Analyses of mental dysfunction-related ACSL4 in Drosophila reveal its requirement for Dpp/BMP production and visual wiring in the brain

Yi Zhang1,2, Di Chen1,2 and Zhaohui Wang1,*

1Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beichen Xilu #1, Beijing 100101, People’s Republic of China and 2Graduate School, Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100039, People’s Republic of China

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Long-chain acyl-CoA synthetases (ACSLs) convert long-chain fatty acids to acyl-CoAs, the activated substrates essential in various metabolic and signaling pathways. Mutations in ACSL4 are associated with non-syndromic X-linked mental retardation (MRX). However, the developmental functions of ACSL4 and how it is involved in the pathogenesis of MRX remain largely unknown. The Drosophila ACSL-like protein is highly homologous to human ACSL3 and ACSL4, and we designate it as dAcsl. In this study, we demonstrate that Dpp production diminished specifically in the larval brain of dAcsl mutants. Consistent with the Dpp reduction, the number of glial cells and neurons dramatically decreased and the retinal axons mis-targeted in the visual cortex. All these defects in Drosophila brain were rescued by the wild-type ACSL4 but not by the mutant products found in MRX patients. Interestingly, expression of an MRX-associated ACSL4 mutant form in a wild-type background led to the lesions in visual center, suggesting a dominant negative effect. These findings validate Drosophila as a model system to reveal the connection between ACSL4 and BMP pathway in neurodevelopment, and to infer the pathogenesis of ACSL4-related MRX.

INTRODUCTION

Long-chain acyl-CoA synthetase (ACSL) is a family of enzymes that activate long-chain (C12–20) fatty acids by adding Coenzyme A to the free fatty acids (1). Acyl-CoA is a potential substrate for diverse cellular processes ranging from lipid metabolism, vesicle trafficking, to signal transduction. There are five members in the mammalian ACSL family, all bearing an AMP-binding domain and a fatty acyl-CoA synthetase (FACS) signature motif (2). They can be further divided into two sub-families based on their homologies to each other, making ACSL3 and ACSL4 in the same sub-family (3).

Mutations in ACSL4, also known as FACL4 (long-chain fatty acyl-CoA ligase 4), have been associated with non-syndromic X-linked mental retardation (MRX) (4–6). ‘Non-syndromic’ means that the patients bearing mutations in ACSL4 display no anomalies other than mental retardation, unlike the syndromic X-linked mental retardation (MRXS) which is usually accompanied by other specific pattern of abnormalities (7). To date, 17 MRX genes have been mapped and cloned (http://www.ggc.org/xlmr.htm), but how they are involved in the development of mental dysfunction is largely unknown.

ACSL4 encodes two protein variants: the shorter one is ubiquituous and the longer one is brain specific (6,8). The enzymatic activity of ACSL4 appears to be critical for the neurodevelopment or maintenance to attain normal mental status, because the mutations associated with MRX reduce the catalytic activity of ACSL4 (4,5). Arachidonic acid is a long-chain fatty acid abundant in the brain and a preferred substrate of ACSL4 (9). It is speculated that arachidonic acid, as an intracellular second messenger or a membrane constituent enriched in brain, may mediate the effect of ACSL4.

To whom correspondence should be addressed. +86 1064807785; Email: zhwang@genetics.ac.cn

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alteration. Additionally, ACSL4 is highly expressed in the hippocampus of both mouse and human, the tissue critical for learning and memory (5,10). A recent report showed that ACSL4 knockdown in rat hippocampal neurons reduced the dendritic spine density and altered the filopodia distribution (8). To this date, however, how ACSL4 is involved in neural development remains elusive.

The model organism Drosophila melanogaster has contributed tremendous knowledge to how organism development is programmed by gene networks. In particular, as a model for neurological disorders, it has provided insights for the pathological development of Parkinson’s, Alzheimer’s, polyglutamine diseases, etc (11–13). Among mental disorders, Fragile X syndrome is the frequent form of inherited mental retardation and has been studied intensively in Drosophila (14–20). In the mushroom body, the learning and memory center of Drosophila, FMRP mediates the sensory-input-dependent axon growth and pruning, indicating its role in development and inferring Fragile X mental dysfunction as a disease of abnormal development (20).

The Drosophila homolog of human ACSL3/4 group is encoded in the genome by a single gene, originally named l(2)44DEa and we designated as dAcsl in this study. We have developed various assays to demonstrate that ACSL4 and dAcsl are functionally conserved. We found in the brain development that dAcsl is required for the expression of decaentaplegic (Dpp), one of the Drosophila BMP homologs. Consistent with the curtailed Dpp expression, glial cells and neurons are much reduced in number and the retinal axons mis-target in the visual center of dAcsl mutants. Intriguingly, all these mutant phenotypes observed in the fly larval brain can be rescued by its human homolog but not by the mutant variants previously found in the MRX patients. Using Drosophila larval brain as an in vivo assay system, we evaluate ACSL4’s function in BMP/Dpp-related brain development, and provide insights into the mechanism by which ACSL4 mutations lead to MRX.

RESULTS

Molecular characterization of dAcsl mutant alleles and the expression patterns of dAcsl

According to the annotations in Flybase (www.flybase.org), there are nine alternatively spliced mRNAs encoding three different protein products of dAcsl, differing in their very N-termini (Fig. 1A1; and see Flybase for details). Multiple mutant alleles were available from the public stock center (Bloomington), but the molecular nature of many was unknown. We molecularly characterized two EMS-induced and one P-element insertion alleles of dAcsl (Fig. 1A and A*). Using the 717 amino acids isoform to describe the mutations, dAcsl<sup>717</sup> carries a point mutation that changes a Val to Asp. This Val is located within a stretch of highly conserved amino acids C-terminal to the FACS signature motif (Fig. 1A, black arrow), which is very essential for the enzyme’s catalytic activity (2). The allele dAcsl<sup>8</sup> contains multiple mutations including K294E, Q419R and W685stop (Fig. 1A, black triangles). K294E is located in the AMP-binding domain; W685stop removes the last 33 amino acids and this truncation was confirmed by the protein analysis (Fig. 1D). The P-element of dAcsl<sup>k10313</sup> is inserted in the first intron of the RB and RD transcripts (Fig. 1A1, sequence submitted to Flybase). dAcsl<sup>58947</sup>, another P-element allele used in this study, is located in the same intron as dAcsl<sup>710312</sup> but slightly more upstream (Fig. 1A1, molecular information available in Flybase). All four alleles are recessively lethal and dAcsl<sup>8</sup> appears to be the strongest based on its lethal stage.

To determine the expression pattern of dAcsl, we examined both transcript and protein in different tissues and at different stages. We found that dAcsl is expressed ubiquitously from embryonic to larval stages (Fig. 1B and C, and Supplementary Material, Fig. S1). The specificity of the antibody was illustrated by spatially specific RNAi and deficiency mutants (Fig. 1B and D). The dAcsl antibody revealed the cytoplasmic signals in embryo and all larval tissues examined (Fig. 1E and F, Supplementary Material, Fig. S1C, and data not shown). Such cytoplasmic localization seemed to depend on the sequence information contained in the N-terminal half of the protein, which was implied by the different intracellular distributions of the N- or C-terminally truncated dAcsl products (Supplementary Material, Fig. S2A).

Because dAcsl is particularly abundant in the fat tissues, we characterized the intracellular distribution of dAcsl in more details in larval fat body cells. Subcellularly, dAcsl co-localized much better with ER than with the Golgi markers (Fig. 1E and F). When human ACSL4 long form was expressed in the larval fat body, it was also co-localized well with the ER marker (Fig. 1G). This subcellular localization of ACSL4 is in accordance with what was observed in rat hippocampal neurons (8). It appears that members of ACSL4 group enzymes are enriched in ER.

Human ACSL4 rescues the lethality of dAcsl mutants

The sequence homology between dAcsl and ACSL4 is high and not restricted to the predicted AMP-binding or the FACS domain (Fig. 1A). There are two isoforms of human ACSL4, the long form has additional 41 amino acids at its N-terminus and is brain specific (6,8). dAcsl (717 amino acids) shares 50% identity/67% similarity to the human ACSL4 short form (ACSL4S, 670 amino acids), 48% identity/64% similarity to the human ACSL4 long form (ACSL4L, 711 amino acids) and 49% identity/65% similarity to ACSL3 (Fig. 1A; ACSL4S and ACSL3 not included in the alignment). The identities in the AMP-binding domain and FACS signature motif are as high as 83 and 74%, respectively.

To examine the functional conservation between dAcsl and human ACSLs, we ubiquitously expressed human ACSL4 or ACSL3 in different dAcsl mutant background. The lethality of dAcsl mutants was rescued by ACSL4, and to a less extent by ACSL3 (Fig. 2 and Supplementary Material, Table S1), and the ACSL4-rescued flies were fertile (data not shown). However, the two MRX-associated forms of ACSL4 (P375L and R570S) did not rescue the lethality of dAcsl mutants, indicating that either of the amino acids is essential for the vital functions of ACSL4. These results demonstrate that human ACSL4 can substitute the function of its fly homolog in the development of the organism.
Figure 1. dAcsl is highly homologous to human ACSL4, ubiquitously expressed and enriched in ER. (A) The longest form of dAcsl shares 48% identity and 64% similarity with human ACSL4 long form (hACSL4). Asterisks denote identical residues; colons denote highly conserved residues; dots denote weakly conserved residues. Black box, the AMP-binding domain; red box, the fatty acyl-CoA synthetase (FACS) signature. The amino acid changes in dAcsl8 allele are marked by black triangles, and the change in dAcsl1 allele by a black arrow. @ symbol denotes stop codon. The stars mark represents the two amino acid changes characterized in MRX families. (A') Schematic illustration of the genomic organization of the dAcsl locus. The translation start sites of the three different isoforms (717, 715 and 707 amino acids) are located in three different exons. The yellow bar and red bars represent the exonal segments corresponding to AMP-binding and FACS domain, respectively. The positions of P-element and EMS alleles used in this study are indicated. Red triangles, EMS hits found in dAcsl8 allele. (B) Larval wing discs stained with dAcsl antibody. Anterior is to the left and ventral up. The dAcsl RNAi was accomplished in the posterior compartment by en-Gal4 (en>dAcsl RNAi). The dAcsl staining was significantly reduced in the posterior compartment of the wing disc where dAcsl was knocked down by RNAi, indicating the specificity of the antibody. (C) Larval brain stained with dAcsl antibody. Anterior is up. (D) Western blot protein analysis of dAcsl in the wild type and the mutants. Embryo extracts of w1118, dAcsl8 and Df(2R)H3E1 (Df) were analyzed, and the protein loadings were revealed by Ponceau S staining. The arrow marks to the position expected for the full-length dAcsl. The black triangle points to the truncated dAcsl protein from dAcsl8 homozygous embryos. Its position is consistent with the predicted size of the truncated form. (E) Fat body cells stained for dAcsl (green) and a Golgi marker (Rabbit anti-GM130, red), and the overlap is poor. (F) Fat body cells stained for dAcsl (green) and an ER marker (Goat anti-GRP78, red). The two signals co-localize very well as indicated by the yellow area in the merged image. (G) Fat body cells stained for ACSL4 long form (green) and an ER marker (red). The ACSL4 long form (ACSL4L) transgene is Myc-tagged at the C-terminus, driven by tub-Gal4, and detected by Myc antibody.
In other words, the functions of ACSL4 and its homolog are highly conserved from fly to human.

**dAcsl is necessary and sufficient for lipid storage, and such function can be substituted by human ACSL4**

dAcsl is predicted to be an acyl-CoA synthetase according to its primary protein sequence. To determine whether or not dAcsl is involved in lipid storage, we compared the neutral lipids deposition in wild type and mutant larval tissues using Oil Red O staining (21). In wild type, the staining was found abundant in the wing primordium, particularly strong in the wing pouch (Fig. 3A). However, the neutral lipids accumulation was blocked in various dAcsl mutants (Fig. 3A). When dAcsl was expressed ubiquitously in the mutant background, the staining of neutral lipids was restored (Fig. 3A, dAcsl8/dAcsl05847:tub>dAcsl). Consistent with the mutant results, when we knocked down dAcsl by RNAi in the posterior compartment, the Oil Red O staining was reduced correspondingly (Fig. 3A). Two independent RNAi lines were used and they exhibited consistent results. In contrast, when we over-expressed the dAcsl in the posterior compartment, the neutral lipids staining was specifically increased in the same area (Fig. 3A). These results demonstrate that dAcsl is both necessary and sufficient for neutral lipids deposition in larval epithelia.

The neutral lipids assay in the larval wing epithelia provided another means for us to explore the functional conservation between dAcsl and ACSL4. Consistent with their ability to rescue dAcsl mutant lethality, expression of ACSL4 or ACSL3 restored the neutral lipids formation in the dAcsl mutant imaginal discs (Fig. 3B). Additionally, ACSL4 expression in the wing posterior induced the neutral lipids accumulation in the corresponding region (Supplementary Material, Fig. S2B, en>ACSL4). In contrast, the expression of either disease-associated form, ACSL4(P375L) or ACSL4(R570S), did not restore the neutral lipids deposition in the wing primordium (Fig. 3B). These results suggest that either of the two amino acids is required for the function of ACSL4 in inducing neutral lipids formation.

To further evaluate how the lipid storage is affected in the whole organism, we quantified the total triacylglycerol (TAG) contents in the pharate adult flies. The TAG contents were lower in both the dAcsl8/dAcsl05847 and Df(2R)H3e1/dAcslk10313 than that in the wild type (Fig. 3C). We also found the TAG content was lower when dAcsl was knocked down by RNAi and was higher when dAcsl was over-expressed (Supplementary Material, Fig. S3C). The TAG reduction in the dAcsl mutants was reversed by expressing ACSL4 (Fig. 3C). The TAG contents correlate well with the fly’s survival under starvation (Supplementary Material, Fig. S3D). Taken together, the data of lipid storage obtained in larval tissues and the whole organism provide additional support for the functional conservation between dAcsl and ACSL4, and imply ACSL4’s function in neutral lipids formation.

**Dpp production in the larval brain is diminished in dAcsl mutant and can be restored by human ACSL4**

The human ACSL4 mutations have been associated with nonsyndromic mental retardations (4–6,8). Since ACSL4 can substitute the functions of dAcsl in the lethality-rescue and lipid-storage assays, we have validated the fruit fly as a model system to investigate whether and how ACSL4 is involved in neurodevelopment. Because the strong dAcsl alleles are embryonic lethal and only certain trans-allelic combinations can survive to pupariation, we focused our study on the brain at the third instar larval stage.

The most obvious abnormality we observed in the dAcsl mutant larval brain was the disordered axon projection coming from the eye primordium (Fig. 4B). In the developing
Figure 3. dAcsl is necessary and sufficient for lipid storage, and human ACSL4 can fulfill the same function. (A and B) Third instar larval wing primordia are oriented anterior to the left and dorsal up. (A) dAcsl is necessary and sufficient for lipid storage in the wing primordium. The neutral lipids in the third instar larval wing primordium were revealed by Oil Red O staining. In wild type, the staining was found in the whole disc and strong in the wing pouch. In either dAcsl^8/dAcsl^05847 or dAcsl^k10313/Df(2R)H3E1, the neutral lipids staining was nearly undetectable. Knocking-down dAcsl in the posterior compartment by en-Gal4>dAcsl RNAi caused neutral lipids reduction, and over-expression of dAcsl by en-Gal4 induced neutral lipids accumulation. When expressing dAcsl ubiquitously by tub-Gal4 in the dAcsl^8/dAcsl^05847 mutant background, the staining was restored in the whole disc. (B) The reduction of neutral lipids in the larval wing primordium in dAcsl mutant can be rescued by the ubiquitous expression of ACSL4 (long and short), or ACSL3, but not by the two MRX-associated ACSL4 variants (P375L or R570S). (C) The TAG contents were reduced in the dAcsl mutant pharates and restored by ACSL4 expression. Total TAG content (µg TAG/mg total protein): (1) wild type; (2) dAcsl^8/dAcsl^05847; (3) dAcsl^k10313/Df(2R)H3E1; (4) dAcsl^8/dAcsl^05847/tub>ACSL4L. Error bars represent standard deviations. There is a significant difference between wild type and the two trans-allelic mutants (*P < 0.05), but not between wild type and dAcsl^8/dAcsl^05847/tub>ACSL4L.
optic system, axons originating from the retinal photoreceptor cells (R1 to R8) grow afferently to make precise contacts with their postsynaptic partners in the optic lobe, the lateral portion of the brain hemisphere. The R1 to R6 axons terminate in the lamina (Fig. 4A); the R7 and R8 in the medulla (Fig. 6B). Consequently, a defined pattern of axon projection was observed in the optic lamina (Fig. 4A), but became obscure and disrupted in the dAcsl mutant (Fig. 4B).

In mammal, BMP serves as a patterning/differentiation signal and axon guidance cues. Dpp, one of Drosophila BMP homologs, is required for the differentiation and migration of glial precursor cells (22). Correctly positioned glial cells in the optic lobe provide the intermediate guidance for the proper projection of the retinal axons (23–26). To find out whether retinal axon mis-targeting was a result of Dpp reduction in the dAcsl mutant, we examined the expression of Dpp revealed by dpp-LacZ in the brain and found that it was significantly reduced in the dAcsl mutant (two dpp-LacZ reporters tested and one is shown in Fig. 4D and E). This is in accordance with the disruption of the optic projections. In the dAcsl mutant background, resupply of dAcsl or human ACSL4, but not the MRX-associated ACSL4 variants, restored the Dpp expression (Fig. 4F and J–L) and re-established the well-ordered retinal projection in the optic lobe (Fig. 4C and G–I). The expression level of the MRX-associated ACSL4 variants was comparable with that of wild type (Supplementary Material, Fig. S4). Notably, Dpp-LacZ expression in the eye or appendage primordia was not detectably altered in the dAcsl mutants (data not shown). It seems that dAcsl is required for Dpp production specifically in the brain.

To address how dAcsl, predicted as an enzyme in lipid metabolism, gets involved in Dpp expression, we made dAcsl mutant

![Figure 4](https://doi.org/10.1093/hmg/ddi355)
clones in the Dpp-expressing domain of the larval brain and examined Dpp expression using two different dpp-LacZ reporters. In general, the homozygous mutant clones were difficult to recover and the survived clones were often very small. Nevertheless, in all mutant clones induced in the Dpp-expressing cells, Dpp-LacZ staining of both reporter lines was apparently normal (Fig. 5A and B). Additionally, expressing dAcsl specifically in the Dpp-domain of dAcsl mutant brain did not restore retinal projection to its precisely ordered pattern (data not shown), unlike ubiquitous expression of dAcsl (Fig. 4B and C). These results suggest that dAcsl is not intrinsically required for Dpp expression in Dpp-expressing cells.

Wingless (Wg) has been demonstrated as an upstream activator of Dpp expression during the pattern formation of the visual center (27). It is expressed in the optic lobe at the posterior-most domains along the dorsal/ventral boundary, and Dpp-positive domains are adjacent to them (Fig. 5D). To see whether Wg expression is compromised in the dAcsl mutant, we examined the Wg protein pattern in the larval brain by immunostaining. Under the exactly same processing and imaging conditions, we could not detect evident reduction of Wg signal in the dAcsl mutant brain relative to that in the wild type (Fig. 5C). Apparently, Wg expression per se is unlikely to account for the diminished Dpp production. However, we cannot exclude the possibility that Wg signaling is compromised at other steps than Wg expression.

dAcsl is required for the assembly of the visual circuitry and can be substituted by human ACSL4 in this process

Precise targeting of the retinal axons requires the interplay with the correctly positioned glial cells in the optic lobe (23–26). Conversely, signals released from the axonal terminals recruit the post-synaptic neurons and trigger the assembly of distinctive glial cell layers (28–30). To better understand how dAcsl is involved in neurodevelopment and also to assess the potentially analogous functions of ACSL4, we analyzed in detail the presence and arrangement of the glia and neurons in the optic ganglia to look for the impairments corresponding to the retinal axon projection in the dAcsl mutants.
Immunostaining of Dachshund (Dac) illuminated the crescent-patterned alignment of the lamina neurons whose number was significantly reduced in \textit{dAcsl} mutants (compare Fig. 6A and D). In wild type, Repo-labeled glial cells align into three layers at the lamina where the retinal axons (R1-R6) terminate and form a densely packed structure called lamina plexus (Fig. 6B and C). In the \textit{dAcsl} mutants, the fine alignment of glial cells disappeared and the lamina plexus was in disarray (Fig. 6E and F). In medulla, the numbers of glial cells decreased greatly in the \textit{dAcsl} mutants (Fig. 6F). Consistently, axon termination in medulla was barely observed (Fig. 6E). All these defects observed in \textit{dAcsl} mutants were consistent with the lack of Dpp expression in the optic lobe (22), and were rescued by the ubiquitous expression of \textit{ACS}L4 (Fig. 6G–I), but not by the MRX-associated form. (C, F, I, L) Repo, a marker of glial cells (cross section, anterior is up). In wild type, the glial cells along the lamina plexus can be divided into three rows: epithelial glial cells (eg), marginal glial cells (mg) and medulla glial cells (meg). These glial cells clumped together in the \textit{dAcsl} mutant, and the medulla neuropil glial cells (mng) layer disappeared (F). This defect was rescued by ACSL4 but not by the MRX-associated form.

**Figure 6.** \textit{dAcsl} is required for the assembly of the visual circuitry and can be substituted by human ACSL4. The genotype of \textit{dAcsl} mutants is \textit{dAcsl}^{dAcsl8/dAcslk10313} in all panels. Image in each panel is a projection of multiple confocal sections. (A, D, G, J) The neurons were visualized using anti-Dachshund (Dac) (lateral view, anterior up). Note that the lamina neurons (in crescent-shaped region) were reduced in the mutant and were rescued by expressing ACSL4, but not by the disease-associated ACSL4(R570S). (B, E, H, K) The photoreceptor axons were visualized by anti-Chp (cross section, anterior is up). In the wild type, different subsets of photoreceptor axons project to the lamina neuropil (lamin) or medulla neuropil (medn). The axon targeting was disrupted in the mutant and was rescued by wild-type human ACSL4, but not by the MRX-associated form. (C, F, I, L) Repo, a marker of glial cells (cross section, anterior is up). In wild type, the glial cells along the lamina plexus can be divided into three rows: epithelial glial cells (eg), marginal glial cells (mg) and medulla glial cells (meg). These glial cells clumped together in the \textit{dAcsl} mutant, and the medulla neuropil glial cells (mng) layer disappeared (F). This defect was rescued by ACSL4 but not by the MRX-associated form.

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**Over-expression of the MRX-associated ACSL4 interferes with the normal Dpp production and wiring in the optic lobe**

An amino acid change (P375L, proline to leucine) in ACSL4 disrupts the enzymatic activity and cosegregates with the mentally challenged individuals in the family carrying this mutation (4). In the various assays described above ranging from lipid metabolism to optic circuit development, ACSL4(P375L) failed to confer the functions that the wild-type ACSL4 achieved. Additionally, when we expressed ACSL4(P375L) in \textit{dAcsl} heterozygous (Fig. 7B–D) or wild-type (Fig. 7E and F) background, we found that the Dpp expression was reduced, glial cell layers did not form properly and retinal axons mis-targeted. The Dpp reduction was more severe when
ACSL4(P375L) was expressed in dAcsl heterozygotes (reduced Dpp-LacZ staining in 18 out of 22 examined, Fig. 7B) than that in the wild-type (reduced Dpp-LacZ staining in 7 out of 16 examined, image not shown). These phenotypes are very similar to those observed in dAcsl hypomorphs. It appears that ACSL4(P375L) may interfere with the normal activity of the endogenous dAcsl. Such effect is quite specific to the optic lobe instead of the peripheral visual system because overexpression of ACSL4(P375L) did not lead to detectable abnormality in the eyes (data not shown). Thus, it suggests that this MRX-associated point mutation (P375L) potentially has a dominant negative effect by interfering with the normal function of the endogenous gene product in the optic lobe. Its relevance to the question of why the heterozygous carriers display mild MRX symptoms is discussed below.

DISCUSSION

Using various analyses in Drosophila development, we have illustrated the functional conservation between ACSL4 and its fly homolog. The larval optic lobe, in particular, provides an accessible, sensitive and relevant readout to reveal the potential functions of ACSL4 in neural development. We found that the Drosophila homolog of ACSL4 is required in the brain optic lobe for the Dpp/BMP production, for the formation of properly aligned glia and neurons, and for the accurate targeting of retinal axons. The correlation between the MRX-associated molecular changes in ACSL4 and their disruption of ACSL4 activity in these assays implies how this protein functions in development, and thus how the mutant products may lead to mental disorder as a result of impaired development.

How is dAcsl involved in the Dpp regulation during the development of visual center?

The reduction of Dpp domain in dAcsl mutant brain can be attributed to the changes in expression levels, cell number and/or cell fate. Besides the Dpp reporters, cellular markers specific for this domain are currently lacking. Thus, we cannot distinguish the possibilities that Dpp reduction in dAcsl mutant is due to reduced cell number and/or change in cell fate, or decreased Dpp expression in each cell. This issue remains to be clarified with additional markers for Dpp-expressing cells in the larval brain.

As indicated by the mutant clonal analysis, dAcsl is not intrinsically required for Dpp expression. This suggests that factors extrinsic to the Dpp-expressing cells may be affected in dAcsl mutants. Clusters of Dpp- and Wg-positive cells are adjacent to each other (Fig. 5D), and Wg has been demonstrated to be the upstream signal required for Dpp activation in the larval optic lobe (27). Because Wg protein in the larval brain did not seem to decrease in dAcsl mutants, we speculate that Wg signaling could be compromised at any step post-translationally.

Palmitate, a 16-carbon saturated fatty acid, is attached to the Wg in the secretory process, and palmitoylation is required for Wg secretion and signaling activity (31, 32). We and others have shown that ACSL4 subfamily enzymes are enriched in ER (Fig. 1E–G) (8). As well as the fact that palmitate is a fatty acid of the length preferred by ACSL family enzymes, it is conceivable that dAcsl could be involved in Wg palmitoylation. Additionally, Wg and lipoprotein particles can be co-purified and the latter is required for Wg signaling in Drosophila wing primordia (33, 34). It is possible that dAcsl contributes to the make-up of the lipid components in

Figure 7. Over-expression of an MRX-associated ACSL4 variant disrupts the optic wiring in the larval brain. Samples in (A) and (C) are dAcsl heterozygous controls. Images in (C–F) are projections of multiple confocal sections. (A and B) Dpp expression revealed by X-gal staining of dpp-LacZBS3.0 was reduced when the ACSL4(P375L) was over-expressed in the dAcsl heterozygous background. (C and D) The retinal axons were markedly reduced in number and the targeting to lamina/medulla was disrupted when the ACSL4(P375L) was over-expressed in the dAcsl heterozygous background. (E and F) The glial cells (E) and the photoreceptor axons (F) were visualized with Repo and Chaoptin, respectively, when the ACSL4(P375L) was expressed in a wild-type background. The number of glial cells along the lamina plexus and in the medulla neuropil (bracket) were severely reduced in the ACSL4(P375L) over-expressing brains (E, compare with the normal pattern in Fig. 6B), and the retinal axon mistargeted (F). eg, epithelial glial cells; mg, marginal glial cells; meg, medulla glial cells.

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lipoprotein particles, and thus gets involved in Wg signaling. In any case, whether or not Wg acylation, trafficking and/or signaling are altered in dAcsl mutants requires further investigations.

**Developmental defects in the brain: the speculated pathogenesis of the ACSL4-associated MRX**

From fly to mammal, BMP and WNT morphogens are crucial for cell growth, differentiation and patterning of the whole organism or various tissues including nervous system. In mammal, BMP and WNT are expressed at the dorsomedial edge of each cerebral cortical hemisphere, and serve as the patterning/differentiation signals and axon guidance cues (35). WNT-3A is required for hippocampus formation (36,37), and BMP helps to maintain WNT signaling and hippocampal development (38). Moreover, a deletion of frizzled 9, one of the WNT receptors, leads to hippocampal and visual-patral learning defects in mice (39). Analogously, Wg and Dpp act together to pattern the visual center of Drosophila brain (27,40). Our analysis of human ACSL4 in Drosophila visual cortex implicates that ACSL4 could play a role in BMP activation during neurodevelopment (Figs 5 and 6). If the close analogy exists between fly and mammal, the reduced ACSL4 activities cause the decrease of BMP expression which would result in un-sustained WNT signaling and hippocampus malformation.

In the families inheriting MRX, some of the female carriers heterozygous for ACSL4 mutations also display mild mental disabilities, although skewed X-inactivation was detected (4,5). This may be explained by the potential dominant negative effect of the mutations in the cells of incomplete gene silencing upon X-inactivation and/or in a tissue mosaic for different X chromosomes (41,42). In support of the former possibility, expression of ACSL4(P375L) in a wild-type background exhibited dominant negative effect and phenocopied the defects of dAcsl mutants observed in larval brain (Fig. 7). Nevertheless, all speculations on ACSL4 and neurodevelopment ought to be tested in mammalian models.

An interesting discrepancy we observed is that the defects in visual cortex formation are more severe in dAcsl mutants than in Dpp pathway mutants (22). It could be a combined effect of Dpp reduction and disrupted lipid metabolism in dAcsl mutants. Neurodegeneration and fatty acids accumulation were observed in the mutants of bubblegum, encoding very long-chain fatty acid CoA synthetase (43). Although we did not detect obvious change in neutral lipids accumulation in dAcsl mutant brain (data not shown), we could not exclude the possibility that the homeostasis of other lipids was disturbed or that critical but subtle changes occurred and exacerbated the existing lesion caused by Dpp reduction.

**Using fly to assess the developmental functions of human ACSL4**

Applying various cellular markers in diverse tissues of Drosophila, we have clearly illustrated the functional conservation between ACSL4 and its fly homolog, and contrasted the wild-type and the MRX-associated ACSL4 variants in every assay ranging from lethality rescue, lipid storage, to the Dpp-induced events in visual cortex development. These findings promise the functional dissection of human ACSL4 in neurodevelopment and lipid metabolism using Drosophila experimental system. These assays can be further explored to screen for other genes involved in the process, to assess the functions of related but different mammalian products, and possibly to be utilized for drug discovery.

**MATERIALS AND METHODS**

**Drosophila stocks and genetics**

dAcsl<sup>8</sup>, dAcsl<sup>12</sup>, dAcsl<sup>k10313</sup>, dAcsl<sup>05847</sup> and Dif(2R)H3E1 were obtained from Bloomington Stock Center. en-Gal4, c754-Gal4, tub-Gal4, dpp-Gal4, Lsp2-Gal4, dpp-LacZ (enhancer trap line) and dpp-LacZ<sup>MP139</sup> (44) lines were described in Flybase. The UAS-dAcsl RNAi (2) line was obtained from the Vienna Drosophila RNAi center (Vienna, Austria; 45).

Mutant clones were generated by the flipase-filippe recognition target method and induced at first or second instar larval stages in 37ºC incubator for an hour. After heat treatment, larvae were allowed to grow at 25ºC for 48 h prior to fixation for antibody staining. All crosses were done at 25ºC unless otherwise specified.

**Molecular characterization of dAcsl mutations**

Genomic DNA was isolated from homozygous mutants, and the complete DNA sequences of the protein coding regions were amplified by PCR and cloned for sequencing to get the molecular information of the two EMS-induced dacsl alleles. The P element insertion site in dAcsl<sup>k10313</sup> was determined by recovering the flanking sequence through plasmid rescue (submitted to Flybase).

**Construction of the transgenes**

dAcsl<sup>717</sup>Myc, dAcsl<sup>715</sup>Myc and dAcsl<sup>707</sup>Myc were constructed by cloning the full-length dAcsl cDNA plus the C-terminal 6xMyc tag into pUAST. UAS-dAcsl<sup>ΔC</sup> removes the C-terminal 163 amino acids and contains 3xFlag tag at the C-terminus of the dAcsl<sup>ΔC</sup> coding region. UAS-MydAcsl<sup>715</sup> and UAS-MyedAcsl<sup>ΔN</sup> contain N-terminal 6xMyc tags, and UAS-MyedAcsl<sup>ΔN</sup> removes the N-terminal 295 amino acids. UAS-ACSL4LMyc, UAS-ACSL4SMyc and UAS-ACSL3Myc were constructed by inserting the human full-length ACSL4, ACSL4S and ACSL3 cDNA into pUAST vectors carrying C-terminal 6xMyc tags.

UAS-dAcsl RNAi (1) was made by insertion of a 648 bp PCR product from the coding region of the dAcsl cDNA into pWIZ vector (46). The PCR primers are: 5'-TCTAGAA GGCACAGATCGTCCGAAT and 5'-CAAATTTACTGT CCTGTCCTGTGCGAAT.

**In situ hybridization and X-gal staining of β-galactosidase activity**

Probes were digoxigenin-labeled sense and anti-sense RNA from dAcsl LP07340 clone. In situ hybridization was carried out as described previously (47).
bands were visualized by 3,3'-diaminobenzidine staining.

**Oil red O and Nile Red staining**

Oil Red O staining was done as described (21). Larvae were dissected in cold PBS fixed in 4% paraformaldehyde/PBS for 20 min. After fixation, specimens were rinsed three times with distilled water and incubated for 25 min in Oil Red O stain (mix of 6 ml of 0.1% Oil Red O in isopropanol and 4 ml distilled water, prepared fresh and centrifuged to remove the precipitation). For the Nile Red staining, the samples were fixed as above and incubated with Nile Red solution (Sigma, 1:1000) for 30 min.

**Immunostaining**

Anti-dAcsl antibody was prepared against the C-terminal 280 amino acids of dAcsl protein expressed in E. coli. The dAcsl coding sequence was cloned to the expression vector pET-28a (Novagen), and the antigen was purified through cutting out the appropriate band from sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gel. Guinea pig was immunized according to the standard protocol and the serum was used directly at a 1:1000 dilution for immunostaining.

All samples were dissected in PBS, fixed and stained as described previously (48). Primary antibodies were used at the following dilutions: mouse anti-Repo (8D12, developed in Corey Goodman’s lab by Bradley W, DSHB) at 1:100; mouse anti-Chaoptin (24B10, developed by Seymour Benzer, DSHB) at 1:100; mouse anti-Wg (4D4, developed in Corey Goodman’s lab by Bradley W, DSHB) at 1:50; mouse anti-Repo (8D12, developed in Corey Goodman’s lab by Bradley W, DSHB) at 1:100; mouse anti-dAcsl (developed by Gerald M. Rubin, DSHB) at 1:100; ER marker goat anti-GRP78 (Santa Cruz) at 1:1000; Golgi marker rabbit anti-GM130 (Santa Cruz) at 1:1000; rabbit anti-β-galactosidase (Cappel) at 1:10 000. AlexaFluor-conjugated secondary antibodies (Molecular Probes of Invitrogen) were used at 1:4000. Fluorescent images were collected by Zeiss ApoTome or Leica Confocal microimaging system.

**Western blot analysis**

Equal number of embryos were ground and analyzed by standard SDS-PAGE. Gel-separated proteins were transferred onto PVDF membrane (Millipore) and washed with PBS including 0.01% Triton X-100 (PBT). Ponceau S staining was carried out according to the standard protocol and served as a loading control. Guinea pig anti-dAcsl serum or peroxidase-conjugated goat anti-guinea pig (Jackson ImmunoResearch, 1:8000) was diluted at 1:8000 in PBT. dAcsl bands were visualized by 3,3'-diaminobenzidine staining.

**TAG assay and starvation assay**

Triacylglycerol measurements were carried out as described previously (49,50). Batches of 10 male flies for each genotype were subjected to thorough homogenization in 0.5% Tween 20 in distilled water and then subjected to a 70°C heat-inactivation step. After centrifugation, the supernatant was used to determine the TAG contents using the Triglycerides Kit (Biosino Biotechnology). The TAG amounts were normalized against the total protein content of the homogenates, determined by the BCA protein assay kit (Beijing Saichi). Experiments were repeated at least three times. Starvation assays were performed as described (49). For each genotype, triplicate batches of 20–25 male flies (~36 h after eclosion) were transferred to vials providing water only.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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**REFERENCES**

ACSL4 plays a role in dendritic spine architecture. *Neuroscience*, **159**, 657–669.


