisher’s protected least significant difference test. The segregation ratio of pup genotypes was tested for significance using the χ² test. A p value <0.05 was defined as significant.

RESULTS

Screening of the ENU mouse library for mutations in coding exons and flanking intron sequences of Impa1 and Impa2 (Fig. 1A) led to the identification of 17 mutations (12 in Impa1 and 5 in Impa2) (Table 1), of which four were missense (nonsynonymous) mutations (Impa1: F81L, T95K, and T96A; Impa2: I282T) (Fig. 1, B and C, and Table 1). The Ile-282 residue is conserved between human and mouse IMPase homologs (Fig. 1B).
1C, green dot), raising the possibility that it impacts the biological function of IMPase 2. However, because Impa2 knock-out (KO) mice lack a detectable phenotype (21), we focused on the three Impa1 mutations. In Fig. 2A, the positions of the mutant amino acid residues are mapped on the crystal structure of the mouse IMPase 1 homodimer (Protein Data Bank 4AS5) (24). The Thr-95 and Thr-96 residues (Fig. 1C, red and yellow dots, respectively) are close to the catalytic site, with Thr-95 being conserved between human and mouse IMPase homologs. The T95K mutation introduces a positive charge, potentially affecting conformation and enzymatic activity. In contrast, the substitution of Leu for Phe-81 may not have a deleterious effect as it is distant from the catalytic site and close to the surface (Fig. 2A). Moreover, Leu occupies a position corresponding to the mouse Impa1 Phe-81 residue in human IMPase 1 and IMPase 2 and in mouse IMPase 2 (Fig. 1C, blue dot). The PolyPhen-2 software tool (25) employs a three-step grading system to predict the impact of given mutations on the biological function of a protein as follows: benign, possibly damaging, and probably damaging. PolyPhen-2 predicted that the three missense mutations, F81L, T95K, and T96A, were benign, probably damaging, and possibly damaging, respectively (Table 1). These analyses strongly support the conclusion that the Lys-95 mutation may exert the strongest effect on biological function.

To test this possibility, wild-type and mutant HA-tagged IMPase 1 recombinant proteins were affinity-purified from cDNA-transfected HEK293T cells (Fig. 2B) and then tested for activity using a published method (8). Consistent with in silico

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**FIGURE 1. ENU-induced mutations in Impa1.** A, schematic representation of the gene-driven ENU mutagenesis system. The library was screened for mutations in the two IMPase genes. B, sequence data for genomic DNA from three Impa1 mutants. Note that each mutant strain (G0) is a heterozygote for the corresponding mutation. Arrows indicate the position of the mutation. C, amino acid sequence alignment of mouse and human IMPases. Identical and conserved amino acids are highlighted in black and gray, respectively. Positions of missense mutations are indicated by colored dots as follows: blue, Impa1 Phe-81; red, Impa1 Thr-95; yellow, Impa1 Thr-96; and green, Impa2 Ile-282.
results, the Lys-95 (T95K) mutant lacked detectable activity (Fig. 2C). In contrast, there was no significant difference between the activity of wild-type and Ala-96 (T96A) proteins. Interestingly, the Leu-81 (F81L) mutant produced higher activity compared with the wild-type protein.

Next, we investigated the phenotypes of mice harboring these three mutations. Heterozygous mice (G (generation) 2 mice shown in Fig. 1A) were generated by in vitro fertilization. We detected no abnormalities in the heterozygotes (data not shown). Therefore, they were backcrossed to the inbred

| Gene | Mutant allele | Stock no. | Position | Exon/intron | Amino acid change | PolyPhen-2 |
|------|---------------|-----------|----------|-------------|-------------------|------------|
| Impa1 | Rgsc01422 | RBRC-GD000153 | c.85G>A | Intron 1 | | |
| | Rgsc01496 | RBRC-GD000155 | c.44T>A | Intron 1 | | |
| | Rgsc01494 | RBRC-GD000154 | c.63+46G>A | Intron 2 | | |
| | Rgsc01411<sup>a</sup> | RBRC-GD000162 | c.197+55C>T | Intron 3 | | |
| | Rgsc00210 | RBRC-GD000158 | c.261T>C | Exon 4 | | |
| | Rgsc001846 | RBRC-GD000159 | c.284C>T | Exon 4 | | |
| | Rgsc001827 | RBRC-GD000160 | c.286A>G | Exon 4 | | |
| | Rgsc01639 | RBRC-GD000157 | c.322T>C | Exon 4 | | |
| | Rgsc01835 | RBRC-GD000161 | c.302+94G>A | Intron 4 | | |
| | Rgsc001418 | RBRC-GD000151 | c.303+4T>A | Intron 4 | | |
| | Rgsc001495 | RBRC-GD000156 | c.458+101A>G | Intron 6 | | |
| | Rgsc001423 | RBRC-GD000152 | c.566+43C>T | Intron 7 | | |
| Impa2 | Rgsc001365 | RBRC-GD000146 | c.103–62C>T | Intron 1 | | |
| | Rgsc001384 | RBRC-GD000149 | c.342–29A>G | Intron 3 | | |
| | Rgsc001373 | RBRC-GD000150 | c.387+21T>C | Intron 4 | | |
| | Rgsc001363 | RBRC-GD000147 | c.496+85C>T | Intron 5 | | |
| | Rgsc001362 | RBRC-GD000148 | c.845T>C | Exon 8 | I282T | Possibly damaging |

<sup>a</sup> Mutant strains are available from RIKEN Bioresource Center.

<sup>b</sup> This mutation was found in other G1 mice derived from the same G0 male, indicating that this mutation existed in the G0 male and was transmitted to G1 mice.
C57BL/6N strain for six generations to dilute unrelated mutations on a homogeneous genetic background (Fig. 1A). Heterozygous males and females from the three strains were mated, and the genotypes of the offspring were determined at weaning. T95K heterozygotes grew normally (Fig. 2D), but only one homozygote (1/84 (1.2%)) was identified (Table 2), implying an essential role for IMPase 1 activity in survival. The lone homozygote, which was undersized and weak, died shortly after weaning (data not shown). In contrast, homozygous and F81L and T96A mice were present at the expected segregation ratio without any visible phenotypic changes (Fig. 2D and data not shown). We therefore focused on the Impa1 T95K (Lys-95) strain.

To determine which Lys-95 homozygous pups die, we again intercrossed heterozygous T95K mice and determined the segregation ratios of offspring genotypes at E18.5. There were significantly fewer homozygous embryos at E18.5 than expected (30/204 (14.7%), $\chi^2 = 0.0435$, $p = 0.83$) (Table 2). The body sizes of the surviving homozygotes (Fig. 3A, denoted as Homo) ranged from being slightly to significantly smaller than those of wild-type (denoted as Wild) and heterozygous (denoted as Het) littermates. The head to body ratio of homozygotes appeared smaller than wild-type controls (Fig. 3A, and data not shown). Surprisingly, the lower jaws of homozygous fetuses were abnormally shorter (mandibular micrognathia) than those of wild-type controls (Fig. 3A, and data not shown), indicating aberrant craniofacial development. Moreover, homozygotes had poorly developed tongues (Fig. 3, A and B) and mandible hypoplasia, revealed by Alcian blue and alizarin red staining (Fig. 3C). These cranial malformations showed 100% penetrance. The severity of mandible hypoplasia did not correlate with body size (Fig. 3A, and data not shown), supporting the conclusion that the mandible phenotype was not a result of delayed growth. Notably, we found no clear defects in the upper jaw or palate closure of homozygotes (Fig. 3, A and B). In addition to lower jaw malformation, ~30% of homozygotes exhibited asymmetric sternum-rib fusion (Fig. 3D). Two homozygous pups exhibited exencephaly (data not shown), and one exhibited a cleft palate (data not shown). However, because the number of these phenotypic abnormalities was not statistically significant, we could not eliminate the possibility that the mutations were not causal. The homozygotes that survived until E18.5 stopped moving within a few minutes after Caesarean section delivery, whereas wild-type and heterozygous littermates continued to move and were resuscitated. Because abnormal peripheral neuronal development is a major cause of paralysis after delivery, we examined neural fiber formation in E10.5 embryos by whole-mount immunohistochemistry, with an anti-neurofilament antibody. We found no detectable differences in neural organization between wild-type and homozygous embryos (Fig. 4A). At E10.5, embryo genotypes from heterozygote intercrossing were present at the expected segregation ratios (Table 2), and there were no visible morphological abnormalities in homozygous embryos (Fig. 4A). These data show that the retardation in mandible formation appears slowly after E10.5 in homozygotes. To investigate this point, we examined heterozygote intercrosses at E14.5. Even at this stage, we were clearly able to discriminate homozygotes from wild-type and heterozygous littermates, based on hypoplasia of the lower jaw (Fig. 4B, yellow arrowheads) and the Meckel’s cartilage (Fig. 4B, red arrowheads), which is laid down prior to mandible formation in the developmental process. We detected no evidence of biased genotype distribution in offspring at E14.5 (Table 2). Importantly, with the exception of the mandible, the gross architecture of the skull, including the maxilla, appeared to be normal (Fig. 4B). Collectively, these results indicate that the cause of mandible hypoplasia is most likely due to abnormal differentiation of the mandibulocranial process from the first pharyngeal arch.

To test whether the Lys-95/Lys-95 phenotypes were caused by a reduction in cellular myo-inositol levels, we supplemented the drinking water of heterozygous females with 2% myo-inositol before mating. myo-Inositol supplementation, which continued until weaning (Fig. 5A), significantly enhanced the survival of homozygotes (127/598, 21.2%) (Table 2). The survivors showed no visible abnormalities in craniofacial development (Fig. 5, B and C) or gross brain structure (Fig. 5D). Moreover, we found no homozygous fetuses with malformation of the rib cage (Fig. 5B). Based on these lines of evidence, we postulate a direct relationship between developmental defects in homozygotes and a relative reduction of cellular myo-inositol. The levels of Impa1 mRNA did not differ in various tissues among the

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**Table 2**

Distribution of offspring’s genotypes by mating between Impa1 Lys-95 heterozygotes

| Stage                      | Wild       | Het       | Homo      | Sum | $\chi^2$ test (df = 1) |
|----------------------------|------------|-----------|-----------|-----|------------------------|
| **Standard diet during pregnancy and lactation** |            |           |           |     |                        |
| E10.5                      | Observed   | 12        | 23        | 11  | 46                     |
|                            | Expected   | 11.5      | 23        | 11.5| 46                     |
| E14.5                      | Observed   | 9         | 20        | 7   | 36                     |
|                            | Expected   | 9         | 18        | 9   | 36                     |
| E18.5                      | Observed   | 68        | 136       | 30ab| 204                    |
|                            | Expected   | 58.5      | 117       | 58.5| 204                    |
| Weaning                    | Observed   | 30        | 53        | 1   | 84                     |
|                            | Expected   | 21        | 42        | 21  | 84                     |
| **Inositol supplementation during pregnancy and lactation** |            |           |           |     |                        |
| E10.5                      | Observed   | 8         | 26        | 12  | 46                     |
|                            | Expected   | 11.5      | 23        | 11.5| 46                     |
| Weaning                    | Observed   | 146       | 325       | 127 | 598                    |
|                            | Expected   | 149.5     | 299       | 149.5| 598                    |

Note: $a,b$ All fetuses/embryos show mandible hypoplasia.

Data include one dead fetus with a normal body size and mandible hypoplasia.
Mouse Model for Intracellular Inositol Depletion

three genotype cohorts fed water, instead of myo-inositol after weaning (data not shown), indicating that the mutation had no effect on the transcription or stability of Impa1 mRNA. Homozygotes and wild-type controls consistently expressed similar levels of IMPase 1 protein (Fig. 5E). Neither Impa2 mRNA nor protein levels were elevated to compensate for deficits in IMPase 1 activity (Fig. 5E and data not shown). The rescued homozygotes died from unknown causes before the wild-type controls (Fig. 5F).

As stated earlier, IMPase 1 is the likely molecular target for lithium when used as a mood stabilizer. Therefore, we explored whether loss of IMPase 1 activity could mimic behavioral changes seen in animals given lithium, using inositol-rescued adult mice (Fig. 5A). We examined mice of both sexes, aged 12 weeks and older, using a comprehensive battery of behavioral tests (Table 3) covering multiple domains of the brain functionality, such as motor function, affective traits, and cognitive and sensorimotor gating functions (26–31). Many researchers have detected antidepressant-like effects for lithium when administered to rodents in various experimental paradigms, including the forced swim and tail suspension tests. Both of these are basic tests to evaluate manic or depressive moods in rodents, where the antidepressant-like effect of lithium is detected as decreased immobility time. Impa1 T95K homozygotes of both sexes exhibited hyperactivity, collectively determined based on the following observations: 1) increased total distances traveled in the dark- and light-box test (Fig. 6A); 2) decreased immobility time in the forced swim test (Fig. 6B); 3) increased entry number in the Y-maze test (Fig. 6C), and 4) reduced freezing behavior on the conditioning day of the fear-conditioning test (Table 3). We also observed a significant increase in the total distance in the elevated plus-maze test and in daytime locomotor activity in the home cage for females, as well as an increased tendency for both measures in males. In addition, we detected increased locomotor activity in the open field test for males (Fig. 6D), whereas there were no significant differences between homozygotes and wild-type controls during the first 10 min of the open field test (Table 3 and Fig. 6D).

Because Impa1 is highly expressed in mouse cerebellar Purkinje cells (8), we examined whether Impa1 Lys-95 homozy-
homozygotes may be prolonged if lithium targets IMPase 1 in vivo. Our homozygotes showed a normal activity rhythm under regular 12-h light:12-h dark conditions (Fig. 7A). Interestingly, we observed that both the onset and offset (Fig. 7B, red and blue arrowheads, respectively) of the free-running activity rhythms of homozygotes were gradually delayed compared with that of the wild types under constant darkness (DD) (Fig. 7B). This trend was clearly visible between DD cycles 11 and 13 (Fig. 7C).

We analyzed this same data set and determined the free-running circadian period, and we found that mutants showed significantly lengthened circadian periods (Fig. 7D). It is highly plausible that the delayed timing of activity rhythms induced by this mutation is the result of prolonged circadian periods. Importantly, a similar prolonging circadian period effect has been reported repeatedly in animals, including rodents treated with lithium, as we will discuss later.

In addition to the beneficial effects on mood, lithium therapy frequently causes multiple side effects, including poisoning, making it essential to monitor and control lithium serum concentrations. Some undesirable effects of lithium can potentially be mediated by inhibiting IMPase. The typical side effects of lithium therapy, including tremor and polyuria, were not exhibited by the homozygote mice (data not shown), and there were no detectable abnormalities in blood cell counts or blood biochemistry (data not shown). Because rescued homozygotes had a shorter life span compared with wild-type controls (Fig. 5F),

**TABLE 3**

**Summary of behavioral test battery of homozygotes**

All the analyses were done using 8–24 mice/genotype. NS means not significant in male and female; ASR means acoustic startle response; CS means conditioned stimulus; US means unconditioned stimulus; PP means prepulse; P means pulse.

| Test                        | Measure            | Difference from wild-type control |
|-----------------------------|--------------------|-----------------------------------|
| Open field (10 min)         | Total distance     | NS*                               |
| Home cage activity          | Total activity (24 h) | NS*                             |
|                             | Day time (12 h)    | NS*                               |
| Elevated plus maze          | Open stay (%)      | Increased*                        |
| Light/dark transition       | Light distance (cm) | NS*                               |
| Tail suspension             | Immobility (%)     | Decreased*                        |
| Forced swim                 | Immobility time (5 min) | Decreased*                     |
| Y maze                      | Alteration (%)     | NS*                               |
| Fear conditioning           | Conditioning       | Decreased*                        |
| (freezing (%))              | Contextual         | NS*                               |
|                            | Cued (pre-CS)      | NS*                               |
|                            | Cued (post-CS)     | NS*                               |
| Prepulse inhibition         | 74/120 (PP/P db) (%) | NS*                          |
|                            | 80/120 (PP/P db) (%) | NS*                          |
|                            | 86/120 (PP/P db) (%) | NS*                          |
| ASR at 120 db               |                    | NS*                               |

* Student’s t test.

* Difference was detected only in males.

* Difference was detected only in females.

* Mann-Whitney’s U test.

* Significant difference was detected in both sexes.

/ Repeated measures two-way analysis of variance (genotype effect).
we cannot exclude the possibility that the premature deaths were related to side effects of lithium therapy.

**DISCUSSION**

In this study, using the powerful and robust RGDMS system, we demonstrated in mice that IMPase 1 activity is required for normal development of the mandible and rib cage, as well as normal brain function. Homozygous Impa1 mutants (Lys-95) rarely survived until weaning, and none survived longer than 7 min after Caesarean delivery at E18.5. It is highly likely that they would still die shortly after birth following a normal gestation period. Of note, Smit1 (sodium myo-inositol transporter 1) KO mice die of congenital central apnea, caused by abnormal respiratory rhythmogenesis (34, 35). Considering the phenotype of Smit1 KO mice, the lack of IMPase 1 activity in Lys-95 mutants could cause central apnea due to abnormal neuronal development, resulting from reduced intracellular myo-inositol concentrations.

We have already reported the spatial expression patterns of Impa1 and Impa2 in embryonic stages (E9.5 and E10.5) (21), as well as brain expression during early post-natal stages (P7 and P21) (8). Those results showed Impa1 is expressed in various areas of the mouse, including the first brachial arch that generates the lower and upper jaws. Although the maxilla (upper jaw) and palate do not appear to be severely affected, the process of mandibular formation is specifically altered in homozygotes. In fact, Meckel’s cartilage, an essential structure for mandibular formation is poorly developed in homozygotes at E14.5 (Fig. 4B). In conjunction with the observation that Smit1 knock-out mice also display a deficiency in osteogenesis (36), these data support a crucial role for free myo-inositol in bone formation. Intriguingly, we observed no abnormalities in limb formation (Figs. 3A and 4), although the expression of Impa1 was relatively high in limb buds during embryogenesis (21). A possible explanation could be that expression of inositol transporters such as SMIT and HMIT masks the deleterious effect of Impa1 deficiency during limb development. Supporting this idea is the observation that Smit1 knock-out mice have shorter limbs (36). Some genetically engineered mice (e.g. Dlx5/6, Zic2, Chd, and Nog KOs) also show abnormal jaw formation (37–39), and some knock-out animals (Dppa4, Ephrin B1, and EphB2/EphB3) exhibit asymmetric fusion of ribs to the sternum (40, 41). Whether and how these morphogenic genes are involved in inositol or inositol phosphate metabolism remain to be determined.

Here, we show that Impa1 Lys-95 homozygotes were more active in various behavioral tests, compared with their wild-type littermates, demonstrating that IMPase 1 activity is required for normal brain function. In keeping with our results, Cryns et al. (42). reported that Impa1 KO mice are hyperactive in the open field test and exhibit shorter immobility times in the forced swim test compared with control mice. The hyperactivity and altered circadian control seen in our homozygotes were not restored by dietary supplementation with 4% myo-inositol during adulthood (Fig. 8, A–C). Although the simplest explanation may be that loss of cellular inositol in the adult brain does not precipitate behavioral deficits, we cannot exclude the possibility that myo-inositol ingested by these adult animals did not sufficiently penetrate the blood-brain barrier, possibly due to its hydrophilic nature. It should be noted that Cryns et al. (42) did not detect a significant reduction in myo-inositol content within the brains of adult Impa1 knock-out mice supplemented with myo-inositol, up to the stage of weaning.
Lithium salts are a first-line drug therapy for bipolar patients, preventing extreme mood swings. In addition, they are often used to treat refractory depression. When administered to normal mice, lithium promotes antidepressant-like effects in multiple behavioral tests, such as the forced swim and tail suspension tests. As stated earlier, Impa1 Lys-95 homozygotes and Impa1 KO mice also exhibit hyper-locomotion. These results support the idea that inhibition of IMPase 1 activity exerts an antidepressant-like effect, similar to that of lithium in rodents and humans. Although mammals express IMPase 2, its activity was only slightly inhibited by lithium in our in vitro assay system (8), and Impa2 KO mice do not exhibit significant behavioral changes (21, 43). Pretreating mice with lithium lowers the threshold for the convulsant effect of pilocarpine, and Impa1 KO mice show greater sensitivity to pilocarpine (42). The Impa1 Lys-95 homozygotes, despite low penetrance, develop an epileptic phenotype without pretreatment with pilocarpine and lithium. In totality, these results support the conclusion that IMPase 1 may serve as a molecular target for lithium, in the lithium-pilocarpine model.

Molecular genetics studies suggest pathophysiological associations between mood disorders and circadian dysregulation, including abnormal period and phase of rest-activity rhythms (44–49). This is the first study showing a potential role for the free inositol-producing system in the regulation of activity rhythm. We discovered that shutdown of the IMPase 1 pathway led to a prolonged circadian period. Although lithium is known to lengthen the circadian period in various experimental systems, until now, this effect was thought to be a result of lithium’s inhibition of GSK3/β (50–52). Our results put forward the novel possibility that IMPase inhibition, especially that of IMPase 1, may at least in part, through the effects of lithium, alter the circadian rhythm and thus be related to the efficacy of this drug. Although it is still unknown how inositol depletion mediates its biological consequences, especially on brain function, research groups have set about trying to answer this question. First, Andreassi et al. (53) found that Impa1 mRNA is the most abundant transcript in the axons of rat sympathetic neurons and that axon-specific down-regulation of Impa1 mRNA induces axon degeneration. Second, IMPase plays an essential role in the generation of...
role in maintaining neuronal polarity as demonstrated by studies on the *Caenorhabditis elegans* gene, *txt-7*, which is the only nematode gene to encode an IMPase. Mutants of *txt-7* are defective in thermotaxis due to disruption of membrane polarity in the RIA neuron (54, 55) and can be rescued by enforced expression of human *IMPA1* or *IMPA2* (21), suggesting that mammalian IMPases and nematode Ttx-7 play similar roles in neurons.

The *Impa1* T95K mutant may therefore serve as a useful tool to dissect the mechanistic action of lithium as a mood stabilizer. Moreover, defining the mechanisms underlying its morphological phenotypes may provide new insights into bone development. Finally, this study illustrates the power of the RGDMS platform to decipher gene function based on the introduction of point mutations, compared with more invasive KO techniques.

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REFERENCES
1. Berridge, M. J., Downes, C. P., and Hanley, M. R. (1989) Neural and developmental actions of lithium: a unifying hypothesis. *Cell* 59, 411–419
2. Williams, R. S., Cheng, L., Mudge, A. W., and Harwood, A. J. (2002) A common mechanism of action for three mood-stabilizing drugs. *Nature* 417, 292–295
23. Kasahara, T., Kubota, M., Niyazaki, T., Noda, Y., Mouri, A., Nabeshima, T., and Kato, T. (2006) mice with neuron-specific accumulation of mitochondrial DNA mutations show mood disorder-like phenotypes. Mol. Psychiatry 11, 577–593.

24. Singh, N., Halliday, A. C., Knight, M., Lack, N. A., Lowe, E., and Churchill, G. C. (2012) Cloning, expression, puriﬁcation, crystallization, and x-ray analysis of inositol monophosphatase from Mus musculus and Homo sapiens. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 68, 1149–1152.

25. Adzhebui, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S., and Sunyaev, S. R. (2010) A method and server for predicting damaging missense mutations. Nat. Methods 7, 248–249.

26. Arguello, P. A., and Gogos, J. A. (2006) Modeling madness in mice: One piece at a time. Neuron 52, 179–196.

27. Baker, M. (2011) Animal models: inside the minds of mice and men. Nature 475, 123–128.

28. Beaulieu, J. M., Zhang, X., Rodriguiz, R. M., Sotnikova, T. D., Cools, M. J., Wetsel, W. C., Gainetdinov, R. R., and Caron, M. G. (2008) Role of GSK3β in behavioral abnormalities induced by serotonin deﬁciency. Proc. Natl. Acad. Sci. U.S.A. 105, 1333–1338.

29. Clapcote, S. J., Lipina, T. V., Millar, J. K., Mackie, S., Christie, S., Ogawa, F., Lerch, J. P., Trimble, K., Uchiyama, M., Sakuraba, Y., Kaneda, H., Shiroyoshi, T., Houslay, M. D., Henkelman, R. M., Sled, J. G., Gondo, Y., Porteous, D. I., and Roder, J. C. (2007) Behavioral phenotypes of Disc1 missense mutations in mice. Neuron 54, 387–402.

30. Del Pino, I., García-Frigola, C., Dehorth, N., Brotons-Mas, J., Ralvez-Salvado, E., Martínez de Lagrán, M., Ciceri, G., Gabaldón, M. Y., Moratal, D., Dierssen, M., Canals, S., Marín, O., and Rico, B. (2013) Erbb4 deletion from fast-spiking interneurons causes schizophrenia-like phenotypes. Neuron 79, 1152–1168.

31. Powell, C. M., and Miyakawa, T. (2006) Schizophrenia-relevant behavioral testing in rodent models: A uniquely human disorder? Biol. Psychiatry 59, 1198–1207.

32. McClung, C. A. (2011) Circadian rhythms and mood regulation: insights from pre-clinical models. Eur. Neuropsychopharmacol. 21, 5683–5693.

33. McClung, C. A. (2013) How might circadian rhythms control mood? Let me count the ways. Biol. Psychiatry 74, 242–249.

34. Berry, G. T., Wu, S., Buccafusca, R., Ren, J., Gonzales, L. W., Ballard, P. L., Golden, J. A., Stevens, M. J., and Greer, J. I. (2003) Loss of murine Na⁺/myo-inositol cotransporter leads to brain myo-inositol depletion and central apnea. J. Biol. Chem. 278, 18297–18302.

35. Chau, J. F., Lee, M. K., Law, J. W., Chung, S. K., and Chung, S. S. (2005) Sodium/myo-inositol cotransporter-1 is essential for the development and function of the peripheral nervous system. FASEB J. 19, 1887–1889.

36. Dai, Z., Chung, S. K., Miao, D., Lau, K. S., Chan, A. W., and Kung, A. W. (2011) Sodium/myo-inositol cotransporter 1 and myo-inositol are essential for osteogenesis and bone formation. J. Bone Miner. Res. 26, 582–590.

37. Inoue, T., Hatayama, M., Tohmoura, M., Itohara, S., Aruga, J., and Miko-shiba, K. (2004) Mouse Zic5 deﬁciency results in neural tube defects and hypoplasia of cephalic neural crest derivatives. Dev. Biol. 270, 146–162.

38. Stöttmann, R. W., Anderson, R. M., and Klingsensmith, J. (2001) The BMP antagonists Chordin and Noggin have essential but redundant roles in mouse mandibular outgrowth. Dev. Biol. 240, 457–473.

39. Depew, M. J., Lufkin, T., and Rubenstein, J. L. (2002) Speciﬁcation of jaw subdivisions by Dlx genes. Science 298, 381–385.
40. Madan, B., Madan, V., Weber, O., Tropel, P., Blum, C., Kieffer, E., Viville, S., and Fehling, H. J. (2009) The pluripotency-associated gene Dppa4 is dispensable for embryonic stem cell identity and germ cell development but essential for embryogenesis. *Mol. Cell. Biol.* 29, 3186–3203

41. Compagni, A., Logan, M., Klein, R., and Adams, R. H. (2003) Control of skeletal patterning by ephrinB1-EphB interactions. *Dev. Cell* 5, 217–230

42. Cryns, K., Shamir, A., Van Acker, N., Levi, I., Daneels, G., Goris, I., Bouwknecht, J. A., Andries, L., Kass, S., Agam, G., Belmaker, H., Bersudsky, Y., Steckler, T., and Moechars, D. (2008) IMPA1 is essential for embryonic development and lithium-like pilocarpine sensitivity. *Neuropsychopharmacology* 33, 674–684

43. Cryns, K., Shamir, A., Shapiro, J., Daneels, G., Goris, I., Van Craenenendonck, H., Straetemans, R., Belmaker, R. H., Agam, G., Moechars, D., and Steckler, T. (2007) Lack of lithium-like behavioral and molecular effects in IMPA2 knockout mice. *Neuropsychopharmacology* 32, 881–891

44. Artioli, P., Lorenzi, C., Pirovano, A., Serretti, A., Benedetti, F., Catalano, M., and Smeraldi, E. (2007) How do genes exert their role? Period 3 gene variants and possible influences on mood disorder phenotypes. *Eur. Neuropsychopharmacol.* 17, 587–594

45. Lamont, E. W., Legault-Coutu, D., Cermakian, N., and Boivin, D. B. (2007) The role of circadian clock genes in mental disorders. *Dialogues Clin. Neurosci.* 9, 333–342

46. McClung, C. A. (2007) Circadian genes, rhythms and the biology of mood disorders. *Pharmacol. Ther.* 114, 222–232

47. Barnard, A. R., and Nolan, P. M. (2008) When clocks go bad: neurobehavioural consequences of disrupted circadian timing. *PLoS Genet.* 4, e1000040

48. Kripke, D. F., Niewerdt, C. M., Joo, E., Shekhtman, T., and Kelsoe, J. R. (2009) Circadian polymorphisms associated with affective disorders. *J. Circadian Rhythms* 7, 2

49. Mendlewicz, J. (2009) Disruption of the circadian timing systems: Molecular mechanisms in mood disorders. *CNS Drugs* 23, 15–26

50. Dokucu, M. E., Yu, L., and Tagbert, P. H. (2005) Lithium- and valproate-induced alterations in circadian locomotor behavior in *Drosophila*. *Neuropsychopharmacology* 30, 2216–2224

51. Lavoie, J., Hébert, M., and Beaulieu, J. M. (2013) Glycogen synthase kinase-3β haploinsufficiency lengthens the circadian locomotor activity period in mice. *Behav. Brain Res.* 253, 262–265

52. Yin, L., Wang, J., Klein, P. S., and Lazar, M. A. (2006) Nuclear receptor Rev-erbα is a critical lithium-sensitive component of the circadian clock. *Science* 311, 1002–1005

53. Andreassi, C., Zimmermann, C., Mitter, R., Fusco, S., De Vita, S., Devita, S., Saiardi, A., and Riccio, A. (2010) An NGF-responsive element targets myo-inositol monophosphatase-1 mRNA to sympathetic neuron axons. *Nat. Neurosci.* 13, 291–301

54. Kimata, T., Tanizawa, Y., Can, Y., Ikeda, S., Kuhara, A., and Mori, I. (2012) Synaptic polarity depends on phosphatidylinositol signaling regulated by myo-inositol monophosphatase in *Caenorhabditis elegans*. *Genetics* 191, 509–521

55. Tanizawa, Y., Kuhara, A., Inada, H., Kodama, E., Mizuno, T., and Mori, I. (2006) Inositol monophosphatase regulates localization of synaptic components and behavior in the mature nervous system of *C. elegans*. *Genes Dev.* 20, 3296–3310