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Parental origin impairment of synaptic functions and behaviors in cytoplasmic FMRP interacting protein 1 (Cyfip1) deficiency mice

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Abstract

CYFIP1 maps to the interval between proximal breakpoint 1 (BP1) and breakpoint 2(AS) and Prader-Willi syndrome. There is only one breakpoint (BP3) at the distal end of deletion. CYFIP1 is deleted in AS patients with the larger class I deletion (BP1 to BP3) and the neurological presentations in these patients are more severe than that of patients with class II (BP2 to BP3) deletion. The haploinsufficiency of CYFIP1 is hypothesized to contribute to more severe clinical presentations in class I AS patients. The expression of CYFIP1 is suggested to be bi-allelic in literature but the possibility of parental origin of expression is not completely excluded. We generated and characterized Cyfip1 mutant mice. Homozygous Cyfip1 mice were early embryonic lethal. However, there was a parental origin specific effect between paternal Cyfip1 deficiency (m+/p-) and maternal deficiency (m-/p+) on both synaptic transmissions and behaviors. In hippocampal CA1 synapses despite no evidence supporting the parental origin difference for the expression. Both m-/p+ and m+/p- showed the impaired input-output response and paired-pulse facilitation. While the long term-potential and group I mGluR mediated long term depression induced by DHPG was not different between Cyfip1 m-/p+ and m+/p- mice, the initial DHPG induced response was significantly enhanced in m-/p+ but not in m+/p- mice. m+/p- but not m-/p+ mice displayed increased freezing in cued fear conditioning and abnormal transitions in zero-maze test. The impaired synaptic transmission and behaviors in haploinsufficiency of Cyfip1 provided the evidence supporting the role of CYFIP1 modifying the clinical presentation of class I AS patients and in human neuropsychiatric disorders.

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Keywords

parental origin

1. Introduction

Cyfp1 [Cytoplasmic FMRP-interacting protein 1 or Specifically Rac1-associated protein 1 (SRA1)] is known to interact with FMRP (fragile X syndrome mental retardation protein), a protein mutated in fragile X syndrome (FXS) with a function of inhibiting protein translation (Bassell and Warren, 2008; Bhakar et al., 2012; Napoli et al., 2008). Cyfp1 also contributes to the formation of WAVE regulatory complex (WRC) which regulates the actin polymerization in synapses (Burianek and Soderling, 2013). Recently, it has been shown that the functions of these two different protein complexes involving Cyfp1 converge on modulating spine morphology and synaptic plasticity (De Rubeis et al., 2013; Pathania et al., 2014). These molecular studies suggest that Cyfp1 deficiency may cause synaptic development and dysfunction (De Rubeis et al., 2013).

Human CYFIP1 is mapped to the proximal breakpoint of chromosomal 15q11-13 common deletion region, an imprinted region that is implicated in the majority cases of human Prader-Willi (PWS) and Angelman syndrome (AS) (Jiang et al., 1998; Nicholls and Knepper, 2001). The deletion of maternal chromosome 15q11-q13 results in the AS that is characterized by absence of speech, epilepsy, and profound intellectual disability (Kishino et al., 1997; Knoll et al., 1989; Matsuura et al., 1997). In contrast, the paternal deletion of the same chromosomal region leads to the PWS, characterized by neonatal hypotonia, childhood obesity, and moderate intellectual disability (Butler et al., 1986; Cassidy et al., 2012; Nicholls et al., 1989). There are two common breakpoints (BP1 and BP2) in the proximal region and one breakpoint (BP3) in the distal region of 15q11-q13. The deletion between BP1 and BP3 is a larger deletion, i.e class I deletion; while the deletion between BP2 and BP3 is denoted as class II deletion (Nicholls and Knepper, 2001). Four genes, NIPA1, NIPA2, CYFIP1 and TUBGCP5 are mapped between BP1 and BP2 in the 15q11.2 region (Chai et al., 2003; Jiang et al., 2008a). There is no evidence supporting that any of these four genes are imprinted in literature. However, these studies did not ruled out a possibility that the imprinted gene expression for these genes may be either cell-type or development-stage specific manner (Bittel et al., 2006; Gregg et al., 2010a; Gregg et al., 2010b). Clinically, it has been reported that the AS patients with class I deletion have more severe phenotypes compared with those of class II deletion (Butler et al., 2004; Sahoo et al., 2007; Varela et al., 2004). These observations suggest that the deficiency of the gene between BP1 and BP2 may contribute to more severe clinical presentation of class I deletion AS patients. Recently, the copy number variation (CNV) in the 15q11.2 region involving the genes of TUBGCP5, CYFIP1, NIPA2, NIPA1 has been reported to associate with a wide spectrum of clinical presentations related to neurodevelopmental or psychiatric disorders (Abdelmoity et al., 2012; Cooper et al., 2011; De Wolf et al., 2013; Leblond et al., 2012; Stefansson et al., 2014). There are also reports indicating a minimal or no pathogenic association of these CNVs in human disease phenotype because the same CNV is also frequently identified in

individuals without apparent health problems (Burnside et al., 2011; Chaste et al., 2014; Leblond et al., 2012).

Among these four genes within the 15q11.2 region, the CYFIP1 gene is clearly an interesting candidate for a possible role of modifying the clinical presentations of PWS and AS in class I deletion as well as a risk factor for other psychiatric or neurodevelopmental disorders due to its expression in brain and its known interaction with FMRP (Bozdagi et al., 2012; De Rubeis and Bagni, 2011; Napoli et al., 2008). In addition, there were early reports suggesting that a small set of FXS patients have features of PWS (called PWS-like like fragile X syndrome) (de Vries et al., 1993; Gillissen-Kaesbach and Horsthemke, 1994; Schrandt-Stumpel et al., 1994). However, it remains unclear whether these observations are simply rare co-occurrences of some non-specific clinical presentations or there is a molecular link between PWS and FXS. The mapping of CYFIP1, an FMRP interacting protein, in the PWS common deleted interval, suggest a plausible molecular link underlying the clinical observations of PWS-like fragile X syndrome.

To determine whether Cyfip1 deficiency has any functional consequences on synaptic function and behavior, and to better delineate the role of CYFIP1 in modifying the clinical presentation of AS, PWS and other neuropsychiatric disorder, we produced and characterized the Cyfip1 mutant mice by conventional gene targeting approach. We focused particularly on examining whether the functional consequence of haploinsufficiency of Cyfip1 is parental origin dependent because Cyfip1 is mapped in an imprinted domain.

2. Results

2.1. Generation of Cyfip1 mutant mice

Cyfip1 mutant mice were produced by gene targeting in mouse 129 SvEv embryonic stem (ES) cells. Targeting construct was obtained from Mutagenic Insertion and Chromosome Engineering Resource (MICER, <http://www.sanger.ac.uk/resources/mouse/micer/>). These constructs are insertional targeting vectors derived from 129 SvEv genomic DNA that were originally designed for chromosomal engineering to produce chromosomal rearrangement (Zheng et al., 1999). However, the single insertional construct have also been used successfully for targeting of single gene (Mills et al., 1999). As diagrammed in Figure 1, the construct include an 8.3 kb genomic fragment containing exon 5 of Cyfip1 as well as a puromycin selectable cassette. The feature of agouti coat color marker and the 3' half of HPRT were designed to facilitate the chromosomal engineering but not used in our study (Fig. 1B). The construct was linearized with NcoI restriction enzyme and electroporated into ES cells using the protocol as previously described (Jiang et al., 1998). The successful insertional event was selected by genomic Southern blot analysis using a 0.6 kb genomic probe between two NcoI sites (Fig. 1A). Germline transmission of targeted ES cells were obtained through the injection of targeted ES cells into C57BL/6J blastocysts. Mutant mice with targeted mutation of Cyfip1 were confirmed by genomic Southern blot analysis (Fig. 1C). Mutant Cyfip1 allele has been backcrossed to C57BL/6J for more than 8 generations prior to electrophysiology study and behavioral testing.

2.2. Early embryonic lethality of mutant Cyfip1 homozygotes

Cyfip1 heterozygous mutant mice (regardless whether the mutant allele is from the maternal or the paternal chromosome) are fertile and do not have apparently developmental defect or behavioral abnormality. However, we were not able to recover any Cyfip1 homozygotes at weaning age after genotyping more than 100 offspring from breeding between Cyfip1 heterozygotes. Similarly, we were not able to recover Cyfip1 homozygous pups at birth which indicated embryonic lethality associated with the deficiency of Cyfip1. We then dissected the stage of embryonic lethality by genotyping the embryos at embryonic day 16, 12, and 9.5 day. We determined that homozygous embryo died before E9.5 day but did not attempt to determine the exact time before E9.5 day. This is consistent with the observation from a different line of Cyfip1 mutant mice reported by Bozdagi et al. (Bozdagi et al., 2012). The homozygous Cyfip1 embryos probably died as early as at the stage of gastrulation as suggested in Cyfip1 mutant in *C. elegans* (Soto et al., 2002). We have not noticed any difference in survival rate between mice on the mixed 129SvEv and C57BL/6J background and on C57BL/6J background after more than 8 generations of backcrossing. This may indicate the essential role of Cyfip1 during the early embryonic development. We then focused on examining the functional consequence of Cyfip1 heterozygotes and compared the difference between the maternal (m-/p+) and paternal (m+/p-) deficiency of Cyfip1.

2.3. Parental origin expression analysis of Cyfip1 in heterozygotes

Because Cyfip1 is mapped within the proximal region of 15q11-q13 imprinted domain, one question is whether Cyfip1 is also subject to tissue-specific imprinted regulation in brains. We examined the expression of Cyfip1 by quantitative RT-PCR in cortex, hippocampus, amygdala, and cerebellum of both Cyfip1 m-/p+ and Cyfip1 m+/p- adult mice (Fig. 1D). This analysis confirmed the haploinsufficiency of Cyfip1 in heterozygous mutant mice and also suggested that the targeted mRNA is unstable. Interestingly, although the difference is mild, the expression level of Cyfip1 mRNA in m-/p+ and m+/p- relative to each WT were significantly different at cortex (m-/p+, $56 \pm 1\%$, n=10 pairs vs m+/p-, $50 \pm 2\%$, n=10 pairs, p=0.031). However, there was not significant difference in hippocampus (m-/p+, $51 \pm 1\%$, n=10 pairs vs m+/p-, $52 \pm 2\%$, n=10 pairs, p=0.659), amygdala (m-/p+, $54 \pm 3\%$, n=10 pairs vs m+/p-, $55 \pm 1\%$, n=10 pairs, p=0.681) and cerebellum (m-/p+, $58 \pm 3\%$, n=10 pairs vs m+/p-, $52 \pm 2\%$, n=10 pairs, p=0.199). These results suggest a mild preferential expression of Cyfip1 from the paternal chromosome.

2.4. Basal synaptic plasticity differed between Cyfip1 m-/p+ and m+/p- mice

Cyfip1 was postulated to be involved in synaptic function based on its interaction with FMRP and WAVE complex. We tested this hypothesis in hippocampal CA1 synapses using slice electrophysiology. Basal synaptic transmissions in the CA1 synapses were affected similarly in both Cyfip1 m-/p+ and m+/p- groups compared with wild type (WT) littermates. The input-output response were significantly reduced in both m-/p+ and m+/p- mice compared to that of WT controls [m-/p+ (n=15 slices, 7 mice) vs WT (n=20 slices, 8 mice), p< 0.001; m+/p- (n=18 slices, 9 mice) vs WT (n=16 slices, 9 mice); p=0.030] (Fig. 2A,B). We also examined paired pulse facilitation (PPF), an indicator for pre-synaptic

originated short-term plasticity (Fig. 2C,D). In m^{-}/p^{+} group, the PPF was overall higher than WT [m^{-}/p^{+} (n=16 slices, 7 mice) vs WT (n=24 slices, 10 mice), genotype, $p=0.022$; genotype $\times$ interval, $p=0.091$]. The differences were significant at intervals of 100 ms ($p=0.046$) and 400 ms ($p=0.001$). In m^{+}/p^{-} group, PPF was significantly higher than WT at 25, 50 and 100 ms [$(p=0.021, 0.003, 0.006$ at 25, 50, 100 ms intervals; genotype $\times$ interval, $p=0.015$; m^{+}/p^{-} (n=24 slices, 10 mice) vs WT (n=29 slices, 12 mice)].

Because of the known interaction between *Cyfp1* and *Fmrp*, we asked whether *Cyfp1* heterozygotes have abnormal plasticity similar to that was reported in *Fmr1* mutant mice. In *Fmr1* mutant mice, the long-term depression (LTD) induced by the group I mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) (DHPG-LTD) is augmented (Huber et al., 2002). In *Cyfp1 m^{-}/p^{+}* mice, the DHPG-LTD magnitude after 60 min was similar to WT [WT, $67 \pm 3\%$, n=9 slice (7 mice) vs m^{-}/p^{+} , $62 \pm 3\%$, n=14 (8 mice), $p=0.238$]. However, during the initial period after DHPG application, the slope decrease of m^{-}/p^{+} was significantly larger than that of WT ($p < 0.001$) (Fig. 2E). In contrast, in *Cyfp1 m^{+}/p^{-}*, both DHPG-LTD [WT, $61 \pm 2\%$, n=13 (8 mice) vs m^{+}/p^{-} , $65 \pm 2\%$, n=13 (6 mice), $p=0.180$] and the initial slope decrease were similar to those of WT ($p=0.881$) (Fig. 2F). This result indicated that mGluR-mediated LTD was kinetically enhanced only when the *Cyfp1* mutation was from maternally inherited (m^{-}/p^{+}).

We also tested the long-term potentiation (LTP) induced by a single high frequency stimulation (100 Hz, 1 sec) in CA1 region. Although there was a trend of reduced plasticity in m^{-}/p^{+} , mice neither m^{-}/p^{+} nor m^{+}/p^{-} showed any statistically significant differences in LTP when compared with WT [WT, $167 \pm 9\%$, n=12 slices (8 mice) vs m^{-}/p^{+} , $159 \pm 8\%$, n=12 slices (7 mice), $p=0.532$; WT, $137 \pm 6\%$, n=14 slices (8 mice) vs m^{+}/p^{-} , $137 \pm 6\%$, n=14 slices (9 mice), $p=0.957$] (Fig. 2G,H).

2.5. Behavioral differences between *Cyfp1 m^{-}/p^{+}* and m^{+}/p^{-} mice

Many human genetics studies have suggested that the copy number loss of 15q11.2 confers the susceptibility of variable clinical presentations of neurodevelopmental and neuropsychiatric disorders (Burnside et al., 2011; Cafferkey et al., 2014; de Kovel et al., 2010; Murthy et al., 2007; Stefansson et al., 2008). However, negative association was also reported in many other studies (Leblond et al., 2012; Milner et al., 2005; Varela et al., 2005). Our brain slice electrophysiology studies have revealed that both m^{-}/p^{+} and m^{+}/p^{-} mutant mice compromised synaptic transmission but only m^{-}/p^{+} mice have altered the initial induction of mGluR-LTD. We therefore examined whether *Cyfp1* heterozygotes have behavioral phenotypes that may be different between m^{-}/p^{+} and m^{+}/p^{-} mice.

In fear conditioning test with a single foot shock, the *Cyfp1 m^{-}/p^{+}* and their WT littermates did not differ in freezing responses in either contextual (WT, $51.8 \pm 3.4\%$ vs m^{-}/p^{+} , $49.6 \pm 4.2\%$, $p=0.693$) or in cued fear-conditioning test (during pre-tone, WT, $19.3 \pm 3.0\%$ vs m^{-}/p^{+} , $15.4 \pm 3.3\%$, $p=0.405$; tone period, WT, $26.6 \pm 3.4\%$ vs m^{-}/p^{+} , $28.4 \pm 3.3\%$, $p=0.707$; n=10 pairs of WT and m^{-}/p^{+}) (Fig. 3A). It was also noted that the overall percentage of freezing was lower in this particular cohort. The *Cyfp1 m^{+}/p^{-}* mice and their WT littermates also showed similar freezing responses in the contextual fear conditioning test (WT, $49.0 \pm 3.5\%$ vs m^{+}/p^{-} , $55.3 \pm 3.6\%$, $p=0.229$, n=10 pairs of WT and m^{+}/p^{-}).

However, in the cued test, *Cyfp1* *m+/p-* showed significantly higher percentage of freezing than WT both during the pretone (WT, $24.0 \pm 3.9\%$ vs *m+/p-*, $40.1 \pm 5.0\%$, $p=0.035$) and during the cued conditioning when tone was presented (WT, $36.8 \pm 5.7\%$ vs *m+/p-*, $59.7 \pm 7.0\%$, $p=0.021$) (Fig. 3B). We repeated fear conditioning in *m+/p-* using a stronger stimulation paradigm of three pairings of electrical shocks and sound (Fig. 3C; $n=13$ pairs). In this protocol, the freezing response of *m+/p-* was again similar to WT in the contextual test (WT, 61.3 ± 4.6 vs *m+/p-*, $63.6 \pm 3.3\%$, $p=0.682$) and in the cued test pretone period (WT, 24.1 ± 5.5 vs *m+/p-*, $32.9 \pm 4.3\%$, $p=0.341$). However, the freezing response in the *+/-* mice remained significantly higher than WT (WT, 44.1 ± 5.1 vs *m+/p-*, $62.8 \pm 3.5\%$, $p=0.006$) during the tone period.

The increased freezing response of *m+/p-* in fear conditioning motivated us to test anxiety behaviors using the elevated zero maze and open field tests (Yen et al., 2012). In elevated zero maze, both *m-/p+* and *m+/p-* heterozygotes showed similar behaviors to WT in terms of the time of staying in open arm (WT, $13 \pm 2\%$ vs *m-/p+*, $13 \pm 1\%$, $p=0.966$, $n=10$ pairs; WT, $10 \pm 1\%$ vs *m+/p-*, $9 \pm 1\%$, $p=0.406$, $n=13$ pairs), or the number of transitions between open and closed arms (WT, 5.5 ± 1.1 vs *m-/p+*, 5.7 ± 1.0 , $p=0.893$; WT, 4.8 ± 1.1 vs *m-/p+*, 4.8 ± 1.3 , $p=1$) (Fig. 3D,E). However, by examining the pattern of transitions manually, we observed that mice frequently attempted to enter the open arm, but did not complete the transitions to the other side closed arm. Instead, they returned to the entering side of closed arm. We referred this behavior as an incomplete transition. The number of incomplete transitions in *m-/p+* mice was similar to that of WT (WT, 6.4 ± 1.3 vs *m-/p+*, 6.5 ± 1.2 , $p=0.955$) (Fig. 3D). However, *m+/p-* mice made significantly fewer incomplete transitions than WT (WT, 9.2 ± 1.2 vs *m+/p-*, 4.3 ± 0.6 , $p=0.002$) (Fig. 3E). This indicates that once out of the closed arm, *m+/p-* were more likely to cross the open arm toward the other side of the closed arm than WT.

In the open field test, the total distance traveled by *m-/p+* mice was significantly shorter than that of WT (WT, 1830.5 ± 103.2 vs *m-/p+*, 1422.0 ± 116.3 , $p=0.017$, $n=10$ pairs), while the time spent in the center were similar (WT, 958.6 ± 41.6 vs *m-/p+*, 981.9 ± 53.4 , $p=0.735$) (Fig. 3F). In contrast, *m+/p-* mice did not differ from WT littermates in either the total distance (WT, 1777.6 ± 155.1 , $n=15$ vs *m+/p-*, 1642.0 ± 117.3 , $p=0.483$, $n=18$) or in center time (WT, 643.6 ± 41.5 vs *m+/p-*, 743.3 ± 43.8 , $p=0.113$) (Fig. 3G).

3. Discussion

In this study, we reported a new line of *Cyfp1* mutant mice by targeting exon 5 using conventional gene targeting approach. The nature of mutation in our report differs from other *Cyfp1* mutant mice recently reported in which mutation was generated by gene trapped strategy at 5' promoter region of *Cyfp1* (Bozdagi et al., 2012; Pathania et al., 2014). However, the complete penetrance of embryonic lethal phenotypes was observed in all lines of *Cyfp1* mutant mice. These support an important role of *Cyfp1* in early development, probably in gastrulation as suggested in *Cyfp1* null allele *C.elegans* (Soto et al., 2002). *CYFIP1* is mapped to an important domain related to PWS/AS in human. However, there is no molecular evidence in literature supporting a parental origin or biased expression of *Cyfp1*. Thus, the *Cyfp1* mutant mice provided a unique opportunity to examine whether

there is a parental origin specific difference for the functional consequence. Our data provided the evidence supporting a parental origin difference in several functional read-outs in *Cyfp1* heterozygotes mice. Intriguingly, the parental origin difference appears bidirectional. Specifically, absence of the maternal *Cyfp1* allele has a more pronounced effect on mGluR-LTD in hippocampal CA1 synapses while absence of the *Cyfp1* on either maternal or paternal allele has a differential effect on behaviors.

Regardless the parental origin effect, our analysis of synaptic transmission and plasticity in *Cyfp1* heterozygotes supported an important role of *Cyfp1* in a selective domain of synaptic function in hippocampal synapses. For the synaptic plasticity, neither *m-/+* nor *m+/p-* mice showed change in the amplitude of LTP or DHPG-LTD compared to their WT littermates. However, the initial decrease of slope (i.e. maximum transient depression, MTD), in the DHPGLTD was significantly larger in *m-/+* than that of WT. The MTD reflects the activation of Group I mGluR (Michalon et al., 2012). Thus, this observation support the involvement of *Cyfp1* in mGluRs mediated synaptic plasticity in response to the DHPG but did not alter the LTD. The enhanced mGluR MTD plasticity is reminiscent of the enhanced DHPG-LTD in fragile X syndrome but the difference is also apparent. Other than LTD, the higher DHPG sensitivity in *m-/+* may lead to more depolarization and increased excitability (Bianchi et al., 2009; Chuang et al., 2002).

The reduced input-output ratio and the mildly altered PPF in both *m-/+* and *m+/p-* support a possibility that the presynaptic neurotransmitter release is affected by the loss of *Cyfp1*. At the axon terminals, *Cyfp1* and *Wave1* are present as a complex (Kawano et al., 2005). *Wave-1* KO mice showed changes in synapse structure, including synaptic vesicle locations in axon terminals as well as in spine morphology abnormalities (Hazai et al., 2013; Pathania et al., 2014). The reduced level of *Cyfp1* may interfere with normal axon growth or axon terminal formation and neurotransmitter release because of the altered function of *Cyfp1*/WAVE complex. This effect is different from the deficiency of *Fmrp* protein in synapse because basal synaptic transmissions were unchanged in *Fmr1* KO mice (Godfraind et al., 1996; Lauterborn et al., 2007; Paradee et al., 1999; Zhang et al., 2009).

The behavioral impairments in *Cyfp1* heterozygotes are subtle but interesting. The *m-/+* and their WT littermates did not differ overall in fear conditioning and zero-maze. In contrast, *m+/p-* showed increased freezing in fear conditioning and abnormal behavior in the zero-maze test. Enhanced cued fear conditioning *m+/p-* mice may indicate change in associative memory or emotional states (Johansen et al., 2011). The significant difference for the reduced number of incomplete transitions in zero-maze test in *Cyfp1* heterozygote is an interesting observation (Fig. 3E). However, the interpretation of this behavioral feature is not straight forward because the time in open area is the main measure for anxiety. A similar phenomenon was observed in G protein-coupled receptor kinase-interactor 2 (GIT2) mutant mice. In *GIT2* KO, the overall time in open area was similar to that of WT, but KO mice made more transitions in a shorter time per transition (Schmalzigaug et al., 2009). The significantly reduced number of incomplete transition in *Cyfp1* *m+/p-* mice may suggest a possibility of anxiety-like behavior or inflexible behaviors. This may recapitulate some of the behavioral features reported in the individuals with PWS and AS or with the CNV loss

of 15q11.2 in individuals with ASD (Cafferkey et al., 2014; Cassidy et al., 2012; Thibert et al., 2013; Walz, 2007).

The molecular mechanism for the parental origin effect remains unclear in this study. The bi-directional effect in the synaptic function and behaviors resulted from the deficiency of Cyfip1 from either maternal or paternal allele does not strongly support the possibility of an imprinted expression of Cyfip1 in brains. Our expression analysis in different brain regions suggests a slightly preferential expression of Cyfip1 from the paternal chromosome in the cortex. However, this observation does not provide immediate support for the mildly impaired synaptic transmission and behaviors in Cyfip1^{+/-} mice. A more complex imprinted expression mechanism such that the Cyfip1 is preferentially expressed from either maternal or paternal alleles in different brain regions respectively may still be considered. In this mechanism, we have previously reported that the expression of Cyfip1 may initiate from different promoters (Jiang et al., 2008b). Therefore, it can be hypothesized that the imprinted expression for maternal or paternal alleles are promoter and developmental specific. The similar mechanisms have been described for Grb10 or Gnas loci (Peters et al., 1999; Sanz et al., 2008). Alternatively, a more likely mechanism is that there is a biased expression of Cyfip1 for either maternal or paternal allele in different brain regions that is stochastic.

Although the embryonic lethal phenotype is consistent among three different lines of Cyfip1 mutant mice (Bozdagi et al., 2012; Pathania et al., 2014), the apparent differences for the synaptic phenotypes are also observed. Bozdagi et al reported significantly enhanced mGluR5-LTD in hippocampal CA1 region of Cyfip1^{+/-} mice carrying a gene trapped mutation in the 5' non-coding region of Cyfip1 (Bozdagi et al., 2012). In addition, the performance in fear conditioning is normal in this line of Cyfip1^{+/-} mice. The reason underlying this discrepancy between these two lines of Cyfip1 mice is not clear. The different mutations or the difference in the age of used mice for studies may be considered and a head to head comparison may be warranted. The nature of mutation in a report by Pathania et al. is not described in detail and the synaptic plasticity and behaviors are not investigated (Pathania et al., 2014).

In summary, our findings support that Cyfip1 is important for early development and synaptic function. The altered mGluR5 mediated LTD in Cyfip1 deficient synapses may support interaction of Cyfip1 and Fmrp in vivo. The behavioral impairments associated with the haploinsufficiency of Cyfip1 provide support that the role of CNVs containing CYFIP1 in the susceptibility of various human neuropsychiatric disorders (Burnside et al., 2011; de Kovel et al., 2010; Doornbos et al., 2009; Leblond et al., 2012; Stefansson et al., 2014). The parent origin difference in synaptic function and behaviors are interesting and may support a role of CYFIP1 in modifying the clinical presentations of PWS/AS in humans (Bittel et al., 2006; Sahoo et al., 2006). However, the mechanism underlying the complex pattern of parental origin difference remains to be investigated further.

4. Experimental Procedure

4.1. Animals

All experiments were conducted with the protocols approved by the Institutional Animal Care and Use Committee at Duke University.

4.2. Generation of *Cyfp1* mice

Targeting construct of *Cyfp1* was requested Chromosome Engineering Resource (MICER, <http://www.sanger.ac.uk/resources/mouse/micer/>) (Zheng et al., 1999). The construct include an 8.3 kb genomic fragment containing exon 5 of *Cyfp1* as well as a puromycin selectable cassette. The construct was electroporated into AB2.1 ES cell and targeted ES cell clones were identified by DNA mini-Southern blot analysis using the *NcoI* restriction enzyme digestion. The corrected targeted clone was injected into C57BL/6J blastocyst to produce the chimaera. Chimeric mice were bred to C57BL/6J to obtain germ-line transmission of targeted *Cyfp1* allele.

4.3. Mouse breeding and genotyping

Cyfp1^{+/-} mice were backcrossed to C57BL/6J for more than 7 generations prior to these experiments. To obtain m⁻/p⁺ pups, male C57 WT were crossed with female *Cyfp1* m⁻/p⁺ whereas for m⁺/p⁻ pups, male *Cyfp1* m⁻/p⁺ were crossed with female C57 WT. For PCR-based genotyping, the primer sequences were as follows: P1, 5'-AGGCCTTCCATCTGTTGCT-3'; P2, 5'-TGCAAGAACTCTTCCTCACG-3'. The PCR condition is as following: 95°C for 30 s, 57°C for 30 s and 70°C for 60 s for 35 cycles.

4.4. Behavior

Mice of 2 to 3 months old littermates were used. Behavioral tests were conducted at the core facility at Duke University in similar methods as in previous studies from the facility (Besnard et al., 2012; Pogorelov et al., 2005; Porton et al., 2010). fear conditioning: In a conditioning day (day 1), during the 30 sec tone period (72 dB, 12 kHz) scrambled footshock (0.4 mA, 2 sec) was presented together during the last 2 sec in the fear conditioning chamber (Med-Associates). For the contextual test (day 2), a mouse was returned to the same chamber for 5 min. For the cued test (day 3), a mouse was placed in a colored plastic box. After 2 min, the conditioned tone was presented for 3 min. Videotaped behavior was analyzed with software (Noldus observer). zero maze: Elevated (43 cm) zero maze was a circular metal platform with two closed and open arms in symmetry. A mouse was placed in the closed arm. The behavior was videotaped for 5 min and later was analyzed with Noldus Observer. open field test: In an open field (AccuScan Instruments), a mouse was monitored over 30 min using VersaMax software. Distance traveled and open area duration were measured in 5 min bins.

4.5. Field potential recording

The age of mice were 6 - 8 weeks for LTP and postnatal day 24 to 35 for LTD. Hippocampus was cut in transverse section at 400 µm. The slicing solution contained (in mM) 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 7 MgCl₂, 0.5

CaCl₂. Slices were recovered at 30 °C at least 2 hours in ACSF containing (mM) 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 1 MgCl₂ and 2 CaCl₂.

Slices were transferred to the submersion recording chamber at 30°C. A glass recording electrode (1–3 MΩ) filled with ACSF were placed in CA1 radiatum. The concentric bipolar tungsten electrode stimulated Schaffer collateral every 30 sec at baseline. The stimulus strength was adjusted to evoke a field excitatory postsynaptic potential (fEPSP) at half of the maximal response. The input-output relationship was obtained by stimulations at 5, 10, 15, 20, 25, 30, 40, 50, 60, 100 μA (200 μsec) using DS301 or Isoflex. For LTP, after stable baseline for 20 min (less than 5 % drift), high frequency stimulation (100 Hz, 1 s) was applied. The drift was calculated from regression slope. For LTD, DHPG (100 μM, 10 min) was applied with ACSF. For the following 60 min, activity to a single pulse was recorded. The slope at 55–60 min were compared to the pre-conditioning baseline response (last 5 min of baseline). Paired-pulse ratios were obtained from the ratio of slope of the second fEPSP to the first, for a range of inter-stimulus intervals (25–2000 ms). Values are expressed in means ± SE.

4.6. RNA isolation and qRT-PCR RNA expression analysis

Brain slices (500 μm) were obtained including basolateral amygdala (bregma –1.0 to –2.0 mm). Basolateral amygdala, hippocampus and cortex were isolated from the slices. Cerebellum was collected without slicing. Total RNA was isolated from the tissues the Trizol method as previously described (Jiang et al. 1998). For RT-PCR analysis, total RNA was reverse transcribed with iScript reverse transcriptase following the manufacturer's recommendations (Bio-Rad, Hercules, CA). Real-time quantitative PCR was carried out using SSoAdvanced SYBR Green (Bio-Rad, Hercules, CA) on a LightCycler 480 Instrument (Roche Diagnostics, Germany). 100 ng cDNA was used in 20 μl PCR reactions at the following cycling conditions: 95 °C for 30s (1X), 95 °C for 5s and 58 °C for 15s (40X). The Cyfip1 primers used were F:ACAAACAGCCTAACGCACA and R: GGCTCTTGACAACCTTCAGC and Gapdh reference primers were F: TCCCACTCTTCCACCTTCGA and R: AGTTGGGATAGGGCCTCTCTTG. All samples and experiments were run in duplicate. Each PCR run also included 2 samples without RNA template as negative controls. Mouse Gapdh RNA expression was used as an internal reference to normalize the quantity of RNA input across samples for quantification.

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Highlights

- We generated and characterized Cyfip1 mutant mice.
- Cyfip1 has been reported to be a non-imprinted gene.
- Parental origin specific effect between paternal and maternal deficiency.

Figure 1.

Generation of *Cyfip1* 1 mutant mice. A. Insertional targeting construct. MICER clone was obtained from MRC. The construct contains an 8.6 kb genomic fragment and 3' half of *Hprt* exon, puromycin, and agouti gene marker. *NcoI* restriction was used to produce a gap and linearized the genomic DNA before electroporation. B. *Cyfip1* targeted allele after insertional event. C. Genotype of *Cyfip1* heterozygous mutant mice by genomic Southern blot analysis using 0.6Kb genomic fragment between *NcoI* sites as diagramed. D. In *m-/p+* and *m+/p-*, the amount of *Cyfip1* mRNA relative to WT were different at cortex. **p*<0.05.

Figure 2.

A&B. Input-Output relationship. A. m^{-}/p^{+} showed reduced responses than WT at 50, 60 and 100 μA . m^{+}/p^{-} showed reduced responses than WT at 40, 50 μA . C&D. In PPF test, facilitation was increased in m^{-}/p^{+} than in WT at 100 and 400 ms interstimulus intervals. In m^{+}/p^{-} , facilitation was larger 25, 50 and 100 ms. E&F. DHPG LTD. E. In m^{-}/p^{+} , the DHPG LTD during the last 5 min was not different from WT. The initial slope decrease after DHPG of m^{-}/p^{+} was larger than WT. F. In m^{+}/p^{-} and WT, DHPG-LTD was not different. G&H. LTP in CA1 with a single high frequency stimulation (100 Hz, 1 sec). No differences in m^{-}/p^{+} and m^{+}/p^{-} for their WT. * $p < 0.05$.

Figure 3.

A-C Fear conditioning. A. m-/p+ did not differ from WT in contextual or cued test. B. m+/p- and WT were similar in contextual test. In cued test, higher freezing in m+/p- than WT during pretone and tone period. C. Fear conditioning with three electrical shocks in m+/p-. To the tone, m+/p- freezing was higher than WT. D&E. Elevated zero maze. D. m-/p+ did not differ from WT in the time of open arm stay, in the transitions between open and closed arms, or in the incomplete transitions. E. The incomplete transition was fewer in the m+/p- than in WT. F&G. Open field test. The total distance was shorter in m-/p+. *p<0.05.

**p<0.01