INTRODUCTION

The Kagami–Ogata and Temple syndromes (KOS14 and TS14) are genomic imprinting diseases caused by aberrant regulation of the imprinted region on human chromosome 14, such as paternal and maternal duplication, maternal and paternal deletion as well as gain and loss of DNA methylation at the intergenic differentially methylated region (IG-DMR), respectively (Kotzot, 2004; Kagami et al., 2005, 2008; Ioannides et al., 2014; Ogata & Kagami, 2016). KOS14 and TS14 are neuromuscular as well as neuropsychiatric diseases caused by irregular CNS RTL1 expression, presumably leading to impaired innervation of motor neurons to skeletal muscles as well as malfunction of the hippocampus-amygdala complex. It is of considerable interest that eutherian-specific RTL1 is expressed in mammalian- and eutherian-specific brain structures, that is, the corticospinal tract and corpus callosum, respectively, suggesting that RTL1 might have contributed to the acquisition of both these structures themselves and fine motor skill in eutherian brain evolution.

KEYWORDS
a eutherian-specific acquired gene, brain evolution, corpus callosum, corticospinal tract, cranial and spinal nerves, KOS14 and TS14, neuromuscular disease, neuropsychiatric disease, RTL1/PEG11
patients exhibit neonatal lethality, presumably due to respiratory problems associated with a small bell-shaped thorax, abdominal wall defects, such as omphalocele and diastasis recti, placentomegaly, polyhydramnios, developmental delay, and/or intellectual disability and feeding disorders (Kagami et al., 2005 and Kagami et al., 2015) (Appendix S1: Table S1). TS14 patients exhibit severe pre- and postnatal growth retardation, hypotonia, early onset of puberty, motor delay, feeding difficulties, and mild intellectual disability (Ioannides et al., 2014; Kagami et al., 2017) (Appendix S1: Table S2).

We previously demonstrated by means of mouse disease models that over-expression and deficiency of retrotransposon Gag like 1 (Rtl1, also called as Peg1) are responsible for placental abnormalities (Sekita et al., 2008; Kagami et al., 2008; Kitazawa et al., 2017) as well as fetal/neonatal muscle defects, especially in the respiratory-related intercostal, abdominal, and diaphragm muscles in these syndromes (Kitazawa et al., 2020). However, the genetic cause(s) of their neurological defects, such as feeding difficulties, motor delay, and developmental delay/intellectual disability, remains elusive.

In this work, we investigated whether Rtl1 is involved in these neurological symptoms in KOS14 and TS14 by conducting analyses of its expression in the nervous system as well as behavioral analyses of two mouse models with over-and under-expression of Rtl1. Although brain Rtl1 expression was present at a low level in humans and mice, we clearly detected a mouse Rtl1 protein in the central nervous system (CNS). Two mouse models with over- and under-expression of Rtl1 exhibited impaired locomotive and cognitive activities. We will discuss the neuromuscular and neuropsychiatric aspects of KOS14 and TS14 caused by irregular Rtl1 expression in the brain based on these results.

Interestingly, Rtl1 expression was observed in the corticospinal tract responsible for fine voluntary skilled muscle movement of limbs, and the corpus callosum responsible for interhemisphere communication. Evolutionarily, the former is unique to mammals (Aboitiz & Montiel, 2003; Mihrshahi, 2006): the nerve bundles originate in the cerebral cortex layer V and pass through the internal capsule and cerebral peduncles of the midbrain, pons and medulla until they reach the spinal cord (Patestas & Gartner, 2016). The corpus callosum is unique to eutherians (Mihrshahi, 2006; Suárez et al., 2014) and is comprised of nerve fibers coming mainly from cerebral cortex layers II and III (the upper layers) and partially from layers V and VI (the lower layers) (Fame et al., 2011). In placental mammals, the commissure systems originate in the same plate in the embryonic brain and the anterior commissure forms first, followed by the hippocampal commissure and then the corpus callosum (Mihrshahi, 2006). Therefore, it is suggested that the evolution of the corpus callosum involved a rerouting of dorsal cortical axons (Suárez et al., 2014). We confirmed that Rtl1 is expressed in the hippocampal and anterior commissures as well as the corpus callosum. We also discuss the role of eutherian-specific Rtl1 in acquisition of the fine motor skill in eutherians by improving the function of corticospinal tract, presumably in association with the emergence of the eutherian-specific corpus callosum.

2 | RESULTS

2.1 | Rtl1 expression in the human and mouse brain

We analyzed human Rtl1 expression by RT-PCR in several adult and fetal tissues as well as the placenta using commercially available human total RNA. Rtl1 was detected in the adult brain and cerebellum as well as the fetal brain at a very low level compared with the placenta and fetal liver: it was detected after 35 cycles of amplification (Figure 1). In the mice, a peak of Rtl1 expression in the fetal brain was observed on embryonic day 12.5 (E12.5), but its level was also very low, as in the human brain, as 35 cycles of amplification were required to detect it. Moreover, its expression was much lower in E10.5 and E14.5 embryos and subsequently. The expression in the medulla oblongata and spinal cord increased in the later fetal stage, and the expression in the latter was observed also in the neonatal and adult stages after 32 cycles of amplification (Figure 1).

In the E12.5 brain (Figure 2), neural stem cells, the dorsal root ganglia (DRG), and Rathke's pouch, from which the anterior pituitary gland develops, were stained with the anti-Rtl1 antibody. In the E14.5 brain, bundles of the nerve fibers were detected from the hindbrain region to the ventrolateral and dorsal spinal cord (Figure 2b,c), indicating that they contain neurons in the reticulospinal tract (RST) that connect the pons and medulla (hindbrain region) with spinal cord (Patestas & Gartner, 2016). In a series of sections of the neonatal brain (postnatal day 0 (P0)), we confirmed that the nerve bundles start from the somatomotor area of cerebral cortex layer V and run through the internal capsule and cerebral peduncles of the midbrain, pons, and medulla until they reach the spinal cord (Figure 2d–f), indicating that Rtl1 is expressed in the pyramidal tract (Figure 2d–f), indicating that Rtl1 is expressed in the pyramidal tract comprising the corticospinal (CST) and corticonuclear (CNT, also called corticobulbar) tracts in addition to the corticoreticular tract (CRT) that connects with the RST (Patestas & Gartner, 2016). The nerve bundles detected in the superior colliculus in the midbrain suggest that Rtl1 is expressed in the corticoreticular tract (CCT) from cerebral cortex layer V, whereas those in the thalamus suggest that it is also expressed in the corticothalamic tract (CThT) from cerebral cortex layer VI, suggesting an involvement in sensory processing (Briggs & Usrey, 2008).

In addition, the Rtl1 protein was also detected in the commissural fibers connecting the corresponding regions in the
right and left hemispheres, such as the corpus callosum, hippocampal, and anterior commissures, as well as other structures in the limbic system, such as the fimbria hippocami (hippocampal fimbria), fornix, and medial amygdala nucleus (Figure 2d–f). We also analyzed RTL1 expression in the two types of disease model mice with paternal and maternal deletion of an Rtl1 allele (Pat-Rtl1Δ and Mat-Rtl1Δ, respectively). In P0 neonates, a similar expression pattern in the brain was observed in the Pat-Rtl1Δ and Mat-Rtl1Δ mice (Figure 2g,h) compared with that of wild-type (WT) mice (Figure 2d–f). In the coronal section, RTL1 was detected in most of the corpus callosum, comprising the neurons from cortex layers V and VI, because the neurons from the cortical areas II and III make up only a minor population at this stage (P0) (Fame et al., 2011). Although the RTL1 protein was detected in Rathke’s pouch in the E12.5 brain, it was not clearly detected in the pituitary gland in the E14.5 (and P0 brains) or P0 brain. The result from the RT-PCR experiment also demonstrated that Rtl1 expression in the pituitary gland is not evident in the postnatal period (Appendix S1: Figure S2).

2.2 | Lack of RTL1 imprinted expression in the neonatal brain

Rtl1 is imprinted in the E14.5 brain, like other tissues and organs, because its expression was completely lost in the Pat-Rtl1Δ brain, whereas being over-expressed in the Mat-Rtl1Δ brain (Appendix S1: Figure S3). However, as shown in Figure 2g, Pat-Rtl1Δ retained brain expression despite of loss of expression in muscle tissues such as the tongue. The Rtl1 mRNA level in the Pat-Rtl1Δ brain was approximately one half that of the wild type, indicating that Rtl1 is also expressed from the maternally derived allele in the P0 brain (Figure 2j), whereas it was approximately two- to threefold higher in the Mat-Rtl1Δ brain than in WTm because of a lack of antiRtl1 that degrades Rtl1 mRNA through six microRNAs via an RNAi mechanism (Figure 2j) (Sekita et al., 2008; Kitazawa et al., 2017 and Kitazawa et al., 2020). Thus, it is likely that Rtl1 imprinting is lost in the neonatal brain.

It should be noted that the two control mice, WTp and WTm (wild-type littermates with Pat-Rtl1Δ and Mat-Rtl1Δ, respectively: see Appendix S1: Figure S1), exhibited different levels of Rtl1 expression: the latter being evidently lower than the former. In this and behavioral experiment, we excluded the neonatal lethal effects due to the B6 genetic background as well as the mother effects in generating the F1 (WTp and Pat-Rtl1Δ mice) and F2 (WTm and Mat-Rtl1Δ mice) generations to as great an extent as possible (Appendix S1: Figure S1). The former were generated by in vitro fertilization using eggs from wild-type females (Sv129) and F10 Pat-Rtl1Δ sperm (basically on a B6 background); then, the fertilized eggs were transplanted to pseudopregnant ICR females. Pups were born via Caesarian section and cared for by ICR surrogate mothers. The latter were generated by in vitro fertilization using eggs from F1 Pat-Rtl1Δ females and wild-type sperm (B6),
then transplanted into pseudopregnant ICR females, and the pups were cared for by ICR surrogate mothers after Caesarian section in like manner as in the former case. Therefore, it is likely that the different Rtl1 expression was due to differences in the egg genotype used to generate the F1 and F2 generations (Sv129 for WTP and Pat-Rtl1Δ, 129/B6 F1 for WTM and Mat-Rtl1Δ), respectively.

2.3 | Reduction of locomotor activity, and incremental increase of anxiety-like and impaired fear conditioning behaviors in Pat-Rtl1Δ and Mat-Rtl1Δ mice

We subjected Pat-Rtl1Δ, Mat-Rtl1Δ mice, and their wild-type littermates (WTP and WTM) to a comprehensive
battery of behavioral tests to evaluate the behavioral effects of Rtl1 under and over-expression, respectively. However, it should be noted that two wild-type mice exhibited different behavioral reactions in several tests (see the Discussion section).

Spontaneous locomotor activity was tested in the open field horizontal activity test. Both the Pat-Rtl1Δ and Mat-Rtl1Δ mice exhibited significant differences compared with the wild type in the parameters of total distance ($p = .0311$, $p = .0165$), average speed ($p = .0286$, $p = .0177$), and...

**Figure 2**  
RTL1 expression in the nervous system during development. (a) A section of the entire E12.5 embryo (upper left) and magnified views of the dorsal root ganglion (upper right), telencephalon and prethalamus regions (lower left), the thalamus and pretectum regions (lower middle), and the hindbrain region (lower right). Scale bars: 500 μm (upper left), 100 μm (others). (b) A sagittal section of the entire E14.5 embryo (upper) and a magnified view of the preptontine hindbrain region (lower). Dc: diencephalon, DRG: dorsal root ganglion, Hb: hindbrain, Mb: midbrain, MO: medulla oblongata, Pa: pallium, Po: pons, RP: Rathke’s pouch, Th: thalamus. Scale bars: 500 μm (top), 100 μm (bottom). (c) A coronal section of the E14.5 embryo (upper) and a magnified view of the spinal cord (lower). Scale bars: 500 μm. (d–f) A series of sagittal sections of the P0 neonatal brain (wild type). AC: anterior commissure, CC: corpus callosum, CP: cerebral peduncle, Hb: hindbrain, HF: hippocampal fimbria, MEA: Medial amygdala nucleus, Mb: midbrain, MO: medulla oblongata, Po: pons, Pu: putamen, T: tongue, VHC: ventral hippocampal commissure, layer V/VI: the fifth and sixth layers of cerebral cortex. (g, h) A sagittal section of the P0 neonatal brain of Pat-Rtl1Δ and Mat-Rtl1Δ, respectively, corresponding to section G (wild type). The RTL1 expression in tongue was much reduced in Pat-Rtl1Δ, as previously reported, whereas its brain expression remained the same and its expression was up-regulated in both the tongue and brain in Mat-Rtl1Δ. Scale bars: 1 mm. (i) A coronal section of the P0 neonatal brain. Upper: immunofluorescent staining of RTL1 (red), CTIP2 (green), and DAPI (blue). Lower: immunofluorescent staining of TBR1 (red), CTIP2 (green), and DAPI (blue). CC: corpus callosum, DF: dorsal fornix, Fi: fimbria, Hi: hippocampus, IC: internal capsule, layer V: the fifth layer of cerebral cortex, layer VI: the sixth layer of cerebral cortex, Th: thalamus, v3: the 3rd ventricle, vl: lateral ventricle. Scale bars: 500 μm. (j) Rtl1 expression levels in P0 brain of Pat-Rtl1Δ (blue, $n = 2$), WTp (black, $n = 2$), WTm (black, $n = 2$), and Mat-Rtl1Δ (red, $n = 2$). Approximately one half of the level of Rtl1 was expressed in the brain in the Pat-Rtl1Δ, indicating a loss of the imprinting regulation of Rtl1 in the brain. * $p < .05$, ** $p < .01$. Two-tailed Student’s t-test was used for the statistical analysis. Error bars indicate stdev.
distance per movement \( (p = 0.0700, p = 0.0212) \), whereas only the Mat-Rtl1Δ mice exhibited a significant difference on total movement duration \( (p = 0.0526, p = 0.0477) \) and the duration per movement \( (p = 0.151, p = 0.0277) \) (Figure 3a). However, we observed an apparent difference between WTP and WTM in the parameters of total movement duration \( (p = 0.0313) \) and total movement episode number \( (p = 0.0412) \) (see Appendix S1: Table S3). This suggests that Mat-Rtl1Δ mice also have an impaired total movement episode number compared with WT(p) mice and the difference in the effect observed on total movement duration seems more intense. In total, both Pat-Rtl1Δ and Mat-Rtl1Δ mice exhibited reduced spontaneous locomotor activity (both distance and speed), and the latter to a noteworthy extent. This trend was similarly observed in the other behavior tests described below.

In the elevated plus maze test, the Pat-Rtl1Δ mice displayed increased anxiety-like behavior. The total distance \( (p = 0.0324) \) and total entry number \( (p = 0.0237) \) were significantly decreased between Pat-Rtl1Δ and WT(p). The percentage of time spent in the open arm and the number of entries into the open arm were significantly decreased \( (p = 0.0313, p = 0.0249) \), respectively. Although the Mat-Rtl1Δ mice displayed no significant difference from WTM in these two parameters \( (p = 0.425, p = 0.521) \), there were significant differences between WTP and WTM in all of the 10 test parameters (see, Appendix S1: Table S4). These results suggest increased anxiety-like behavior in the Pat-Rtl1Δ, Mat-Rtl1Δ mice as well as the WTM mice (Figure 3b).

In the light/dark transition test, there was no significant difference between the Pat-Rtl1Δ and WTP in the distance in the dark or light chamber \( (p = 0.0565, p = 0.0757) \), but there was a significant difference between Mat-Rtl1Δ and WT(m) \( (p = 0.00284, p = 0.0436) \). There was no significant difference between the genotypes in terms of the time spent in the light chamber \( (p = 0.234, p = 0.0774) \) (see, Appendix S1: Table S5), but the number of transitions between the chambers was significantly decreased \( (p = 0.0429, p = 0.0432) \). These results suggest that anxiety-like behavior is mildly increased in both the Pat-Rtl1Δ and Mat-Rtl1Δ mice (Figure 3c).

In the fear conditioning test, there was also significant difference in total distance \( (p = 0.0406, p = 0.00117) \) but no significant difference between the genotypes and the WT in total freeze percentage \( (p = 0.162, p = 0.0775) \). In the contextual test (hippocampus-dependent memory retrieval) on the 2nd day, total freeze time was significantly increased between Mat-Rtl1Δ and WT(m) \( (p = 0.0243) \), but there was no significant difference between Pat-Rtl1Δ and WT(p) \( (p = 0.276) \). However, it should be noted that an apparent difference exists between WT(m) and WT(p) \( (p = 0.0002) \) that appears to be related to the difference between Mat-Rtl1Δ and WT(m) (see the Discussion section). In contrast, in the second cued test (amygdala-dependent memory retrieval) on the 3rd day, the total freeze percentage was significantly increased in both the Pat-Rtl1Δ and Mat-Rtl1Δ mice \( (p = 0.0389, p = 0.0284) \) (Figure 3d and Appendix S1: Table S6). This clearly indicates that both the Pat-Rtl1Δ and Mat-Rtl1Δ mice have impaired amygdala-dependent memory retrieval.

Hippocampal-dependent learning, induced acquisition of spatial memory, and long-term spatial memory were tested in the Morris water maze test. There were significant differences in total distance and moving speed between Mat-Rtl1Δ and WTM \( (p = 0.0168, p = 0.00376) \). Importantly, Mat-Rtl1Δ mice did not exhibit any improvement during the period of the first and fourth day of the experiments in terms of the time to reach the platform, indicating that the Mat-Rtl1Δ mice had severely impaired spatial memory in addition to reduced locomotor activity, whereas Pat-Rtl1Δ displayed an excellent performance on this test, indicating that Pat-Rtl1Δ have better memory than WTP, or, alternatively, they may have increased escape behavior (Figure 3e and Appendix S1: Table S7).

3 | DISCUSSION

We found that Rtl1 expression in the fetal brain was very low by RT-PCR analysis, but the immunohistological analyses using an anti-RTL1 protein clearly illustrated the precise RTL1 expression profiles in the CNS from E12.5 to P0, demonstrating that RTL1 is actually expressed in the pyramidal tract comprising CST and CNT as well as CRT and RST that regulate the motor and sensory neurons in the limbs and trunk from cortex layer V (Patestas & Gartner, 2016) as well as in the CTT, which together with the CRT works in parallel with the CST to influence motoneurons in the spinal cord both directly and indirectly via the brainstem descending pathways (Fregosi et al., 2019). RTL1 is also expressed in the CThT from layer VI, an integral part of thalamo-cortico-thalamic circuit which intimately interconnects the thalamus and cortex for sensory processing (Briggs & Usrey, 2008).

RTL1 is also expressed in the interhemispheric commissural structures, such as the corpus callosum, hippocampal, and anterior commissures that communicate with the corresponding right and left hemispheric sites (Mihrshahi, 2006; Suárez et al., 2014), and the hippocampal fimbria and fornix, the major output route of hippocampal-related memory formation (Warburton et al., 2000; Aggleton & Nelson, 2015), and the medial amygdala nucleus, a structure involved in emotion-related behavior (Pardo-Bellver et al., 2012). Therefore, we think it is highly likely that the resulting impairment of these brain and spinal functions leads to the neurological defects that are observed in KOS14 and TS14 patients, although it remains to be determined whether humans have the same RTL1 expression profiles in the nervous system. In general, it is very challenging to reproduce human neurological phenotypes (i.e., symptoms) solely with data from behavioral
FIGURE 3  (Continued)
Concerning the motor delay, the two mouse models exhibited a severe reduction in locomotive activity in most of the behavioral tests. Therefore, this is highly likely to be associated with the impairment of CST, CRT, and RST because they are responsible for the voluntary muscle movement of the trunk and limbs (Lemon, 2008; Patentes & Gartner, 2016), such as specific skilled muscle movement (i.e., CST) (Iwaniuk & Whishaw, 2000) and diverse specific motor function (i.e., CRT and RST) (Perreault & Giorgi, 2019) including escape, micturition, reaching and grasping, sleep, respiration, vomiting, and locomotion. Impairment of the corpus callosum is also likely to be involved in the motor delay because it connects the right and left hemispheres and is critically involved in information exchange, especially in cortex layer V.

In our most recent published report, we discussed the fact that skeletal muscles defects, especially in the muscles used in respiration, such as the diaphragm, intercostal, and abdominal muscles, are the major cause of respiratory problem leading to neonatal lethality in KOS14 patients. However, the findings in this work, that Rtl1 expression was clearly

analyses of KO mice, even when they display the same genetic defects. In an effort to overcome the evident limitation of the behavior tests using the Mat- and Pat Rtl1ΔKO mice, it is reasonable to consider the symptoms of KOS14 and TS14 in the context of the results from these KO mice with the help of the comprehensive RTL1 expression profiles in the CNS described above.

Most KOS14 patients exhibit developmental delay/intellectual disability and feeding difficulties, whereas approximately 2/3 of TS14 patients exhibit hypotonia and feeding difficulties, and some exhibit auditory impairment (Kotzot, 2004; Kagami et al., 2005, 2008, 2015, and Kagami et al. 2016; Ioannides et al., 2014; Ogata & Kagami, 2016). As expected, it has proven to be very difficult to straightforwardly account for most of these symptoms only from the behavioral abnormalities observed in the KO mouse models with over- and under-expression of Rtl1. However, the feeding difficulties and delayed speech might be explained by the impairment of certain cranial nerves, such as the trigeminal (the fifth of cranial nerve, V), glossohypophyseal (IX), vagus (X), and hypoglossal (XII) nerves, and the auditory impairment by the vestibulocochlear (VIII) nerves because the CNT directly innervate these cranial nerves (Pateas & Gartner, 2016). It may be possible that the impairment of the oral cavity as well as tongue muscles linked to these symptoms, as we previously reported that RTL1 is expressed in fetal/neonatal skeletal muscles. However, it is also possible that impairment of cranial nerves regulating the oral and tongue muscles plays a role in these symptoms. It is yet again possible that some combination of both is the case.

In terms of developmental delay/intellectual disability, it is possible that RTL1 expression in the hippocampal fimbria and fornix as well as medial amygdala nucleus is related to this symptom. The fimbria and fornix are the main output routes of hippocampus memory (Warburton et al., 2000;
Aggleton & Nelson, 2015), and the medial amygdala nucleus is a key structure in the emotional response by relaying olfactory information to the hypothalamic nuclei involved in reproduction and defense (Pardo-Bellver et al., 2012; Keshavarzi et al., 2014). Memory and social behavior are essential parts of behavioral development as well as intellectual disabilities (Darling-Hammond et al., 2020). This characterization is consistent with the finding that the two mouse models exhibited increased anxiety, as well as impaired amygdala- and possibly hippocampus-dependent fear responses. It is also supported by the evidence that most knockout mice related to the intellectual disability genes in humans exhibited impaired score in the fear conditioning test (Zhang et al., 2015; Aincy et al., 2018). In the behavioral tests, WTp and WTm mice exhibited different phenotypes, especially in that WTm mice exhibited severe responses in the elevated plus maze and fear conditioning tests compared with the WTp mice. As mentioned above, there is an egg genotype difference between WTp (Sv129) and WTm (Sv129/B6 F1). However, it is unlikely that such a difference would affect only the results of the elevated plus maze and fear conditioning tests. As the Mat-Rtl1Δ pups displayed much more severe responses in the fear conditioning test than the WTm mice, we assume that this was the result of the Mat-Rtl1Δ pups having been raised together with the WTm mice before weaning at 4 weeks of age. They may suffer from exposure to the abnormal behavior of the Mat-Rtl1Δ pups in a specific manner, especially in terms of fear conditioning. Rtl1 expression in the WTm mice was lower than the WTp mice, it is possible that this may be related to their sensitivity to the abnormal behavior of their littermates.

Concerning the postnatal growth retardation observed in the TS14 patients, the Pat-Rtl1Δ pups maintained a lower weight than the control WTp pups (Sekita et al., 2008; Kitazawa et al., 2017). Similarly, KO mice with delta-like noncanonical Notch ligand 1 (Dlk1) in the same imprinted region exhibited postnatal growth retardation (Moon et al., 2002; Cheung et al., 2013). Therefore, it is highly likely that both RTL1 and DLK1 are related to the postnatal growth retardation of TS14, although the crown-rump length of Pat-Rtl1Δ pups recovered to the same level in the period from weaning period to 8 weeks (Kitazawa et al., 2017) and the effect of Dlk1 KO was slight concerning the body length in the period from weaning to 14 weeks (Cheung et al., 2013). TS14 patients exhibit a postnatal reduction in both body weight and length (Ioannides et al., 2014; Kagami et al., 2017), suggesting that the effects of RTL1/Rtl1 and DLK1/Dlk1 are different in humans and mice or that other maternally expressed imprinted genes, such as MEG3, MEG8, and MEG9, may contribute to the postnatal growth retardation as well as other symptoms in TS14 and KOS14. Alternatively, concomitant loss of both RTL1 and DLK1 genes may be necessary to explain the reduced postnatal body length. Rtl1 expression in the pituitary gland was not as evident in mice (Figure 2j), whereas Dlk1 exhibits apparent pituitary gland expression in the pre- and postnatal periods (Yevtodyienko & Schmidt, 2006; Cheung et al., 2013; Falix et al., 2013). Dlk1KO mice also exhibit a mild reduction in pituitary growth hormone (GH) content in the postnatal period, although it is believed that such mild GH reduction is inadequate to explain the postnatal growth retardation in Dlk1 KO mice (Cheung et al., 2013). Thus, the real cause of postnatal growth retardation in both Rtl1 and Dlk1 KO mice remains to be elucidated.

It is of interest that eutherian-specific RTL1 (Charlier et al., 2001; Edwards et al., 2008; Kaneko-Ishino & Ishino, 2012, 2015) is expressed in both the corticospinal tract and corpus callosum, that is, mammalian- and eutherian-specific brain structures, respectively (Aboitiz & Montiel, 2003; Mihrshahi, 2006; Suárez et al., 2014). The corticospinal tract is responsible for fine muscle movement, and it is reported that the eutherian corticospinal tract system is functionally superior to that in marsupials by comparing the paw skills of rats and opossums (Frost et al., 2000; Ivanco et al., 1996), implying that RTL1 may play a role in the evolution of this system as an eutherian-specific gene. The corpus callosum connects the left and right brain hemispheres, so it is highly probable that the emergence of corpus callosum also contributed to the improvement of skilled motor function along with other behaviors (Mihrshahi, 2006; Suárez et al., 2014). It is comprised of nerve fibers derived mainly from the cerebral cortex layers II and III in the upper layer as well as in the lower portion of cerebral cortex layers V and VI in adults (Fame et al., 2011), where RTL1 is specifically expressed. It is of great interest that RTL1 is also expressed in the hippocampal commissure, implying that RTL1 might have been involved in the evolutionary emergence of this structure, because it has been suggested that the evolution of the corpus callosum involved a rerouting of dorsal cortical axons. This rerouting involves axons crossing through the anterior commissure that employ the same embryonic substrate as the hippocampal commissure and the fact that the nerve fibers in the hippocampal commissure connecting the left and right fornix help the nerve fibers in the corpus callosum pass though the midline of the brain on embryonic development day 15.5 (E15.5) (Mihrshahi, 2006; Suárez et al., 2014).

Rtl1 imprinting is lost in the P0 brain, so one half of the RTL1 expression, from the maternal allele, was observed in the P0 Pat-Rtl1Δ brain. Importantly, Rtl1 is apparently imprinted in the E14.5 brain, like other tissues and organs, because its expression was completely lost in the Pat-Rtl1Δ brain whereas being over-expressed in the Mat-Rtl1Δ brain (Appendix S1: Figure S2). A similar result was previously reported in which Dlk1 imprinting is lost in postnatal stem cells and the niche astrocytes regulating neurogenesis
suggesting that the postnatal loss of genomic imprinting in the brain might be a characteristic of the DLK-DIO3 imprinted region. Although the molecular function of RTL1 in the brain as well as the details in the neuronal impairments that result from its irregular expression remain to be elucidated, these findings suggest that KOS14 and TS14 should be regarded as neuromuscular as well as neuropsychiatric diseases caused by over- and under-expression of RTL1, although the hypotonia in TS14 is transient and neonate-specific. It also implies that eutherian-specific RTL1 might be a key gene in the functional evolution of eutherian nerve system. Thus, RTL1 is another good example of acquired genes from LTR-retrotransposons and/or retroviruses functioning in the eu- therian brain, such as Arc (Campillos et al.; 2006; Ashley et al.; 2018; Pastuzyn et al.; 2018), Sirh7/Ldoc1 (also termed Sirh8/Rgag1 Rtl5) (sub- termed), and (also termed Sirh11/Zcchc16 Rtl7), and another good example of acquired genes from LTR-retrotransposons and/or retroviruses functioning in the eutherian brain, such as Arc (Campillos et al.; 2006; Ashley et al.; 2018; Pastuzyn et al.; 2018), Sirh7/Ldoc1 (also termed Sirh8/Rgag1 Rtl5) (sub- termed), and (also termed Sirh11/Zcchc16 Rtl7), and

4 | EXPERIMENTAL PROCEDURES

4.1 | Mice

All animals and experimental procedures were approved by the Animal Ethics Committees of Tokyo Medical and Dental University. The Rtl1 KO mice were generated by using ES cells (CCE) of 129/SvEv mouse origin, as previously described (Sekita et al., 2008). The Rtl1 KO lines were main- tained by continuous crossing with male and female C57BL/6J mice (WT), and mice in the F1 (Rtl1 KO: Pat-Rtl1Δ) and F2 (antiRtl1as KO: Mat-Rtl1Δ) generations were used for behav- ioral analyses, whereas the F8 (Pat-Rtl1Δ) and F9 (Mat-Rtl1Δ) generations were used for immunohistochemical analyses.

4.2 | Analysis of the expression of RTL1/Rtl1

The human total RNA set was purchased from TOYOBO (Japan, Master Panel II, Clontech catalog no. 636,643). The cDNA was synthesized from 1 μg of total RNA using ReverTra Ace® qPCR RT Master Mix (TOYOBO) according to the manufacturer’s protocol. For the RT-PCR analysis of RTL1, 10 ng of cDNA in a 25 μl reaction mixture containing 1x ExTaq buffer (TAKARA), 200 μM of each dNTP, prim- ers and 0.5 units of ExTaq HS (TAKARA) were subjected to 35 cycles at 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s using a C1000 Touch thermal cycler (Biorad). The following primer sequences were used: a RTL1 forward primer, 5’- AACCCACTTGTGAAGGCCAA-3’; a reverse primer, 5’- CCTCAGTGATGTCACCTCCG-3’. The genomic DNA and total RNA samples were prepared from fetuses, neo- nates, and 1- and 4-week-old mice using TRIZol Regent (Life Technologies). The cDNA was synthesized from 5 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen) with the following oligo-dT + Adaptor primer: 5’-CTGATC TAGAGGTACCGGATCCGACTCGAGTCGACATCGTT TTTTTTTTTTTTTTTTTTTTTT-3’. For the RT-PCR analysis of Rtl1, 10 ng of cDNA in a 25 μl reaction mixture containing 1x KOD FX buffer (KFX-101, TOYOBO), 200 μM of each dNTP, primers, and 0.5 units of KOD FX was subjected to 30 cycles at 98°C for 15 s, 69°C for 30 s, and 74°C for 30 s using a C1000 Touch thermal cycler (Biorad). The following primer sequences were used: Rtl1, 5’-TCCAAAGGAGCATCTCGAC GTACCAGTGTGACTTACC-3’; an adaptor primer for both genes, 5’-AGAGGTACCGGATCCGACTTCGACTCGACA TCG-3’; and Gapdh, 5’-CACTCTTCCATCCATGTCGC-3’ and 5’-CTTGTGGTCGATGTCCCTTG-3’.

4.3 | Quantitative PCR assay

The quantitative real-time PCR was performed by using 5 ng of cDNA in a THUNDERBIRD SYBR qPCR Mix (QPS-201, TOYOBO). The cycle conditions were 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 10 s using the LightCycler 480 apparatus (Roche). The gene expression levels were normalized to Gapdh. The following primer sequences were used for this study: Rtl1, 5’-GAGTACTGTCGACTCGACAT CGACA TCG-3’; and Gapdh, 5’-CACTCTTCCATCCATGTCGC-3’ and 5’-CTTTGTGGTCGATGTCCCTTG-3’.

4.4 | Immunostaining (paraffin sections)

Mouse fetuses and neonates were fixed using SUPER FIX (KURABO), soaked in 5% formic acid in 70% ethanol at 4°C overnight for two nights, dehydrated in 70% and 90% ethanol for 2 hr each, 100% ethanol for 2 hr three times, and xylene for 2 hr four times, and finally embedded in par- affin wax. The paraffin blocks were sectioned at a 5-μm thickness with a microtome and mounted on Superfrost Micro Slides (Matsunami Glass). The sections were de- paraffinized 3 times in xylene for 20 min, 3 times in 100% ethanol for 5 min, and in 90% and 70% ethanol for 5 min each. For antigen retrieval, the sections were boiled in 0.01 M Citrate Buffer pH 6.0 at 98°C for 40 min and then immersed (dehydrated) in cold methanol at –30°C over- night. After being air dried, the sections were blocked with 10% goat serum, 5% bovine serum albumin (BSA: Sigma
For the immunohistochemical analysis, an anti-Rtl1 antibody (1:200) was used as the primary antibody and was prepared in 5% BSA and 0.1% Triton-X 100 in PBS at 4°C overnight (more than 20 hr). This primary reaction was developed with a biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories) and then incubated with an alkaline phosphatase (AP) complex (1:200; Vector Laboratories) for 1 hr. The histochemical detection of the alkaline phosphatase activity was performed with BCIP/NBT (Vector Laboratories) in 100 mM Tris-HCl at a pH of 9.8 and mounted with VectaMount AQ Mounting Medium (Vector Laboratories). The images were captured using a BIOREVO microscope (KEYENCE).

4.5 Immunostaining (Cryosections)

Mouse neonate brains were corrected and fixed in 4% paraformaldehyde (PFA: Nacalai tesque), 10% and 25% sucrose at 4°C overnight each and finally embedded in OCT compound (Sakura Finetek). The OCT blocks were sectioned at a 12-μm thickness with a cryostat (MICROTOME) and mounted on Superfrost Micro Slides (Matsunami Glass). The cryosections were fixed in 4% PFA for 10 min at room temperature and washed three times with PBS for 5 min. For antigen retrieval, the sections were boiled in 0.01 M Citrate Buffer pH 6.0 at 80°C for 10 min and then immersed (dehydrated) in cold methanol at −30°C 7 min. After being air dried, the sections were blocked with 10% goat serum, 5% bovine serum albumin (BSA: Sigma Aldrich), and 0.1% Triton-X 100 (WAKO) in PBS at room temperature for 1 hr.

For the immunofluorescence staining, anti-Rtl1 antibody (1:200), anti-Ctip2 antibody (1:500, abcam), and anti-Tbr1 antibody (1:500, abcam) were used as the primary antibody and was prepared in 5% BSA and 0.1% Triton-X 100 in PBS at 4°C overnight (approximately 16 hr). Alexa Fluor 488-conjugated anti-mouse IgG (1:1,000, Thermo Fisher Scientific) and 555-conjugated anti-rabbit IgG (1:1,000, Thermo Fisher Scientific) were used as the secondary antibodies and stained with DAPI (1:1,000, WAKO) for 1 hr. The slides were mounted with VectaShield (Vector Laboratories). The images were captured using a BIOREVO microscope (KEYENCE).

4.6 Open field test

Locomotor activity was measured using an open field test. Each mouse was placed in the corner of the open field apparatus (40 x 40 x 30 cm; Accuscan Instruments, O’hara & Co., LTD.). The chamber of the test was illuminated at 100 lux. Total distance travelled (in cm), vertical activity, and time spent in the center area were recorded. Data were collected for 10 min using a video camera attached to a computer and were calculated by the Image LD program.

4.7 Elevated plus maze test

The elevated plus maze consisted of two open arms (25 x 5 cm) and two closed arms of the same size with 15 cm high transparent walls (O’hara & Co., LTD.). The arms and central square were made of gray plastic plates and were elevated 50 cm above the floor. The behavior testing room (170 x 210 x 200 cm) was soundproof, and the illumination level was maintained at 100 lux. Each mouse was placed in the central square of the maze with its head toward a closed arm. Data were recorded for 10 min using a video camera attached to a computer and were calculated by the Image EP program. The number of entries into each arm and the time spent in the open arms were recorded, and these measurements served as an index of anxiety-like behavior.

4.8 Light/dark transition test

The apparatus used for the light/dark transition test comprised a cage (21 x 42 x 25 cm) divided into two sections of equal size by a partition with a door (O’hara & Co., LTD.). One chamber was brightly illuminated (400 lux), whereas the other chamber was dark. Each mouse was placed into the dark side and allowed to move freely between the two chambers with the door open for 10 min. The total number of transitions between the chambers, the time spent in each, the latency to first enter the light chamber, and the distance travelled in each chamber were recorded using a video camera attached to a computer and calculated by the Image LD program.

4.9 Contextual and cued fear conditioning test

On the 1st day, each mouse was placed into a test chamber (26 x 34 x 29 cm) with a stainless-steel grid floor inside a sound-attenuated chamber and allowed to explore freely for 2 min. A 60 db white noise, which served as the conditioning stimulus (CS), was presented for 30 s, followed by a mild foot shock (2 s, 0.5 mA), which served as the unconditioned stimulus (US). Two more CS-US pairings were presented with a 2 min interstimulus interval. On the 2nd day, a context test was conducted in the same chamber as the conditioning test and data were recorded for 2 min. On the 3rd day, a cued test was conducted in the white opaque plastic chamber and the same 60 db white noise as used on the 1st day was presented for 30 s and data were recorded for 2 min using a
video camera attached to a computer. In each test, the movement freezing percentage and distance travelled were calculated automatically by the Image FZ program.

4.10  |  Morris water maze test

The apparatus used for the Morris water maze test was a circular pool with a diameter of 100 cm and depth of 50 cm. Four high contrast spatial cues were placed about the room (above the pool). A 10-cm diameter clear plexiglass platform was placed in the pool and the pool filled with water until the platform was 1 cm above the water surface. The water was allowed to equilibrate to room temperature (22°C) and the white paint melted so as to make the water in the pool cloudy. Each mouse was put into a location which the computer had specified. During the 1st to 4th days, the maximum trial time was set at 60 s and if mouse found the platform before this time, the program software stopped the trial. On the 5th day, 1 trial was created with no platform zone and the mouse was allowed to swim for 60 s. Data were recorded using a video camera attached to a computer, and the data were calculated automatically by the Image MWM program.

ACKNOWLEDGMENTS

We thank Drs. Y. Sekita (Kitasato University, School of Science), D. Endo (Nagasaki University, Graduate School of Biochemical Science), H. Shiura (the University of Yamanashi) and J. Lee (TMDU) for their helpful advice. Pacific Edit reviewed the manuscript prior to submission.

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

T.K.-I. and F.I. conceived and designed the experiments; M.K. and A.S. performed the experiments; M.K. analyzed KO mice and performed the immunofluorescence analyses; M.K., A.S., T.K.-I., and F.I. analyzed the data and drafted the manuscript.

ORCID

Tomoko Kaneko-Ishino https://orcid.org/0000-0002-2566-9961
Fumitoshi Ishino https://orcid.org/0000-0001-8458-6069

REFERENCES

Aboitiz, F., & Montiel, J. (2003). One hundred million years of interhemispheric communication: The history of the corpus callosum. Brazilian Journal of Medical and Biological Research, 36, 409–420.
Aggleton, J. P., & Nelson, A. J. D. (2015). Why do lesions in the rodent anterior thalamic nuclei cause such severe spatial deficits? Neurosci Biobehavior Reviews, 54, 131–144.
Ainey, M., Meziane, H., Herault, Y., & Humeau, Y. (2018). Synaptic dysfunction in amygdala in intellectual disorder models. Prog Neuropsychopharmacol and Biol Psychiat, 84, 392–397.
Ashley, J., Cordy, B., Lucia, D., Fradkin, L. G., Budnik, V., & Thomson, T. (2018). Retrovirus-like Gag Protein Anc1 Binds RNA and Traffics across Synaptic Boutons. Cell, 172, 262–274.
Briggs, F., & Usrey, W. M. (2008). Emerging views of corticothalamic function. Current Opinion in Neurobiology, 18, 403–407.
Campillos, M., Doerks, T., Shah, P. K., & Bork, P. (2006). Computational characterization of multiple Gag-like human proteins. Trends in Genetics, 22, 585–589.
Charlier, C., Segers, K., Wagenaar, D., Karim, L., Berghmans, S., Jaillon, O., & Georges, M. (2001). Human–Ovine Comparative Sequencing of a 250-kb Imprinted Domain Encompassing the Callipyge (clpy) Locus and Identification of Six Imprinted Transcripts: DLK1, DAT, GTL2, PEG11, antiPEG11, and MEG8. Genome Research, 11, 850–862.
Cheung, L. Y. M., Rizzoti, K., Lovell-Badge, R., & Le Tissier, P. R. (2013). Pituitary phenotypes of mice lacking the notch signalling ligand delta-like 1 homologue. Journal of Neuroendocrinology, 25, 391–401.
Darling-Hammond, L., Flook, L., Cook-Harvey, C., Barron, B., & Osher, D. (2020). Implications for educational practice of the science of learning and development. Applied Developmental Science, 24(2), 97–140.
Edwards, C. A., Mungall, A. J., Matthews, L., Ryder, E., Gray, D. J., Pask, A. J., & Ferguson-Smith, A. C. (2008). The evolution of the DLK1-DIO3 imprinted domain in mammals. PLoS Biology, 6, e135.
Falix, F. A., Tjon-A-Loi, M. R. S., Gaemers, I. C., Aronson, D. C., & Lamers, W. H. (2013). DLK1 Protein Expression during Mouse Development Provides New Insights into Its Function. ISRN Developmental Biology, 2013, 628962.
Fame, R. M., MacDonald, J. L., & Macklis, J. D. (2011). Development, Specification, and Diversity of Callosal Projection Neurons. Trends in Neurosciences, 34, 41–50.
Ferrón, S. R., Charalambous, M., Radford, E., McEwen, K., Wildner, H., Hind, H., & Ferguson-Smith, A. C. (2011). Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis. Nature, 475, 381–385.
Fregosi, M., Contestabile, A., Badoud, S., Borgognon, S., Cottet, J., Brunet, J.-F., & Rouiller, E. M. (2019). Corticocortical Projections From the Premotor or Primary Motor Cortex After Cortical Lesion or Parkinsonian Symptoms in Adult Macaque Monkeys: A Pilot Tracing Study. Frontiers in Neuroanatomy, 13, 50.
Frost, S. B., Milliken, G. W., Plautz, E. J., Masterton, R. B., & J. Nudo, R. J. (2000). Somatosensory and motor representations in cerebral cortex of a primitive mammal (Monodelphis domestica): A window into the early evolution of sensorimotor cortex. The Journal of Comparative Neurology, 421, 29–51.
Ioannides, Y., Lokulo-Sodipe, K., Mackay, D. J., Davies, J. H., & Temple, I. K. (2014). Temple syndrome: Improving the recognition of an underdiagnosed chromosome 14 imprinting disorder: An analysis of 51 published cases. Journal of Medical Genetics, 51, 495–501.
Irie, M., Yoshikawa, M., Ono, R., Iwafune, H., Furuse, T., Yamada, I., & Kaneko-Ishino, T. (2015). Cognitive Function Related to the Sirh11/Zcchc16 Gene Acquired from an LTR Retrotransposon in Eutherians. PLoS Genetics, 11, e1005521.
Irie, M., Koga, A., Kaneko-Ishino, T., & Ishino, F. (2016). An LTR Retrotransposon-Derived Gene Displays Lineage-Specific
control of contextual fear expression is affected in a model of intellectual disability. *Brain Structure and Function*, 220, 3673–3682.

**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.