A Drosophila model of GSS syndrome suggests defects in active zones are responsible for pathogenesis of GSS syndrome

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We have established a Drosophila model of Gerstmann−Sträussler−Scheinker (GSS) syndrome by expressing mouse prion protein (PrP) having leucine substitution at residue 101 (MoPrP P101L). Flies expressing MoPrP P101L, but not wild-type MoPrP (MoPrP 3F4), showed severe defects in climbing ability and early death. Expressed MoPrP P101L in Drosophila was differentially glycosylated, localized at the synaptic terminals and mainly present as deposits in adult brains. We found that behavioral defects and early death of MoPrP P101L flies were not due to Caspase 3-dependent programmed cell death signaling. In addition, we found that Type 1 glutamatergic synaptic boutons in larval neuromuscular junctions of MoPrP P101L flies showed significantly increased numbers of satellite synaptic boutons. Furthermore, the amount of Bruchpilot and Discs large in MoPrP P101L flies was significantly reduced. Brains from scrapie-infected mice showed significantly decreased ELKS, an active zone matrix marker compared with those of age-matched control mice. Thus, altered active zone structures at the molecular level may be involved in the pathogenesis of GSS syndrome in Drosophila and scrapie-infected mice.

INTRODUCTION

Prion diseases (PrDs) are a variety of fatal neurodegenerative diseases including Gerstmann−Sträussler−Scheinker (GSS) syndrome, familial fatal insomnia (FFI), Creutzfeldt−Jakob disease (CJD) and kuru in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in mule deer and elk (1,2).

Key pathological features of PrD are progressive degeneration of neurons without classical inflammation, formation of protease-K (PK)-resistant pathological prion protein (PrP\textsuperscript{Sc}) deposits in neurons and spongiform encephalopathy (2). Although the majority of PrD cases in humans are classified as sporadic forms of CJD, molecular cloning of the prion protein gene (PRNP) linked to inherited PrD (IPrD) has provided tremendous contributions to understanding pathogenesis of PrD. IPrDs caused by mutations in PRNP have been annotated in GSS, FFI and familial CJD (3).

More than 30 different pathogenic mutations in PRNP can be divided into three molecular categories: a point mutation leading to the substitution of an amino acid or an early stop codon or the insertion of extra octapeptide repeats (4).

At least 10 different pathogenic mutations in PRNP have been shown to induce clinical symptoms and pathological features belonging to GSS syndrome which is characterized by prominent progressive cerebellar ataxia with dementia and multicentric amyloid plaques in the cerebellum and cerebral cortex (4−8).

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PK-susceptible human PrP (HuPrP<sup>C</sup>) to PK-resistant PrP (HuPrP<sup>Sc</sup>) (7–9, 13). Studies in the largest kindred with P102L GSS syndrome showed that phenotypic heterogeneity was present and preclinical silence periods may be influenced by an SNP at codon 129 of PrP (13). Phenotypic heterogeneities reported for GSS syndrome and other PrD patients suggested that pathogenesis is the result of a complex series of steps or interventions of pathways and that these events might be influenced by unknown environmental and/or genetic risk factors.

Recent studies aimed at identifying pathways involved with onset and progression of PrD suggested that programmed cell death (PCD) and synaptic dysfunction may contribute to clinical manifestation of PrDs (14–16). Apoptotic cell death was consistently observed in brains with PrDs, especially retina and cerebellum in which spongiform degeneration was minimal or absent (17). Autophagic vacuoles in different sizes and stages of formation in PrD brains suggested that expansion of autophagic vacuoles may contribute to spongiform encephalopathy (16). Pre-synaptic bouton degeneration and dendritic atrophy were observed from PrD brains at terminal stages (18–21). More detailed studies using mouse PrD models showed that synaptic dysfunction may represent pre-symptomatic alterations in brains (22–26). Compared with recordings from pyramidal cells of age-matched control mice, attenuated Shaffer collateral-evoked EPSP, depolarized resting membrane potentials and an increased membrane resistance were recorded from pyramidal cells from scrapie-infected mice (27). Those studies indicate that pre-symptomatic alteration of synaptic structures and functions may be one of the key steps responsible for the manifestation of clinical symptoms.

To understand molecular and cellular pathophysiologies underlying GSS syndrome, transgenic (TG) mouse models were generated by expressing mouse PrP having leucine substitution at residue 101 (P101L), homologous to P102L mutation in human PrP. The TG mice over-expressing mouse (Mo) PrP<sup>P101L</sup> recapitulated key pathological features of GSS syndrome in humans. For example, MoPrP<sup>P101L</sup> TG mice showed spontaneous spongiform degeneration and gliosis in the brains with spontaneously developed neurological dysfunction in old age (28,29). However, knock-in TG mice expressing MoPrP<sup>P101L</sup> under the control of endogenous promoter do not manifest pathological phenotypes and live a normal life span (30,31). Thus, the exact mechanisms underlying onset and progression of P102L GSS syndrome are still unknown.

*Drosophila* has been extensively utilized as a genetic model for elucidating unknown molecular and cellular etiologies underlying human diseases. Evolutionarily conserved molecular components at synapses and basic neuronal signaling pathways between *Drosophila* and humans allow us to investigate molecular and cellular pathophysiologies underlying various neurological disorders in humans (32,33). In this study, we have generated *Drosophila* models of GSS syndrome by transgenically introducing MoPrP<sup>P101L</sup>. We found that MoPrP<sup>P101L</sup> flies phenocopied several key behavioral symptoms and pathological hallmarks reported from human and TG mice with GSS syndrome. Those symptoms were not suppressed by the expression of P35, a viral apoptotic inhibitor. In addition, MoPrP<sup>P101L</sup> induced altered synaptic architectures in larval neuromuscular junctions and progressive reduction of an active zone marker, Bruchpilot (Brp), and a synaptic scaffolding protein, Discs large (DLG), in adult brains. Since similar alteration of an active zone marker was confirmed in scrapie-infected mouse brains, the alteration of active zones at synapses may be an important molecular pathogenic mechanism underlying various PrDs.

**RESULTS**

**Development of a *Drosophila* model of GSS syndrome**

To develop a model of GSS syndrome in *Drosophila*, we utilized the Gal4/UAS bi-partisan system to induce the expression of mouse PrP cDNAs encoding mouse wild-type PrP tagged with human/hamster 3F4 epitope at residue 109M–112M (MoPrP<sup>3F4</sup>) or MoPrP<sup>3F4</sup> with additional proline to leucine substitution at residue 101 (MoPrP<sup>P101L</sup>) in transgenic flies, since 3F4 epitope tagging to MoPrP does not distort the normal topology or functions of PrP and allows us to detect MoPrP using mouse monoclonal anti-PrP3F4 antibodies (MAb-PrP<sup>3F4</sup>) (34,35).

To characterize UAS-MoPrP transgenic flies, three independent lines of UAS-MoPrP<sup>3F4</sup> or UAS-MoPrP<sup>P101L</sup> were crossed with Tubulin-Gal4 driver (Tub-Gal4) flies. Western blot analysis using mouse monoclonal anti-PrP<sup>3F10</sup> antibodies (MAB-PrP<sup>3F10</sup>) (Fig. 1A) clearly detected expressed MoPrP<sup>3F4</sup> and MoPrP<sup>P101L</sup> in transgenic *Drosophila*. No band was observed in larvae carrying the driver (Tub-Gal4/+ ) alone, even though a comparable amount of α-tubulin was detected by MAb-α-tubulin (Fig. 1A). There were no significant differences in the amount of expressed MoPrPs among tested transgenic lines when they were normalized with loading controls (Fig. 1B). MoPrP<sup>3F4</sup> and MoPrP<sup>P101L</sup> appeared as at least three major bands of 25–35 kDa due to the differentially glycosylated forms recognized by both MAb-PrP3F10 and PrP3F4 (Figs 1A and 4A). Additional two or three bands of 18–20 kDa of MoPrP were detected only by MAb-PrP<sup>3F10</sup> (Fig. 4A).

**Expression of MoPrP<sup>P101L</sup> in *Drosophila* induces severe defects in climbing ability**

To determine whether flies expressing MoPrP<sup>3F4</sup> or MoPrP<sup>P101L</sup> in all tissues by *Tub-Gal4* driver showed defects in climbing ability, we tested the climbing ability of age-matched flies expressing MoPrP<sup>3F4</sup> or MoPrP<sup>P101L</sup>. Flies staying at the bottom of vials after agitation of vials on a vortex mixer for 10 s followed by 20 s without agitation (Supplementary Material, Movie S1) were considered to have behavioral defects. More than 70% of 30-day-old MoPrP<sup>3F4</sup> flies were able to climb and stay at the top or wall of the vials. However, MoPrP<sup>P101L</sup> flies showed severe climbing defects even at day 10. Less than 20% of flies were able to climb the vials. Those defects were even more severe at 20 or 30 days of age (Fig. 1C). Three independent lines expressing MoPrP<sup>P101L</sup> showed severe climbing disabilities, whereas three independent lines expressing MoPrP<sup>3F4</sup> did not show any defect. Thus, MoPrP<sup>P101L</sup> induces significant behavioral defects in *Drosophila*. 
Two different forms of MoPrPs in Drosophila moved or targeted to the synapses

The localization patterns of two different forms of MoPrPs in Drosophila larval brains were examined by performing double-labeling immunocytochemistry using MAb-PrP 3F4 and rabbit anti-Drosophila synaptotagmin (DSypt) antibodies (Fig. 2A–C). The larval central nervous system (CNS) and ventral nervous system (VNS) of control (Tub-Gal4/+) flies did not show any PrP immunoreactivity, even though synaptic vesicle pools in the CNS and the VNS were clearly detected by DSypt antibodies (Fig. 2A). In contrast, in Drosophila transgenic for MoPrP3F4 or MoPrP P101L, PrP immunoreactivity was detected in the CNS and the VNS. In addition, stronger PrP immunoreactivity was localized at the synaptic regions of mushroom bodies in the CNS and the neurophil regions of the VNS which were revealed by DSypt immunoreactivity (Fig. 2B and C).

To test whether two different MoPrPs were targeted to the synaptic terminals, Type 1 glutamatergic synaptic boutons in larval neuromuscular junctions (NMJs) expressing either MoPrP3F4 or MoPrP P101L were examined by performing double-labeling immunocytochemistry utilizing MAb-PrP 3F4 together with one of several antibodies specific to synaptic molecular components. When MoPrPs were expressed at the pre- and the post-synaptic terminals, strong PrP immunoreactivity was observed from the pre- and post-synaptic terminals of Type 1 glutamatergic synaptic boutons (Fig. 2D–I). Synaptic vesicles at the pre-synaptic terminals were partially colocalized with PrP immunoreactivity (Fig. 2D and G). Immunoreactivity of DLG that is present mainly at the post-synaptic terminal subsynaptic reticulum structures (36) was overlapped with PrP immunoreactivity (Fig. 2E and H). The post-synaptic density structures revealed by rabbit anti-Drosophila p21-activated kinase antibodies were encompassed by PrP immunoreactivity (Fig. 2F and I). These double-labeling immunocytochemistry results suggested that expressed MoPrPs were targeted or moved to the synaptic terminals of Type 1 glutamatergic larval NMJs.

By utilizing the neuronal-specific C155-Gal4 driver and the muscular-specific C57-Gal4 drivers, we further examined whether the motor neuron-specific or the muscular-specific expression of MoPrPs are enough to target or move MoPrPs to the synaptic terminals. When MoPrPs were expressed at the motor neurons, PrP immunoreactivity was completely enclosed by the immunoreactivity of a neuronal membrane-specific marker, goat anti-Horse Radish Peroxidase (HRP) antibodies (Fig. 2J and K). When MoPrPs were expressed at the muscles, PrP immunoreactivity surrounded HRP immunoreactivity (Fig. 2L and M) and mostly overlapped with DLG immunoreactivity (Fig. 2N and O). These results suggested that the motor neuron-specific or the muscular-specific expression of MoPrPs were enough to target or move MoPrPs to the pre- or post-synaptic terminals of Type 1 glutamatergic NMJs, respectively.

Expressed MoPrP P101L accumulated in Drosophila adult brains, but was resistant to PK digestion and enzymatic deglycosylation

To determine whether the amount of MoPrPs in the adult brains in Drosophila was progressively increased or not, we employed
Figure 2. Two different MoPrPs were localized at the synapses in Drosophila larvae. Single-slice confocal images taken from the larval brains of control larvae (A), MoPrP^{3F4} larvae (B) or MoPrP^{P101L} larvae (C) depict that the two different MoPrPs are expressed in Drosophila larvae. (A) There was no PrP immunoreactivity in the larval CNS and VNS of the control larvae, even though strong DSypt immunoreactivity was clearly detected. (B and C) Similar localization patterns of two MoPrPs at the Type 1 glutamatergic synaptic boutons in larval NMJs were revealed by several antibodies. MoPrPs are partially colocalized with synaptic vesicle pools and were revealed by anti-DSypt antibodies. (D and G) DLG immunoreactivity, present mainly at the postsynaptic terminals of Type 1 glutamatergic NMJs, overlapped with MoPrPs (E and H). Anti-DPak, the post-synaptic density marker, was completely enclosed by MoPrP immunoreactivity (F and I). MoPrPs, targeted or moved to the pre-synaptic terminals of motor neurons, were completely enclosed by neuronal membranes labeled with a neuronal membrane-specific marker, anti-HRP (J and K). MoPrPs expressed at the postsynaptic terminal muscles were surrounded by neuronal membrane structures labeled by anti-HRP (L and M) and overlapped with DLG immunoreactivity (N and O).
UAS-MoPrP<sub>3F4</sub> (1–3) flies and UAS-MoPrP<sub>P101L</sub> (2–3) flies expressing similar amounts of MoPrP at the third instar larvae to further studies (Fig. 3A and B). Protein extracts from 1-, 10-, 20- and 30-day-old adult heads were analyzed using MAb-PrP<sub>3F10</sub> and MAb-α-tubulin for quantifying the amount of MoPrPs in brains. The amount of MoPrPs in brains expressing MoPrP<sub>3F4</sub> and MoPrP<sub>P101L</sub> gradually increased as flies aged (Fig. 3A). There were no significant differences in the amount of MoPrPs between MoPrP<sub>3F4</sub> and MoPrP<sub>P101L</sub> flies when they emerged (Fig. 3A). However, the amount of MoPrP<sub>P101L</sub> in brains of 10-, 20- and 30-day-old MoPrP<sub>P101L</sub> flies was significantly more than that of MoPrP<sub>3F4</sub> in age-matched MoPrP<sub>3F4</sub> flies (Fig. 3A and B). To verify that expression-level differences of Prnp mRNA in MoPrP<sub>3F4</sub> (1–3) flies and MoPrP<sub>P101L</sub> (2–3) flies did not contribute to different amounts of MoPrPs in aged adult heads, we extracted total RNA from 12-h-old embryos, first larva (24 h after egg laying), second instar larva (48 h after egg laying), early third instar larva (72 h after egg laying), late third instar larva (96 h after egg laying), 1-day-old adult flies and 10-day-old adult flies and then processed for semi-quantitative RT–PCR (Q-RT–PCR). (C) RT–PCR products of Prnp mRNAs from 15, 30, 45 cycles were compared. Similar Prnp mRNA levels were detected during development. Actin-5C was used as loading control. Asterisk indicates significant differences at P < 0.01. Double asterisks indicate significant differences at P < 0.005. ‘W’ represents MoPrP<sub>3F4</sub> and ‘M’ represents MoPrP<sub>P101L</sub>.

**Figure 3.** Enhanced accumulation of MoPrP<sub>P101L</sub> in *Drosophila* adult brains. The amounts of expressed PrP in *Drosophila* heads were examined by MAb-PrP<sub>3F10</sub> blotting (A) and quantified using MAb-α-tubulin as a loading control (B). One-day-old flies did not show significant differences. However, significantly more MoPrP<sub>P101L</sub> was detected from 10-, 20- and 30-day-old flies. To examine expression levels of MoPrP mRNAs from the two transgenic lines, UAS-MoPrP<sub>3F4</sub> (1–3) and UAS-MoPrP<sub>P101L</sub> (2–3), total mRNAs were extracted from embryos (12 h after egg laying), first larva (24 h after egg laying), second instar larva (48 h after egg laying), early third instar larva (72 h after egg laying), late third instar larva (96 h after egg laying), 1-day-old adult flies and 10-day-old adult flies and then processed for semi-quantitative RT–PCR (Q-RT–PCR). (C) RT–PCR products of Prnp mRNAs from 15, 30, 45 cycles were compared. Similar Prnp mRNA levels were detected during development. Actin-5C was used as loading control. Asterisk indicates significant differences at P < 0.01. Double asterisks indicate significant differences at P < 0.005. ‘W’ represents MoPrP<sub>3F4</sub> and ‘M’ represents MoPrP<sub>P101L</sub>.

Expression of MoPrP<sub>P101L</sub> in cholinergic neurons was sufficient to induce climbing defects and early death in *Drosophila*

To identify which circuits in the nervous systems or tissues in *Drosophila* are responsible for age-dependent progressive motor defects in MoPrP<sub>P101L</sub> flies, we utilized several previously characterized Gal4 driver flies. We employed Tub-Gal4 and Actin-5C (Act-5C)-Gal4 driver flies for ubiquitous expression of MoPrP<sub>3F4</sub> or MoPrP<sub>P101L</sub> because PrP and PRNP mRNA are present in neuronal and non-neuronal tissues in humans and mammals (41). We also employed C155-Gal4 driver flies for pan-neuronal expression, Choline acetyltransferase (Cha)-Gal4 driver flies for cholinergic
neuronal expression, 3,4-dihydroxyphenylalanine-\textit{L}-decarboxylase (\textit{Ddc})-\textit{Gal4} driver flies for mainly dopaminergic neuronal expression, \textit{Gliotactin} (\textit{Glio})-\textit{Gal4} drivers flies for expressing in subsets of glial cells, \textit{C57-Gal4} driver flies for muscular-specific expression and \textit{201Y-Gal4} driver flies for mushroom body expression to examine important tissues or circuits for manifesting severe behavioral symptoms. Genetic background carrying only \textit{Gal4}-driver (\textit{Gal4}/+ or transgenes (\textit{UAS-MoPrP}/+) was used as a control for this study. We examined two \textit{UAS-MoPrP} controls, \textit{UAS-MoPrP}_{3F4}/+ and \textit{UAS-MoPrP}_{P101L}/+, neither of which showed any significant difference in climbing ability and survival rates (Supplementary Material, Fig. S1).

The striking differences in behavior were observed when \textit{MoPrP}_{P101L} was expressed in all tissues or cholinergic neurons (Fig. 5). When \textit{Tub-Gal4} and \textit{Act-5C-Gal4} were used for ubiquitous expression of \textit{MoPrP}_{3F4} or \textit{MoPrP}_{P101L}, \textit{MoPrP}_{P101L} flies showed severe defects in climbing ability even at day 5. After 20 days of age, more than 90% of \textit{MoPrP}_{P101L} flies were not able to climb (Supplementary Material, Movie S1, Mutant). In contrast, more than 50% of \textit{MoPrP}_{3F4} flies and controls were able to climb the vials (Supplementary Material, Movie S1, Wild). Interestingly, when \textit{Cha-Gal4} driver was employed, more than 90% of flies failed to pass the climbing test at 20 days of age. A similar, but delayed, climbing defect was observed when pan-neuronal \textit{C155-Gal4} driver was used. After 55 days of age, more than 90% of flies were incapable of climbing the vials. Flies expressing \textit{MoPrP}_{P101L} in mainly dopaminergic neurons (\textit{Ddc-Gal4}), mushroom body (\textit{201Y-Gal4}), subsets of glial cells (\textit{Glio-Gal4}) and muscles (\textit{C57-Gal4}) did not show any obvious climbing disability (Supplementary Material, Fig. S2).

Expression of \textit{MoPrP}_{P101L} in all tissues by \textit{Tub-Gal4} or \textit{Act-5C-Gal4} significantly induced the early death of flies (Fig. 6A and B). Even though half of the controls and half of the \textit{MoPrP}_{3F4} flies lived until 70 days of age, more than 50% of flies expressing \textit{MoPrP}_{P101L} generated by \textit{Tub-Gal4} or \textit{Act-5C-Gal4} were dead at 25 and 30 days of age, respectively (Fig. 6A and B). When \textit{MoPrP}_{P101L} was expressed in all neurons (Fig. 6C), ~50% of \textit{MoPrP}_{P101L} flies were dead at 55 days of age, even though ~50% of controls or \textit{MoPrP}_{3F4} flies were alive until ~70 days of age. By 25 days of age, more than 60% of flies expressing \textit{MoPrP}_{P101L} in cholinergic

![Figure 4](https://academic.oup.com/hmg/article-abstract/19/22/4474/2527272)
neurons were dead. In contrast, more than 50% of controls and flies expressing MoPrP3F4 in cholinergic neurons survived by 70 days of age (Fig. 6D). Similar to climbing test results, flies expressing MoPrP P101L in muscles, glial cells, dopaminergic neurons or mushroom bodies did not show significant early death compared with those of controls (Supplementary Material, Fig. S2). These results suggested that MoPrPP101L in neurons, especially in cholinergic neuronal circuits, may...
be responsible for progressive loss of motor control and early death of flies.

**MoPrP**<sup>P101L</sup> forms deposits and induces vacuoles in *Drosophila* adult brains

To determine whether live MoPrP<sup>P101L</sup> flies showing severe climbing defects have PrP deposits and/or degeneration in neurons in adult brains, we examined the brains of 20-day-old flies expressing MoPrP<sup>3F4</sup> or MoPrP<sup>P101L</sup>. At this stage, more than 90% of MoPrP<sup>P101L</sup> flies showed climbing defects, but less than 25% of flies were dead (Figs 5A and 6A). Only live flies were processed for immunohistochemical and pathological analysis. When midline sections from heads of MoPrP<sup>3F4</sup> flies were examined, PrP immunoreactivity was diffusely distributed all over the brains (Fig. 7A, C and E). In contrast, strong PrP immunoreactivity, mainly presenting as deposits, was observed in the midline brain section of MoPrP<sup>P101L</sup> flies (Fig. 7B, D and F).

We further analyzed the degeneration of neurons by performing hematoxylin and eosin (H&E) staining. The adult brains of MoPrP<sup>3F4</sup> flies did not show any obvious vacuoles (Fig. 7A, G and I). Various sizes of vacuoles, indicated by black arrows from several parts, were observed in the brains of MoPrP<sup>P101L</sup> flies (Fig. 7B, H and J). We counted the number of vacuoles bigger than 5 μm in diameter from 20-day-old flies expressing MoPrP<sup>3F4</sup> or MoPrP<sup>P101L</sup>. MoPrP<sup>P101L</sup> flies had a 5-fold increase in the number of vacuoles compared with MoPrP<sup>3F4</sup> flies (Table 1). Vacuoles were more often observed at the superior medial protocerebrum (Fig. 7H), the lobula and the lobula plate in adult heads (Fig. 7J). These results suggested that MoPrP<sup>P101L</sup> in adult heads formed deposits and induced vacuoles.

**Expression of MoPrP<sup>P101L</sup> induced altered synaptic architecture of Type I glutamatergic larval NMJs**

In this study, we have shown that MoPrPs were localized at the synaptic terminals in Type 1 glutamatergic larval NMJs (Fig. 2). To determine whether MoPrP<sup>P101L</sup> expression resulted in any structural defects at synapses, we further examined Type 1 glutamatergic synapses in larval NMJs by staining with a neuronal membrane-specific marker, anti-HRP antibody. Type 1 glutamatergic NMJs expressing MoPrP<sup>P101L</sup> in all tissues or neurons had more small-sized satellite boutons (Fig. 8, white arrows) than those of controls or MoPrP<sup>3F4</sup> NMJs (Fig. 8J and K). However, the muscular-specific expression of MoPrP<sup>P101L</sup> did not increase the number of satellite boutons (Fig. 8L). These results were consistent with behavioral analysis results and suggested that the neuronal...
expression, but not the muscular expression, of MoPrP<sup>P101L</sup> induced abnormal development of synaptic architectures.

**Progressive declines of Brp and DLG in MoPrP<sup>P101L</sup> adult flies**

To further characterize the synaptic defects associated with MoPrP<sup>P101L</sup> expression, we compared the localization patterns of several synaptic molecular components including DSypt, Brp, Dpak, and DLG in brains and synaptic boutons of MoPrP<sup>P101L</sup> larvae with those of MoPrP<sup>F34</sup> larvae. Among tested synaptic markers, Brp immunoreactivity in the brains and Type 1 glutamatergic synaptic boutons was significantly reduced in MoPrP<sup>P101L</sup> larvae, compared with that in MoPrP<sup>F34</sup> larvae (Fig. 9A–D). Relative Brp-specific intensities observed from synaptic boutons and brains of MoPrP<sup>P101L</sup> larvae were 50 and 80%, respectively, of intensities seen in MoPrP<sup>F34</sup> larvae (Fig. 9E and F). We did not find any significant alteration in the localization patterns or amounts of other synaptic molecular components in the brains and the synaptic boutons of MoPrP<sup>P101L</sup> larvae (data not shown).

We further examined by western blot analysis whether Brp in adult heads of MoPrP<sup>P101L</sup> flies was also altered. Two Brp-specific bands around 150 kDa were significantly reduced in 30-day-old MoPrP<sup>P101L</sup> flies compared with those of age-matched MoPrP<sup>F34</sup> flies (Fig. 9G and H). In addition, the amount of DLG in adult heads was also significantly decreased in 30-day-old flies (Fig. 9G and I). These results suggested that the amounts of Brp and DLG in the adult head are continuously decreasing as flies age and that significantly reduced amounts of Brp and DLG in the synapses of MoPrP<sup>P101L</sup> brains may be the basis of severe phenotypic abnormalities.

**Reduced ELKS, a component of cytoskeletal matrix assembled at active zones and Synaptotagmin I in scrapie-infected mice**

To examine whether the amount of ELKS, a component of cytoskeletal matrix at active zone (CAZ) in the hippocampi of scrapie-infected mouse brains, was altered, ELKS in scrapie-infected mice hippocampi was compared with that in the control mouse hippocampi. There was a 20% reduction in the level of ELKS in the hippocampi of scrapie-infected mice compared to control hippocampi (Supplementary Material, Fig. S4A). In addition, the amount of Sypt I in scrapie hippocampi was reduced to ~50% of the level in...
control mice (Supplementary Material, Fig. S4A). These results suggested that scrapie-infected mouse brains also showed a reduced amount of CAZ and synaptic vesicle pools at the synaptic terminals.

Expression of P35, viral anti-apoptotic proteins did not suppress abnormal behavior

To test whether PCD contributed to severe defects in climbing ability and early death of MoPrP P101L, P35, a viral Caspase 3 inhibitor, was expressed together with MoPrP P101L in cholinergic neurons (Figs 5D and 6D). The numbers of climbing flies between MoPrP P101L alone or together with P35 were similar (Fig. 5D). These results suggested that the climbing disability observed from MoPrP P101L flies was not due to activation of Caspase 3-dependent PCD pathways.

The concomitant expression of P35 and MoPrP P101L in flies significantly induced early death of flies compared with controls, MoPrP3F4 flies, P35 flies and MoPrP P101L flies (Fig. 6D). These synergistic effects of P35 and MoPrP P101L in the early death of flies suggested that the death of MoPrP P101L flies was regulated by one or more unknown pathways that are independent upon Caspase 3.

DISCUSSION

A Drosophila model of GSS syndrome recapitulates key pathological hallmarks of GSS syndrome

The major goal of the present study was to develop a Drosophila model of GSS syndrome to gain insight into the mechanisms by which GSS mutant PrP induces clinical symptoms of GSS syndrome. Recent studies utilizing TG animal models have shown that highly over-expressed wild-type PrPs have given rise to phenotypic abnormalities that were similar to those of PrD models. For example, the highly over-expressed wild-type hamster PrP in flies resulted in degenerative phenotypes in a dose-dependent manner (42). In addition, TG mice over-expressing MoPrP3F4 ~10-fold, but not ~5-fold, developed spontaneous neurodegenerative symptoms such as tremor, paralysis and early death (43). Even though Gavin et al. (44) have shown that the expression of MoPrP P101L in flies induced accelerated accumulation of MoPrPs and spongiform degeneration in brains, the expressed amount of MoPrP P101L was >20-fold more than MoPrP3F4. Thus, their results are not enough to verify that the phenotypic abnormalities observed from MoPrP P101L are not due to differences in the amount of expressed PrPs. Therefore, to verify that any pathological abnormalities observed from flies expressing MoPrP P101L are not due to differences in the amount of expressed PrPs, we generated and examined the amount of expressed MoPrPs to select transgenic flies with similar amount of PrPs. Six independent transgenic flies did not show significant differences in the amount of expressed PrPs throughout development (Fig. 3). Thus, our most significant finding in this study is that the expression of MoPrP P101L, but not MoPrP3F4, either in all tissues or in the nervous systems of Drosophila, results in severe defects in climbing behavior and early death (Figs 5 and 6).
Since *Drosophila* does not have a gene showing significant homology to MoPrP (data not shown; a search result from http://flybase.org/blast), it is not possible to quantify the exact amount of over-expressed MoPrPs. Flies expressing MoPrP<sup>3F4</sup> ubiquitously, but not specifically, at neuronal circuits or non-neuronal tissues showed weak but significant loss in climbing abilities compared with those of controls (but less loss than seen for MoPrP<sup>P101L</sup>) (Fig. 5 and Supplementary Material, Fig. S2), suggesting that over-expressed MoPrP<sup>3F4</sup> in non-neuronal tissues may cause unknown negative effects in locomotor controls in flies ubiquitously expressing MoPrP<sup>3F4</sup>. Nevertheless, the survival rates of all examined MoPrP<sup>3F4</sup> flies were not significantly different from those of controls (Fig. 6 and Supplementary Material, Fig. S3). In addition, MoPrP<sup>3F4</sup> flies do not have vacuoles, which were observed in the adult brains of MoPrP<sup>P101L</sup> flies (Fig. 7 and Table 1).

Different from TG mouse and fly models over-expressing PrP<sup>P101L</sup>, knock-in TG mice expressing wild-type levels of MoPrP<sup>P101L</sup> under control of endogenous *Prnp* promoter do not exhibit spontaneous neurodegenerative symptoms (30,31). Instead, the incubation periods of those knock-in mice after inoculated with several scrapie strains were significantly altered (30,31). One possible explanation for this phenomenon is that knock-in TG mice may not live longer enough to develop spontaneous neurological symptoms since reported onset ages of GSS syndrome in humans are over 40 years (30,31). Another possibility is that more MoPrP<sup>P101L</sup> are required for manifesting spontaneous symptoms in knock-in mice models for GSS syndrome. Taken together, the amount of expressed normal or mutant PrPs in animals may be a critical factor for generating more appropriate animal models for GSS syndrome.

Similar to what has been observed in TG mouse models (28) and human cells over-expressing HuPrP<sup>P101L</sup> (38), we found that MoPrPs in *Drosophila* are differentially glycosylated (Figs 1, 3 and 4) and localized at the synapses (Fig. 2). In addition, MoPrP<sup>P101L</sup> showed increased resistance to PK digestion and deglycosylation compared with those of MoPrP<sup>3F4</sup> (Fig. 4). In addition, significantly more MoPrP<sup>P101L</sup> accumulated in adult brains than did MoPrP<sup>3F4</sup>, even though the amount of the two MoPrPs in adult heads is progressively increasing (Fig. 3). Furthermore, MoPrP<sup>P101L</sup> in adult brains is mainly present as deposits (Fig. 7), which are reminiscent of multincentric amyloid plaques in human or TG mouse brains with GSS syndrome (29,46). Taken together, the expression of MoPrP<sup>P101L</sup> in flies is enough to recapitulate several key behavioral, biochemical and pathological hallmarks observed in human or TG mouse models with GSS syndrome.

**Involvement of cholinergic neural circuits in *Drosophila***

**GSS syndrome**

Another interesting finding in this study is the demonstration of the involvement of the cholinergic neural circuit in the induction of GSS syndrome in flies. Similar to findings in other neurodegenerative disorders in humans, certain types of neurons or parts of brains manifest pathological defects in PrDs, even though causative PrPs are expressed in tissues and cells other than brain neurons (47–49). For example, MoPrP<sup>P101L</sup> TG mouse brains with phenotypic abnormalities showed severe vacuolation in the globus pallidus, cerebellum, substantia niagra, neocortex and caudate (29). Even though it is still not clear whether cholinergic neurons in human brains with GSS syndrome are affected or not, a recent study in sporadic and variant CJD cases showed that dopaminergic neurons and striatal outflow neurons, but not cholinergic neurons, in the nigrostriatal pathway are significantly damaged (50). How can we reconcile those differences between this report and our finding? One possibility is that the basic locomotor control neural circuits between *Drosophila* and mammals may be different, because they diverged at very early stages of animal evolution. Since many neurodegenerative disorders in humans, such as polyglutamine repeat diseases (51), Parkinson’s disease (52,53), Alzheimer’s disease (54,55), early-onset torsion dystonia (56), amyotrophic lateral sclerosis (57) etc., have been successfully modeled using *Drosophila*, we do not support this possibility. Rather, the unique pathogenic feature of GSS syndrome may give some clue to reconcile this difference. The major components of aggregated and accumulated fibrillar amyloid deposits in the brains with GSS syndrome is a ~7 kDa PrP fragment, spanning from residues 81–82 to 144–153 of PrP (58) which polymerizes into protease-resistant fibrils and triggers the cell death of murine cortical neurons (59). A recent report has shown that acetylcholinesterase (AChE) promotes the fibrillization of pathogenic 7 kDa PrP fragments with GSS mutation *in vitro* in a dose-dependent manner (60). Thus, it may be that AChE plays a pivotal role in the pathogenesis of the *Drosophila* model of GSS syndrome by triggering the fibrillization of MoPrP<sup>P101L</sup>, even though any alteration of AChE or cholinergic neurons in brains with GSS syndrome in humans has not as yet been reported. Thus, the second possibility is that MoPrP<sup>P101L</sup> expressed in cholinergic neurons in flies may interact with endogenous AChE at the synaptic clefts to become toxic pathogenic PrP deposits, resulting in phenotypic abnormalities. Therefore, it will be intriguing to examine any genetic interaction between MoPrP<sup>P101L</sup> flies and available *Drosophila* AChE (Ace) mutant animals to verify the contribution of Ace in MoPrP<sup>P101L</sup>-induced pathogenesis.

**Altered synaptic and molecular architecture of Type 1 glutamatergic synaptic boutons in larval NMJs of MoPrP<sup>P101L</sup> flies**

Synaptic dysfunctions clarified by the selective loss of presynaptic elements, degeneration of synapses and altered neuronal activities are observed in human and mouse brains with PrDs (24,26,61). In this study, we also found several synapse-associated defects in MoPrP<sup>P101L</sup>larvae and adults; first, the small-sized satellite boutons in Type 1 glutamatergic larval NMJs were significantly increased when MoPrP<sup>P101L</sup> is present at the pre-synaptic terminals or the pre- and post-synaptic terminals (Fig. 8). Expression of MoPrP<sup>P101L</sup> only at the post-synaptic terminal muscles did not induce any structural changes, suggesting that MoPrP<sup>P101L</sup> at the pre-synaptic terminal is necessary for inducing structural changes. Even though the alteration of synaptic morphology in the brains of...
the humans and TG mice with GSS syndrome are not well characterized, a growing body of evidence from other PrD models suggests that the progressive alteration of sizes and numbers of synapses may contribute to development and pathological changes in the PrD brains (62).

Second, Brp immunoreactivity is decreased at the synapses in the brains and Type 1 glutamatergic NMJs in MoPrP<sup>P101L</sup> larvae (Fig. 9). In addition, MoPrP<sup>P101L</sup> flies showed a progressive reduction of expressed Brp in adult brains (Fig. 9). Recent studies on loss of function in Brp-mutant flies have shown that Brp is localized at the active zones required for excitatory synaptic transmission and associated with locomo-tor controls in adult flies (63,64). We also found reduced DLG, which is responsible for orchestrating synaptic architecture by regulating localization of ion channels, neuronal cell adhesion molecules and receptors (65) in the adult heads (Fig. 9). Thus, our results on changes at the molecular level help explain why MoPrP<sup>P101L</sup> flies manifested a progressive loss in climbing ability. We also showed that those synaptic component defects may not be restricted in MoPrP<sup>P101L</sup> flies, since hippocampi of scrapie-infected mice showed significantly reduced expression of ELKS, a component of CAZ (Supplementary Material, Fig. S4).

**Inhibition of Caspase 3-dependent apoptotic neuronal cell death pathway did not suppress the onset of GSS symptoms in flies**

Even though the premature, primary death of neurons underlies the clinical symptoms of PrDs, the cellular pathways leading to this neuronal cell death are not completely understood at this time (16). PCD has been proposed to be involved with neuronal loss and vacuole formations in brains with PrDs (16,66–72). Even though DNA fragmentation and over-expressed Bax has been reported in both naturally and experimentally induced PrDs (14–16), activated Caspase 3 immunoreactivity was found in only a few cells in scrapie-infected mice (73), but not in scrapie-affect ed sheep (70). However, genetic manipulation of neuronal apoptotic cell death combining IPrD mouse models or BSE-infected mouse models with a Bax deletion mutation shows that Bax-dependent PCD pathway may not be involved with synaptic degeneration and manifestation of neurological symptoms in those PrD mouse models (66,74). Recently, it has been shown that human PrP with P102L GSS syndrome mutation, but not other GSS syndrome-associated mutations, lost the anti-Bax function that prevent Bax-mediated chromatic condensation or DNA fragmentation in primary human neurons (75). These previous reports suggested that different point mutations associated with GSS syndrome may induce the loss of neurons and the vacuole formations by activating different types of PCD pathways or by interfering with unknown signaling pathways. In this study, we used a molecular test to investigate whether Caspase 3-dependent PCD pathway is involved with the loss of locomotor abilities and early death of MoPrP<sup>P101L</sup> flies by expressing P35, a viral Caspase 3 signaling inhibitor which has previously been shown to suppress retinal cell death in rd<sup>C</sup> and nine<sup>E</sup>(H127)<sup>+</sup> flies (76) and neuronal toxicities of poly-glutamine proteins in ommatidia (77). Concomitant expression of P35 and MoPrP<sup>P101L</sup> did not suppress the early loss in the climbing ability of flies, but significantly induced early death of flies compared with that of MoPrP<sup>P101L</sup> or P35 flies (Figs 5 and 6). These results establish that the early loss of motor controls and premature death in MoPrP<sup>P101L</sup> flies are not caused by Caspase 3-dependent PCD pathway in *Drosophila*. Other Caspases or autophagic cell death pathways may contribute to the onset of GSS symptoms in MoPrP<sup>P101L</sup> flies.

In this study, we provided several lines of evidence that key behavioral, biochemical and pathological hallmarks of GSS syndrome observed from MoPrP<sup>P101L</sup> flies were not due to differences in the amount of expressed PrP in flies. In addition, we found that two important synaptic molecular components, Brp and DLG, were progressively reduced in MoPrP<sup>P101L</sup> flies. We also provided evidence that the activation of Caspase-3-dependent PCD is not associated with the onset and progression of behavioral defects in MoPrP<sup>P101L</sup> flies. As the studies reported here demonstrate, further studies in this *Drosophila* model for GSS syndrome should provide unique opportunities to obtain additional novel information about the underlying cellular and molecular mechanisms of this disorder.

**MATERIALS AND METHODS**

**Fly genetics**

Flies were raised on a standard medium at 25°C.

**Generation of MoPrP<sup>3F4</sup> and MoPrP<sup>P101L</sup> transgenic flies.** The open-reading frames of wild-type mouse PrP tagged with a human/hamster 3F4 epitope at residues 109–112M (MoPrP<sup>3F4</sup>) and MoPrP<sup>3F4</sup> having leucine substitution at residue 101 (MoPrP<sup>P101L</sup>) (78,79), were cloned into pUAST-germline transformation vectors. DNA sequences were confirmed again, and typical germline transformation technique procedure was followed (56). Several independent fly lines were generated for each construct. Similar levels of expression and phenotypic properties were observed for independent isolates.

**Gal4 drivers.** Expression of UAS-MoPrP constructs was driven by several Gal4 drivers including a Tubulin (Tub)-Gal4 driver, an *Actin-5C-Gal4* driver, a *Choline acetyltransferase (Cha)-Gal4* driver, a *3,4-dihydroxyphenylalanine-1-decarboxylase (Ddc)-Gal4* driver, a *glutacin-Gal4* driver, a *201Y-Gal4* driver and a *C57-Gal4* driver (56).

**Protein extraction and immunoblot analysis**

Five *Drosophila* larvae or 10 adult heads were homogenized in RIPA buffer (20 mM HEPES, 1% Triton X-100, 1% deoxycho-late, 0.1% SDS, 150 mM NaCl, pH 7.2; Sigma-Aldrich, St Louis, MO, USA) with protease inhibitors (Halt<sup>TM</sup> protease inhibitor cocktail, Thermo Fisher Scientific, Rockford, IL, USA). The protein concentration of homogenates was determined by using the BCA protein assay kit (Thermo Fisher Scientific) and prepared for SDS—PAGE. Total proteins (50–60 μg) were separated on 8%–12% SDS—PAGE and transferred to nitrocellulose membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The blots were probed with MAb-PrP<sup>3E10</sup>...
Animals and scrapie infection

Four- to six-week-old female C57BL/6 mice were purchased from SLC INC (Hamamatsu, Japan). They were bred and maintained in the animal facility of the Ilsong Institute of Life Science at Hallym University, Republic of Korea. The scrapie strain ME7 was kindly provided by Alan Dickinson of AFRC and MRC Institute (Edinburgh, Scotland, UK). The mice were inoculated intracerebrally with 50 μl of AFRC and MRC Institute (Edinburgh, Scotland, UK). Life Science at Hallym University, Republic of Korea. The brains were immediately frozen and stored at −80°C until immunoblot analysis. All animal experimental procedures were examined and approved by the Animal Research Ethics Committee at the Hallym University, Republic of Korea.

Behavioral assays

Climbing ability test. Each group of flies tested for climbing abilities consisted of 10 females. Climbing assay was modified from those of Ganetzky and Wu (80). After each group of flies was transferred into a vial, the vial was agitated by a Vortex-Genie bench mixer (Scientific Industries, Inc., Bohemia, NY) at the highest speed for 10 s and then left for 20 s, before counting the number of climbing flies. Flies retaining climbing abilities were considered to be those walking on the sides or tops of vials, rather than being immobilized or remaining on the bottom. Tested flies were collected from three independent crosses. MoPrP3F4-expressing flies were compared with MoPrP101L-expressing flies or genetic backgrounds of GAL4 drivers. Ten independent tests were performed for each genotype. All flies were transferred to fresh food every 2–3 days.

Survival rate analysis. Survival rates of examined flies were estimated through counting the number of dead flies every 5 days. One group, consisting of 10 age-matched adult females, was transferred to fresh food every 2–3 days. Two-sample t-tests were performed by using SigmaStat 3.1 software (Systat Software, San Jose, CA, USA).

Protease resistance assay

Ten fly heads were homogenized in RIPA buffer as described above. Equal amounts of brain homogenate were treated with different concentrations of PK (Qiagen, Hilden, Germany) at 37°C for 30 min. The PK was inactivated by the addition of phenylmethylsulfonylfluoride (Sigma-Aldrich) to a final concentration of 2 mM and proteins were immediately boiled in 1× sample buffer for 5 min and subjected to immunoblot analysis utilizing MoPrP3F10 and MoPrP3F4 as described above.

Enzymatic deglycosylation

For deglycosylation, brain homogenates were mixed with 0.1 volume of 10× denaturing buffer (5% SDS, 0.4 M DTT) and boiled for 10 min at 95°C. Samples were deglycosylated with PNGase F (New England BioLabs, Boston, MA, USA) in 50 mM sodium phosphate buffer (pH 7.5, 1% Nonidet P-40) at 37°C for 18 h. Proteins were dissolved in sample buffer and used in SDS–PAGE immunoblotting.

Preparation of total RNA and semi-quantitative RT–PCR

Total RNA was extracted from eggs, larvae or fly heads using TRizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). After treating with DNase I (Invitrogen), complementary DNAs (cDNAs) were synthesized from 4 μg of total RNA using cDNA synthesis kits (Promega, Madison, WI, USA) according to the manufacturer’s instruction. To semi-quantify the expression of Prnp mRNA during Drosophila development, Prnp-specific primers (5'-CACATGCGAAGCTTAGCTC-3' and 5'-TCATCCACACATCAGGaAT-3') and Actin-5C-specific primers (5'-CATCTTTCTACGGTTGC-3' and 5'-AAGGACTCGTACGTTGGTG-3') were used. Reactions were carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using the following parameters: 15–45 PCR cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min). Products were run on a 1.0% agarose gel and stained with ethidium bromide. The sequences of amplified DNAs were confirmed.

Immunocytochemistry and confocal microscopy

Third star larvae were dissected in Ca2+-free saline (128 mM NaCl, 2 mM KCl, 4 mM MgCl2, 35.5 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.2; Sigma-Aldrich) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Body wall muscle preparations were incubated with MAb-PrP3F4 (1:200), MAb-Synapsin (1:1000) [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA, USA], rabbit-anti-DSypt antibodies (1:2000), rabbit-anti-Drosophila P21-activated kinase antibodies (DPak, 1:2000) (81), MAb-Brp (nc82, 1:50, DSHB), rabbit-anti-DLG (1:400) or goat anti-HRP-FITC (1:50) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Donkey anti-rabbit IgG Alexa-568, donkey anti-mouse IgG Alexa-568 and donkey anti-mouse IgG Alexa-488 (Invitrogen) were used as secondary antibodies at a 1:200 dilution. A Zeiss Laser Scanning Microscope 510 (LSM510; Zeiss, Jena, Germany) was used to take serial or single-slice confocal immunofluorescence images. Digital images were processed using LSM image browser or Adobe Photoshop 6.0 (Adobe Systems, Foster City, CA, USA).
Systems, San Jose, CA, USA). The numbers of boutons in NMJs in muscles 6 and 7 in the third abdominal segment in larvae were counted under an epifluorescence microscope. A small-sized synaptic bouton attached to the synaptic boutons in the main branches of NMJs was considered to be a satellite bouton. One-way ANOVA in SAS program (SAS Institute Inc., Cary, NC, USA) was used to perform statistical analysis.

Quantification of the intensity of Brp immunoreactivity
Larvae used for intensity analysis were processed simultaneously for MAb-PrP immunocytochemistry, and confocal images were acquired under identical settings in LSM 510 (Zeiss). Single-slice confocal images were taken from several Type 1b synaptic boutons labeled with MAbs in larvae expressing MoPrP3F4 or MoPrP101L. The mean intensity of each MAbs-specific spot in synaptic boutons or neurrophils was automatically calculated by the wand tool and the histogram functions in Adobe Photoshop program. SigmaStat software was used for the two-sample t-tests.

MAb-PrP immunocytochemistry and H&E staining of adult heads
Twenty-day-old adult heads of MoPrP3F4 or MoPrP101L flies were fixed with 4% paraformaldehyde and processed for paraffin-embedding protocol as described previously (82). Five microns serial paraffin sections were collected on a slide and then processed for MAbs immunocytochemistry. Donkey anti-mouse IgG-Peroxidase and 3, 3’-diaminobenzidine were used for visualizing PrP localization of adult heads. Five microns serial paraffin sections were collected on a slide and then processed for MAb-PrP3F4 immunocytochemistry and H&E staining. Digital images of serial sections of adult brains were acquired under identical settings in LSM 510 (Zeiss). Single-slice confocal images were taken from several Type 1b synaptic boutons labeled with MAb-Brp in larvae expressing MoPrP3F4 or MoPrP101L. The mean intensity of Brp immunoreactivity of adult heads was compared with two-sample t-tests using SigmaStat software.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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