

Figure 4. Relationship between *dPGAM5* and the mitochondrial fusion/fission genes. (A–F) *dPGAM5* inactivation failed to rescue the mitochondrial fragmentation caused by *mfn* knockdown (*mfn RNAi*) or introduction of an extra copy of the *drp1* gene (*drp1⁺*). To visualize the mitochondria under a fluorescence microscopy, we used the muscle-specific *MHC-GAL4* driver to induce expression of a mitoGFP (green) transgene in 5-day-old adult flies with the indicated genotypes. Muscle tissue was counterstained with phalloidin (red). Scale bar = 2 μm. (G) The average length of the mitochondria in the direction of the myofibrils was measured from wild-type ($n=343$ from 7 adult flies), *PGAM5¹* ($n=390$ from 8), *mfn RNAi* (VDRC40478, $n=305$ from 6; VDRC105261, $n=372$ from 8), *mfn RNAi* (VDRC40478); *PGAM5¹* ($n=355$ from 7), *mfn RNAi* (VDRC105261); *PGAM5¹* ($n=237$ from 5), *drp1⁺*

($n=245$ from 5) and *drp1⁺; PGAM5¹* ($n=247$ from 5) as shown in (A–F). Data are shown as means \pm SE (** $p<0.01$; N.S., not significant). The genotypes are as follows: +/Y; *MHC-GAL4>mitoGFP* (A, wild-type), *PGAM5¹/Y; MHC-GAL4>UAS-mitoGFP* (B, *PGAM5¹*), +/Y; *MHC-GAL4>UAS-mitoGFP; UAS-mfn RNAi* (VDRC40478) (C, *mfn RNAi*), *PGAM5¹/Y; MHC-GAL4>UAS-mitoGFP; UAS-mfn RNAi* (VDRC40478) (D, *mfn RNAi; PGAM5¹*), +/Y; *MHC-GAL4>mitoGFP; drp1⁺* (E, *drp1⁺*), *PGAM5¹/Y; MHC-GAL4>mitoGFP; drp1⁺* (F, *drp1⁺; PGAM5¹*). doi:10.1371/journal.pgen.1001229.g004

tested if Keap1/Nrf2 signaling modulates *PINK1* phenotypes. Removal of a copy of the *keap1* gene (Gene ID: 42062) in *dPINK1* knockdown flies, wherein the *dPINK1* RNAi was expressed in the muscle tissues, failed to rescue the abnormal wing posture (Figure 8A). However, *Keap1* heterozygosity is beneficial to survival of aging *dPINK1* knockdown flies, supporting a previous report suggesting that oxidative stress is partly involved in the *PINK1* pathology (Figure 8B) [33,34].

Discussion

The event of fusion/fission is required for maintenance of a healthy mitochondrial population. Mitochondrial fusion is believed to require the interchange of a set of internal components, including copies of the mitochondrial genome, respiratory proteins and metabolic products. Mitochondrial fission has been proposed to play a role in disposal of damaged mitochondria, such as those with a reduced mitochondrial membrane potential, via mitophagy [35]. A role for *PINK1* in the regulation of mitochondrial fission/fusion dynamics has recently been demonstrated in *Drosophila* [10–12]. The *PINK1*/Parkin pathway appears to promote fission and/or inhibits fusion, likely through an indirect mechanism. Indeed, loss of *dPINK1* or *dParkin* produces swollen or enlarged mitochondria in tissues with high-energy demands, such as the muscles, which is suppressed by reduced fusion activity or increased fission activity after genetic manipulation of the mitochondrial fission/fusion machinery. Namely, either reducing the activity of the mitochondrial fusion proteins OPA1 and Mfn, or increasing the activity of a mitochondrial fission protein, Drp1, can partially rescue *PINK1* and *parkin* mutant phenotypes.

We identified *PGAM5* as a *PINK1*-binding protein and went on to show that *dPGAM5* can modulate *dPINK1* mutant phenotypes. Loss of *dPGAM5* activity had little effect on the lifespan of a *dPINK1* RNAi fly strain in our initial *in vivo* test (Figure 1C). However, we found that loss of *dPGAM5* does significantly extend lifespan of *PINK1^{B9}* mutant flies (Figure 5J). We speculate that continuous expression of the short hairpin RNA in the RNAi-based test confers additional toxicity, leading to a shorter lifespan in a sequence-independent manner, such that the suppressive effect of *dPGAM5* mutations cannot be detected in the *PINK1* RNAi flies.

dPGAM5 appears to be dispensable for mitochondrial homeostasis in *Drosophila*, as overall, flies homozygous for a null allele of *dPGAM5*, *PGAM5¹*, appear to be normal. It has previously been reported that ectopic expression of *PGAM5* leads to perinuclear aggregation or small fragmentation of mitochondria in mammalian cultured cells, which suggested that *PGAM5* has a role in regulation of mitochondrial fission/fusion process or mobility [24]. Our study also observed alteration of mitochondrial morphology in *Drosophila* with different *dPGAM5* activities. Transgenic expression of *dPGAM5* or *dPGAM5-2* leads to fragmentation of mitochondria both in the *TH⁺* neurons and indirect flight muscles (Figure 3E, 3F, 3I and 3J). By contrast, *dPGAM5* LOF moderately increases mitochondria with a longer tubular or a swollen morphology (Figure 3D and 3K). Our genetic tests with the

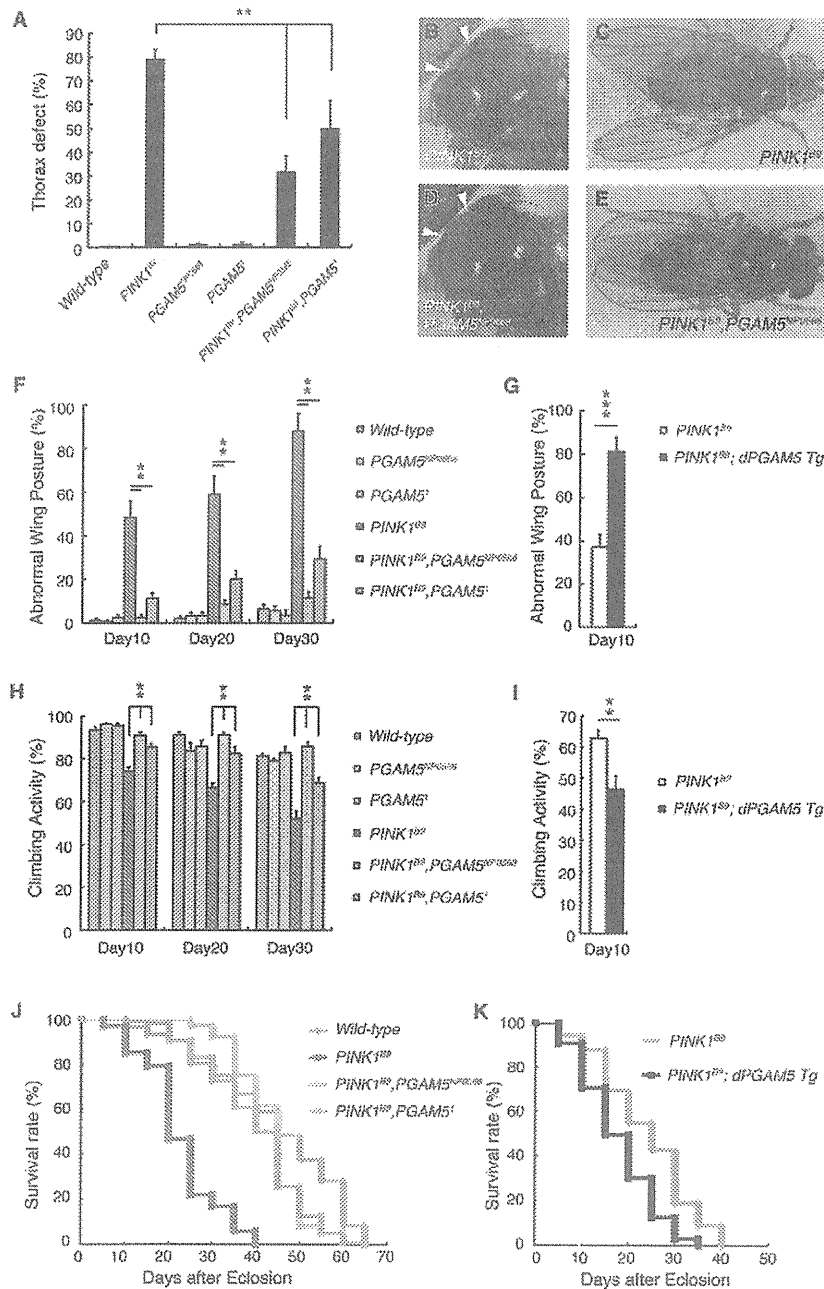


Figure 5. Loss of *dPGAM5* suppresses *dPINK1* mutant phenotypes in *Drosophila*. A thorax defect (B, arrowheads) and abnormal wing posture (C) caused by loss of *dPINK1* activity are suppressed in *dPGAM5* mutant genetic backgrounds (A, D and E). (F) Percentage of 10-, 20- and 30-day-old male flies showing abnormal wing postures. Error bars show S.E. from three experiments. (G) Percentage of 10-day-old male *PINK1^{BP}* and *PINK1^{BP}* ubiquitously overexpressing *dPGAM5* flies showing abnormal wing postures. Error bars show S.E. from three experiments. (H, I) Percentage of locomotor activity. Error bars show S.E. from three repeated experiments. (J) Lifespan of adult male flies. Loss of *dPGAM5* partially improved the reduced lifespan seen in *PINK1^{BP}* fly (*PINK1^{BP}* vs. *PINK1^{BP}; PGAM5^{NP0568}* or *PINK1^{BP}; PGAM5¹*, $p < 0.001$; wild-type vs. *PINK1^{BP}; PGAM5^{NP0568}* or *PINK1^{BP}; PGAM5¹*, $p < 0.01$ by the log rank test). (K) Lifespan of adult male *PINK1^{BP}* and *PINK1^{BP}* ubiquitously overexpressing *dPGAM5* flies. Overexpression of *dPGAM5* further reduced the lifespan (*PINK1^{BP}* vs. *PINK1^{BP}; dPGAM5 Tg*, $p < 0.001$). The same flies were used in (A–F, H and J) and in (G, I and K). The genotypes and the number used in the assays are; wild-type (*PINK1^{RV}/Y*, $n = 161$), *PGAM5^{NP0568}* (*PGAM5^{NP0568}/Y*, $n = 161$), *PGAM5¹* (*PGAM5¹/Y*, $n = 161$), *PINK1^{BP}* (*PINK1^{BP}/Y*, $n = 101$), *PINK1^{BP}; PGAM5^{NP0568}* (*PINK1^{BP}; PGAM5^{NP0568}/Y*, $n = 162$) and *PINK1^{BP}; PGAM5¹* (*PINK1^{BP}; PGAM5¹/Y*, $n = 160$) in (A–F, H and J), *PINK1^{BP}* (*PINK1^{BP}/Y*; *Da-GAL4/+*, $n = 162$) and *PINK1^{BP}; dPGAM5 Tg* (*PINK1^{BP}/Y*; *Da-GAL4* > *UAS-dPGAM5*, $n = 161$) in (G, I and K). doi:10.1371/journal.pgen.1001229.g005

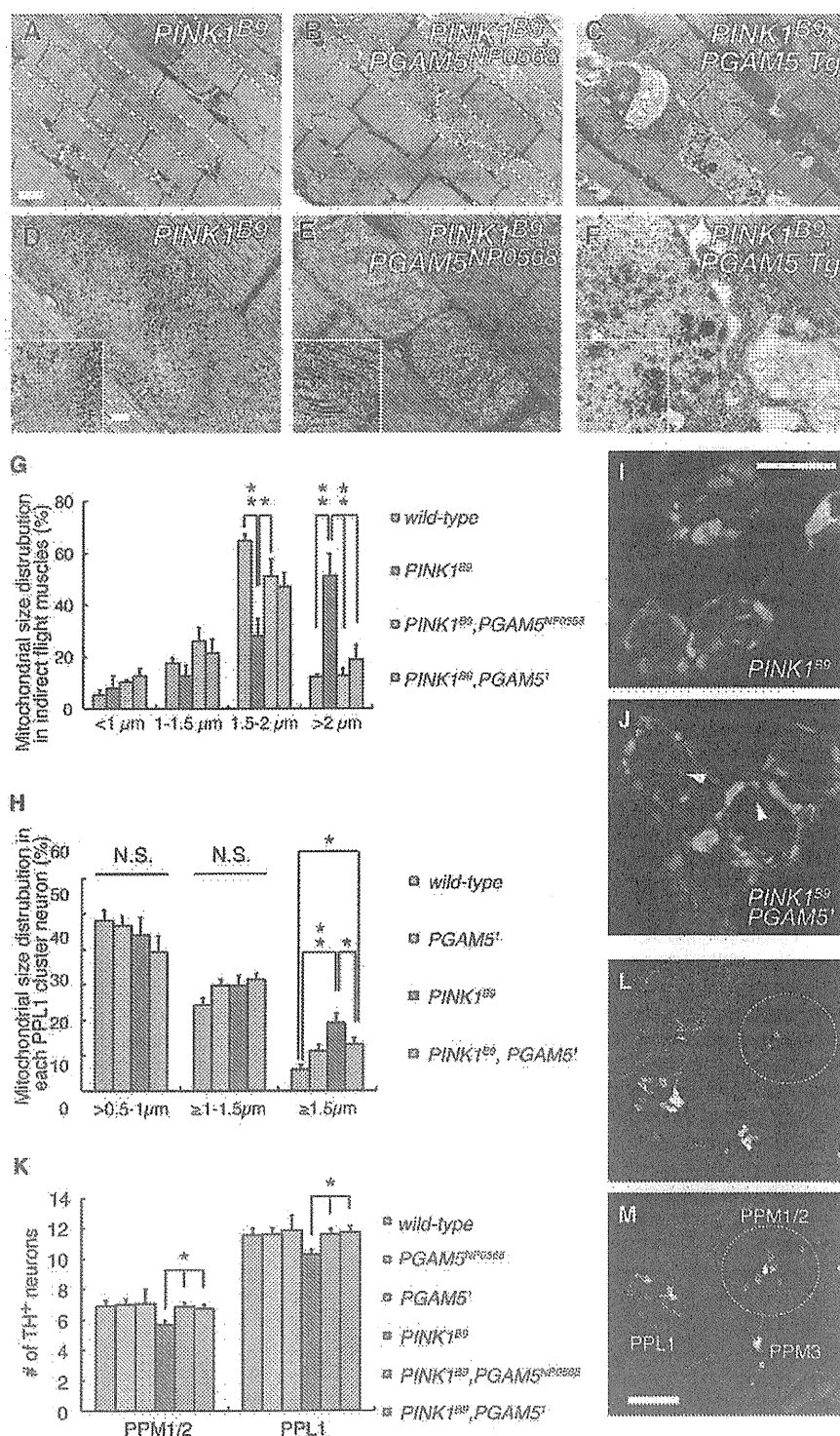


Figure 6. Loss of *dPGAM5* improves degeneration of the mitochondria and DA neurons caused by *dPINK1* inactivation in *Drosophila*. (A–F) TEM analysis of the indirect flight muscle and morphology of mitochondria in 2-day-old adult flies with the indicated genotypes. In A and B, some mitochondria are outlined with broken lines. The insets in D–F show representative mitochondria matrices. Scale bars = 1 μm in A–C and 200 nm in D–F. (G) Quantification of the percentage of mitochondrial size distribution in the indirect muscle tissue from *wild-type* ($n = 136$ from 5 adult flies), *PINK1^{BS}* ($n = 96$ from 5), *PINK1^{BS}, PGAM5^{NP0568}* ($n = 116$ from 5), *PINK1^{BS}, PGAM5¹* ($n = 111$ from 5) as shown in Figure 3K. Data are shown as means \pm SE (* $p < 0.05$, ** $p < 0.01$). (H–J) Quantification of the percentage of cytoplasmic mitochondrial aggregates with diameter of 0.5–1.0, 1.0–1.5 or ≥ 1.5 μm in each PPL1 TH⁺ neuron from *wild-type* ($n = 373$ from 18 adult flies), *PGAM5¹* ($n = 356$ from 18), *PINK1^{BS}* ($n = 231$ from 11), *PINK1^{BS}, PGAM5¹*

flies ($n=235$ from 13). Mitochondrial morphology was revealed by mitoGFP as shown in Figure 3L–3O. Data are shown as means \pm SE (*, $p<0.05$; **, $p<0.01$; N.S., not significant). Tubular or reticular mitochondria were excluded from the estimation due to difficulty in the counting. However, the ratio of mitochondria with that morphology was also increased in *PINK1*^{B9}*PGAM5*¹ flies (J) compared with that in *PINK1*^{B9} flies (I). Arrowheads in (J) indicate representative tubular or reticular mitochondria. Scale bar in (I) = 5 μ m. (K) Quantification of TH⁺ DA neuron number in the PPM1, PPM2 and PPL1 clusters in 25-day-old males. PPM1 and PPM2 cluster neurons were counted together. Data are shown as means \pm SE (*, $p<0.05$; **, $p<0.01$; n = 16). (L, M) Representative images of PPM1/2, PPM3 and PPL1 clusters of *PINK1*^{B9} (L) and *PINK1*^{B9}, *PGAM5*^{N⁰⁵⁶⁸} flies (M) visualized with anti-TH antibody. Scale bar in (M) = 50 μ m. The genotypes are: *PINK1*^{RV}/*Y* (wild-type), *PINK1*^{B9}/*Y* (*PINK1*^{B9}), *PGAM5*^{N⁰⁵⁶⁸}/*Y* (*PGAM5*^{N⁰⁵⁶⁸}), *PGAM5*¹/*Y* (*PGAM5*¹), *PINK1*^{B9}, *PGAM5*^{N⁰⁵⁶⁸}/*Y* (*PINK1*^{B9}, *PGAM5*^{N⁰⁵⁶⁸}), *PINK1*^{B9}, *PGAM5*¹/*Y* (*PINK1*^{B9}, *PGAM5*¹), *PINK1*^{B9}/*Y*; *D α -GAL4* > *dPGAM5* (*PINK1*^{B9}, *PGAM5*¹), *PINK1*^{B9}/*Y*; *TH-GAL4* > *mitoGFP* (wild-type), *PINK1*^{B9}/*Y*; *TH-GAL4* > *mitoGFP* (*PINK1*^{B9}), *PGAM5*¹/*Y*; *TH-GAL4* > *mitoGFP* (*PGAM5*¹), *PINK1*^{B9}, *PGAM5*¹/*Y*; *TH-GAL4* > *mitoGFP* (*PINK1*^{B9}, *PGAM5*¹) in (H–J). doi:10.1371/journal.pgen.1001229.g006

known mitochondrial fusion/fission machinery suggested that PGAM5 acts upstream of them or in an independent pathway (Figure 4). Given that PGAM5 is involved in mitochondrial fission, loss of PGAM5 would be expected to enhance the *PINK1* mutant phenotype in *Drosophila*, similar to the interaction between *PINK1* and the mitochondrial fusion/fission machinery [10–12]. Interestingly, the number of large aggregated mitochondria, which are frequently seen in *PINK1*^{B9} flies, was mildly decreased in TH⁺ neurons of *PINK1*^{B9}*PGAM5*¹ flies (Figure 6H–6J). Moreover, loss of *dPGAM5* also modulated the mitochondrial morphology of *dParkin* mutant fly without suppressing the mitochondrial degeneration (Figure 7H–7L). Based on these observations, it could be speculated that PGAM5 does not directly regulate mitochondrial fission but instead, modulates the *PINK1* pathway in a different way. Recent studies have proposed two different models for the mechanism of mitochondrial morphological changes through the *PINK1*/*Parkin* pathway in *Drosophila* and mammals. Ziviani *et al.* and Poole *et al.* have demonstrated that *dParkin* promotes degradation of Mfn in a *dPINK1*-dependent manner, which leads to mitochondrial fragmentation in *Drosophila* [16,36]. Our current results demonstrated that the loss of *dPGAM5* activity does not affect mitochondrial fragmentation caused by reduction of Mfn activity, suggesting that *dPGAM5* might not contribute to the proposed *PINK1*/*Parkin* pathway (Figure 4). Sandebring *et al.* have proposed that accumulation of damaged mitochondria by *PINK1* inactivation results in mitochondrial calcium efflux, which activates Drp1 through Calcineurin-mediated dephosphorylation of Drp1 in human cells [37]. This model well explains the observation that loss of *PINK1* indirectly promotes mitochondrial fragmentation in mammalian cells and the indication that *PINK1* is not a core component of the fusion/fission machinery in a *Drosophila* study [11]. However, most of *Drosophila* studies do not support a result that loss of *PINK1* leads to mitochondrial fragmentation in mammals. Thus, it still remains a question for further investigation how PGAM5 modulates the mitochondrial dynamics.

Although the property of PGAM5 to physically interact with *PINK1* appears to be conserved between human and *Drosophila*, the functional significance of this binding remains to be established. *PINK1* and PGAM5 have kinase and phosphatase activities, respectively. However, there is no evidence to suggest that *PINK1* directly phosphorylates PGAM5, or that PGAM5 dephosphorylates *PINK1* (Figure 2G and 2H) [18,25,26]. The *PINK1* protein levels are maintained at very low level under steady-state conditions by constitutive processing and subsequent degradation through the ubiquitin-proteasome pathway [15]. Recent studies suggested that *PINK1* selectively translocates from cytosol to mitochondria with low membrane potential, at which *PINK1* is stabilized [14,15,38]. The accumulated *PINK1* on the depolarized mitochondria further recruits *Parkin* to induce mitophagy [13–15,38]. However, *Parkin* does not seem to be the target of *PINK1* kinase activity [15], and *PINK1* does not

seem to activate *Parkin* E3 activity directly (data not shown). Based on these findings, it is possible that PGAM5 may promote a selective recruitment of *PINK1* to the outer membrane of the damaged mitochondria, or that PGAM5 may regulate *PINK1* stabilization. Our molecular analysis, however, did not support the idea that PGAM5 stabilizes *PINK1* (data not shown). In addition, because loss of *dPGAM5* partially suppresses *dPINK1* null phenotypes, it seems likely that *PINK1* negatively regulates PGAM5 function (Figure 9). PGAM5 was originally identified as a Bcl-xL-binding protein, and in itself can be toxic to cells, promoting mitochondrial fragmentation, when expressed at high levels (Figure 3B). Therefore, it seems possible that PGAM5 modulates a cell protective or a mitochondrial morphogenetic activity of the Bcl-2 family member Bcl-xL downstream of *PINK1* but in a pathway that is independent from *Parkin* (Figure 9A) [39]. In this context, *PINK1* may suppress the cell toxic action of PGAM5 through an indirect mechanism where an unidentified substrate of *PINK1* inactivates PGAM5. Interestingly, a recent report suggests that the *dPINK1* phenotype can be partially suppressed by transgenic expression of *Drosophila* Bcl-2 protein Buffy [8]. Alternatively, PGAM5 may be one of components of a negative regulator complex against *Parkin* E3, downstream of *PINK1* (Figure 9B). Matsuda *et al.* have reported that *Parkin* E3 activity is activated only at the depolarized mitochondria, suggesting the existence of its negative regulator(s) [38]. E3 activity of *Parkin* may be released when *PINK1* associates with the negative regulator complex via PGAM5 and suppresses its function by phosphorylation of another complex component(s). This idea might be partly supported by our observation that loss of *dPGAM5* had little effect on *dparkin* mutant flies.

Although the primary cause of the mitochondrial degeneration by the loss of *PINK1* remains less obvious, the growing evidence suggests that *PINK1* eliminates oxidatively damaged mitochondria in cooperation with *Parkin*, the failure of which leads to tissue degeneration. Supporting for this hypothesis, the mitochondrial phenotypes of *dPINK1* and *dParkin* mutant flies are primarily exhibited in similar tissues that require higher energy demands [7–9]. Although *dPGAM5* might not regulate *Keap1* function in *Drosophila*, the *Keap1*/*Nrf2* pathway appears to be conserved in *Drosophila* [32], and activation of the *Keap1*/*Nrf2* pathway by genetic manipulation effectively suppressed the short-lifespan phenotype by *dPINK1* inactivation. This finding may also support the above idea that the accumulation of oxidatively damaged mitochondria leads to degeneration of specific tissues, providing a hint of therapeutic strategies for *PINK1*-associated PD.

In conclusion, the results of our genetic study demonstrate that the mitochondrial-localized protein PGAM5 modulates the *PINK1* pathway in *Drosophila*. However, further work will be required to determine how PGAM5 regulates the *PINK1* pathway at the molecular level, as well as to determine if manipulation of PGAM5 activity might provide a therapeutic advantage in treatment of *PINK1*-associated PD.

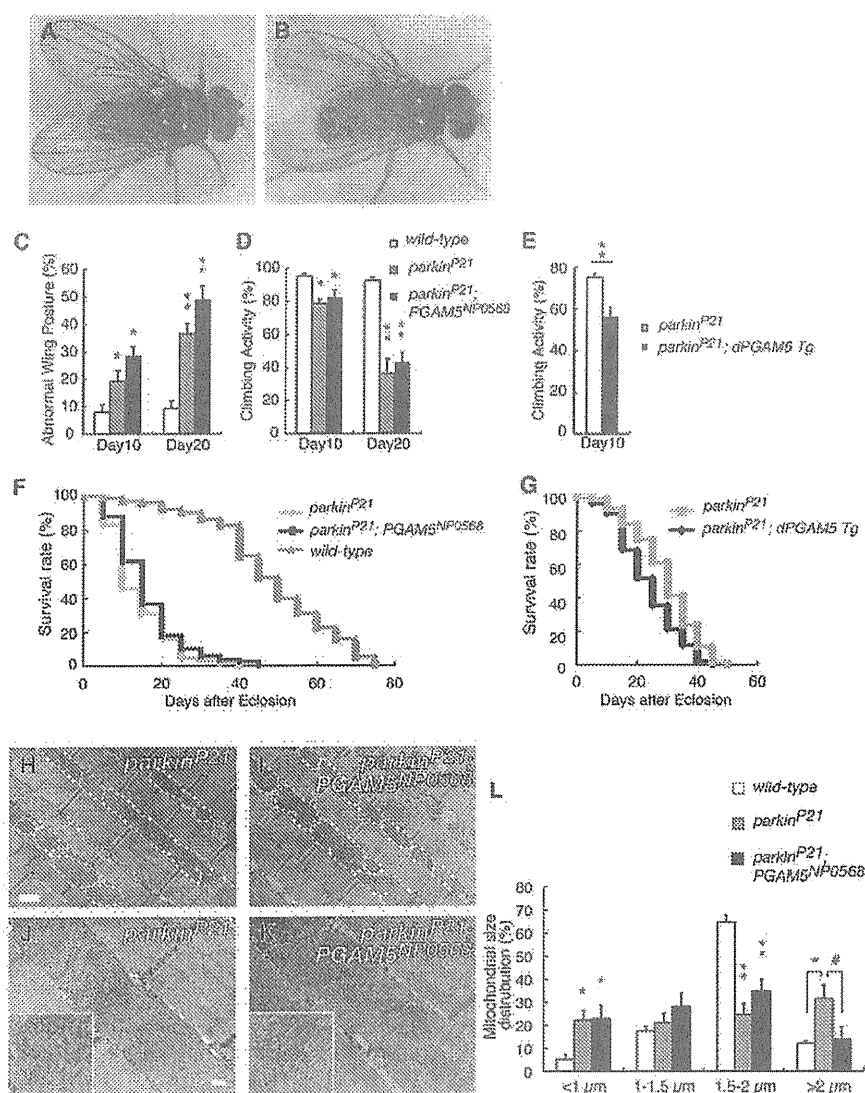


Figure 7. Disruption of *dPGAM5* fails to suppress the mitochondrial phenotype caused by *dParkin* inactivation in *Drosophila*. The abnormal wing posture caused by a homozygous *dParkin* mutation (A) was not suppressed by removal of the *dPGAM5* gene (B). (C) Percentage of flies with abnormal wing posture among 10- and 20-day-old male wild-type ($n = 105$), *parkin^{P21}* ($n = 102$) and *PGAM5^{NP0568}; parkin^{P21}* ($n = 109$) flies. Error bars show S.E. from three repeated experiments. *, $p < 0.05$; **, $p < 0.01$ vs. *dParkin*(+/+). (D) Percentage of flies showing locomotor activity among 10- and 20-day-old male *parkin^{P21}* ($n = 86$), *parkin^{P21}* ($n = 73$) and *PGAM5^{NP0568}; parkin^{P21}* ($n = 78$) flies. Error bars show S.E. from twenty repeated experiments. *, $p < 0.05$; **, $p < 0.01$ vs. *dParkin*(+/+). (E) Locomotor activity of 10-day-old male *parkin^{P21}* ($n = 153$) and *parkin^{P21}* ubiquitously overexpressing *dPGAM5* flies (*parkin^{P21}; dPGAM5 Tg*, $n = 155$) flies. Error bars show S.E. from twenty repeated experiments. **, $p < 0.01$. (F) Lifespan of adult male wild-type ($n = 104$), *parkin^{P21}* ($n = 102$) and *PGAM5^{NP0568}; parkin^{P21}* ($n = 91$) flies. *PGAM5^{NP0568}; parkin^{P21}* vs. *parkin^{P21}*, $p = 0.191$; wild-type vs. *parkin^{P21}*, $p < 0.001$ by log rank test. (G) Lifespan of adult male *parkin^{P21}* ($n = 153$) and *parkin^{P21}; dPGAM5 Tg* flies ($n = 155$) flies. *parkin^{P21}* vs. *parkin^{P21}; dPGAM5 Tg*, $p < 0.001$ by log rank test. (H–K) TEM analysis of the indirect flight muscle and mitochondrial morphology in tissue from flies of the indicated genotypes. The long tubular mitochondrial phenotype seen in *parkin^{P21}* flies can be rescued by *dPGAM5* inactivation (H and I). However, the mitochondrial matrix still appears degenerated (insets in J and K). Scale bars = 1 μm in H and I and 200 nm in J and K. (L) Quantification of the percentage of mitochondrial size distribution in the indirect muscle tissue from wild-type ($n = 136$ from 5 adult flies), *parkin^{P21}* ($n = 89$ from 5) and *parkin^{P21}; PGAM5^{NP0568}* flies ($n = 84$ from 5) as shown in (H–K). The length of the mitochondria in the direction of the myofibrils was measured. Data are shown as means \pm SE (* $p < 0.05$, ** $p < 0.01$ vs. wild-type; # $p < 0.05$ vs. *parkin^{P21}; PGAM5^{NP0568}*). The genotypes are: +/Y (wild-type), +/Y; *parkin^{P21}*/*parkin^{P21}* (*parkin^{P21}*), *PGAM5^{NP0568}*/Y; *parkin^{P21}/parkin^{P21}* (*parkin^{P21}; PGAM5^{NP0568}*). doi:10.1371/journal.pgen.1001229.g007

Materials and Methods

Purification of PINK1-Binding Proteins

HEK293 cells stably expressing hPINK1-FLAG or parent cells were grown in suspension culture (Joklik-modified Eagle's

minimum essential medium with 5% fetal bovine serum). The cell pellet (2.4×10^8 cells) was homogenized in lysis buffer (50 mM Tris pH 7.4, 120 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton-X100) supplemented with Complete Protease Inhibitors (Roche Diagnostics). The soluble fraction of the suspension was

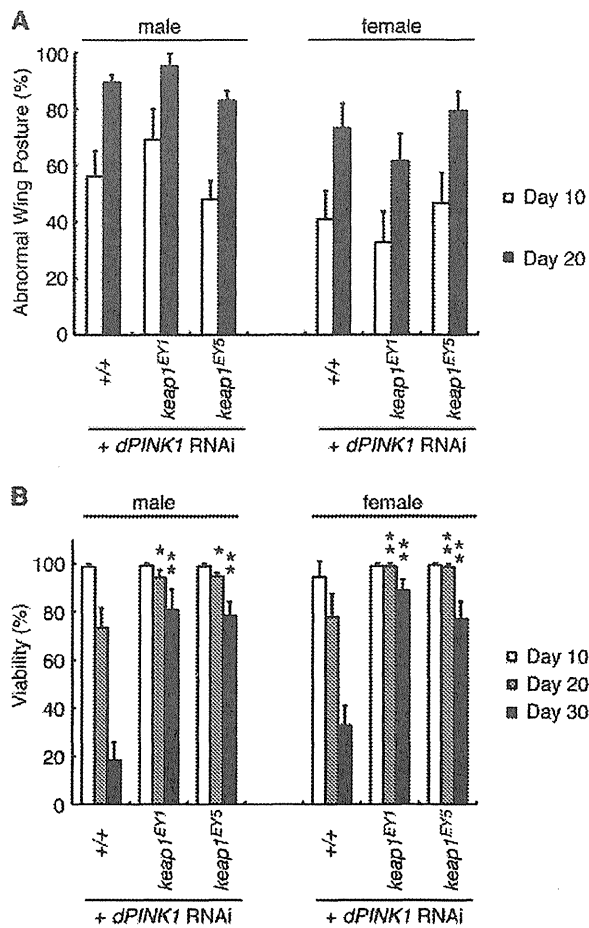


Figure 8. Reduction of Keap1 activity improves the lifespan of *dPINK1* RNAi flies. Removal of one copy of *Drosophila keap1* had no effects on the wing phenotype of *dPINK1* RNAi flies (A) but improved viability (B). *, $p < 0.05$; **, $p < 0.01$ vs. age-matched *dPINK1* RNAi group. The genotypes are as follows: *MHC-GAL4 > dPINK1^{RNAi} (+/+)*, *MHC-GAL4 > dPINK1^{RNAi}/Keap^{EY1}* (*Keap^{EY1}*), *MHC-GAL4 > dPINK1^{RNAi}/Keap^{EY5}* (*Keap^{EY5}*). Flies were raised at 29°C. doi:10.1371/journal.pgen.1001229.g008

immunoprecipitated with anti-FLAG M2 agarose (Sigma-Aldrich) and washed five times in lysis buffer. The fractions eluted with 200 μ g/ml 3x FLAG peptide were resolved by SDS-PAGE. Specific bands detected by silver staining were excised for in-gel digestion. The digest extracted from the gel was subjected to online HPLC-MS/MS, followed by informatics-based identification of the proteins (Nippon Proteomics).

Drosophila Genetics

Fly culture and crosses were performed on standard fly food containing yeast, cornmeal and molasses, and flies were raised at 25°C unless otherwise indicated. To generate *UAS-dPGAM5* transgenic lines, cDNA for *dPGAM5* and *dPGAM5-2* obtained by RT-PCR from adult *Drosophila* total RNA was subcloned into the *pUAST* vector. Introduction of transgenes into *Drosophila* germ line and establishment of transgenic lines into a *w* background were performed by BestGene Inc. (Chino Hills, CA). A *P*-element insertion line for *dPGAM5* mutant, *PGAM5^{NP0568}* obtained from Kyoto *Drosophila* Genetic Resource Center, expresses a reduced

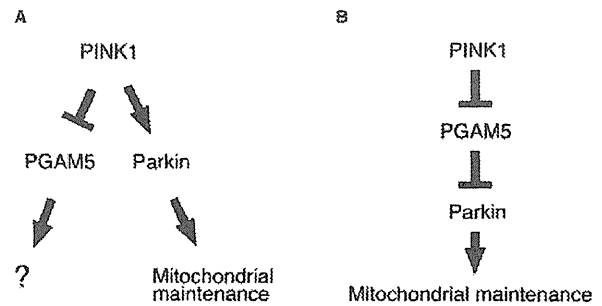


Figure 9. Schematic of the proposed *PINK1*/*PGAM5* pathways in *Drosophila*. (A) *PGAM5* has a role in mitochondrial activities independently of *Parkin* downstream of *PINK1*. (B) *PGAM5* negatively regulates *Parkin* downstream of *PINK1*. doi:10.1371/journal.pgen.1001229.g009

level (~25%) of mRNA, in which *NP0568* element is integrated 50 bp downstream of the *dPGAM5* translational start site (Figure S1B). We could not detect any *dPGAM5* protein signal in *PGAM5^{NP0568}* homozygous flies (Figure S1C). The *PGAM5^{NP0568}* line was backcrossed to *w* for six generations to remove background mutations, and used for most experiments as a *dPGAM5* mutant line. *UAS-dPGAM5* RNAi (VDR C 51655) and *UAS-mfn* RNAi (VDR C 105261 and VDR C 40478) strains were obtained from the Vienna *Drosophila* RNAi Center. To generate *PGAM5^Δ*, the KG09727 *P*-element insertion (obtained from the Bloomington *Drosophila* Stock Center) was mobilized using $\Delta 2-3$ transposase, and the entire *dPGAM5* coding region was deleted by imprecise excision, as shown in Figure S1A. All other fly stocks and *GAL4* lines used in this study were obtained from the Bloomington *Drosophila* Stock Center and have been previously described: *UAS-dPINK1 RNAi* [9]; *PINK1^{B9}* and revertant *PINK1^{RV}* [8]; *parkin^{P21}* [30]; *keap1^{EY1}* and *keap1^{EY5}* [32]; *Drp1²* and *Drp1⁺* [40].

RT-PCR and Plasmids

For quantitative RT-PCR analysis, reverse transcription and PCR reactions with total RNA extracted from fly heads were performed using a Superscript VILO cDNA synthesis kit (Invitrogen) and SYBR GreenER qPCR SuperMix (Invitrogen), respectively. Full-length cDNAs corresponding to *hPGAM5* (GenBank NP_001170543) and a short isoform of *hPGAM5* (*hPGAM5-S*, GenBank NM_138575) were amplified by RT-PCR from total RNA purified from HEK293 cells or a cDNA clone (RIKEN clone ID: IRAK003D15), and was cloned in *pcDNA3-Myc*, *pGEX6P-1* and *pGEX4T-1* vectors. Expression plasmids for *hPINK1-FLAG* and *pUAST-dPINK1-Myc* have been reported elsewhere [9,20].

Antibodies

Rabbit anti-human *PGAM5* polyclonal antibody was raised against recombinant GST-tagged *PGAM5* domain (89–289 aa) produced in the *E. coli* strain BL21(DE3)pLysS (Novagen), and was affinity-purified against the antigen. Rabbit anti-*dPGAM5* polyclonal antibody was raised against the peptide ELLTN-RIPRDVKNVV. Anti-*hPINK1* antibody (BC100-494), anti-Myc (4A6) and anti-FLAG (M2) antibodies were purchased from Novus, Millipore and Sigma-Aldrich, respectively. Mouse anti-TH monoclonal antibody was purchased from ImmunoStar, and rabbit anti-*Drosophila* TH polyclonal antibody was described previously [9].

Cell Culture, Immunoprecipitation, and Immunoblot Analysis

Transfection of mammalian cultured cell, immunopurification of FLAG-protein from transfected cell lysate and immunoblot analysis was performed as described previously [4,41]. For the hPGAM5 RNAi experiment, HEK293 cell lysate transfected with 20 μ M stealth RNAi reagent against hPGAM5 or a control RNAi reagent (Invitrogen), was analyzed 72 hrs after transfection. To detect an endogenous interaction between PINK1 and PGAM5, we treated HEK293 cells (2×10^7 cells) with 20 μ M carbonyl cyanide 3-chlorophenylhydrazone for 24 hrs to induce a sufficient level of human PINK1 protein for the study. The treated cells were subjected to immunoprecipitation using a Rabbit TrueBlot kit combined with rabbit anti-human PINK1 or rabbit anti-Delta (Santa Cruz) as a species-matched control. For the preparation of fly samples for immunoblot analysis, fly heads were directly homogenized in 20 μ l/head of SDS sample buffer using a motor-driven pestle. After centrifugation at 16,000 g for 10 min, the supernatant was used in SDS-PAGE.

In Vitro Phosphorylation Assay

Recombinant 2x GST-dPINK1 (153–709 aa), which has an N-terminal GST-tag and a C-terminal GST/6x His tag, was produced in the *E. coli* strain pG-KJE8/BL21 (TAKARA) and purified by a sequential purification with Ni-NTA agarose and glutathione sepharose. GST-hPGAM5-S (1–255 aa) and GST-hPGAM5 (1–289 aa) were incubated with 2x GST-dPINK1 as described in Figure 2.

Whole-Mount Immunostaining and Transmission Electron Microscopic (TEM) Analysis

Counting of TH-positive neurons was performed by whole-mount immunostaining of brain samples as described previously [9]. TEM images were obtained at the Biomedical Research Core of Tohoku University Graduate School of Medicine. All histochemical analyses were performed using DeltaVision microscope system (Applied Precision) or LSM5 PASCAL laser scanning microscope system (Carl Zeiss). The images obtained by DeltaVision system were deconvolved through 10 iterations using the DeltaVision deconvolution software (Applied Precision). Area calculation of the mitochondria was performed following established criteria for classification [12] using softWoRx (Applied Precision) or Image J software from the US National Institute of Health (<http://rsb.info.nih.gov/ij/>).

Lifespan Assay and Quantification of Wing Phenotypes and Climbing Ability

For lifespan studies, twenty female adult flies per vial were maintained at 25°C, transferred to fresh fly food, and scored for survival every 4 or 5 days. To control for isogeny, the *PGAM5*^{NP0568}, *PINK1*^{B9} and *PGAM5*^I, *PINK1*^{B9} alleles were backcrossed to *PINK1*^{B9} for six generations, and *parkin*^{P21} and *PGAM5*^{NP0568}; *parkin*^{P21} were backcrossed to *w*⁻ wild-type background for six generations, *UAS-dPGAM5* transgenic flies were generated in the *w*⁻ genetic background and thus have matched genetic backgrounds. The lifespan of *PGAM5*^I was compared in the *y*⁻ genetic background. The number of flies exhibiting defective abnormal wing posture (held-up or drooped) was determined for each genotype [9]. A climbing assay was performed as described previously [42].

Statistical Analysis

One-way repeated measures ANOVA was performed to determine significant differences among multiple groups unless otherwise indicated. If a significant result was achieved ($p < 0.05$), the mean of the control and the specific test groups was analyzed using the Tukey-Kramer test. For lifespan assays, the Kaplan-Meier analysis with log-rank test was performed.

Supporting Information

Figure S1 *dPGAM5* mutant alleles. (A) *PGAM5*^{NP0568} and *PGAM5*^I mutant alleles are depicted. Boxes, exons of the *dPGAM5* gene; triangles, the positions of the transposon *NP0568* and *KG09727* insertions. Coding regions and the transcript are depicted by black and yellow boxes, respectively. (B) Quantitative RT-PCR of the *dPGAM5* transcript in homozygous *dPGAM5* mutant and RNAi lines. Expression of the *dPINK1* RNAi was induced via the *Da-GAL4* driver. Primer-binding sites for PCR are shown as arrows in (A). (C) Immunoblot analysis of *dPGAM5* in the homozygous *dPGAM5* mutant, RNAi and transgenic lines. LE, longer exposure. (D) Alignment of the amino acid sequences of PGAM5 orthologues. Putative transmembrane domains are underlined in green for mammalian PGAM5 and blue for *Drosophila* PGAM5. A red arrowhead indicates the point of insertion of the transposon *NP0568*. Red underlining, sequences corresponding to the reported keap1-binding motif in human PGAM5. Found at: doi:10.1371/journal.pgen.1001229.s001 (0.69 MB TIF)

Figure S2 Quantitative RT-PCR of the *mfn* transcript in the *mfn* RNAi lines. Expression of the *mfn* RNAi was induced via the *Da-GAL4* driver, and total RNA was purified from 3rd instar larvae because *mfn* RNAi flies exhibited a pupation-defect phenotype. Found at: doi:10.1371/journal.pgen.1001229.s002 (0.18 MB TIF)

Figure S3 Loss of *dPGAM5* improved mitochondrial degeneration of the indirect flight muscles caused by dPINK1 inactivation. To visualize the mitochondria under a fluorescence microscopy, we used the *MHC-GAL4* driver to induce expression of a mitoGFP (green) transgene in 5-day-old adult flies with the indicated genotypes. Muscle tissue was counterstained with phalloidin (red). Integrity of the mitochondria in *PINK1*^{B9} flies was partially restored by removal of *dPGAM5* as shown by recovery of the mitoGFP signal (green) in *PINK1*^{B9}*PGAM5*^I flies. The genotypes are as follows: *MHC-GAL4>MitoGFP* [wild-type], *PGAM5*^I/*Y*; *MHC-GAL4>UAS-mitoGFP* [*PGAM5*^I], *PINK1*^{B9}/*Y*; *MHC-GAL4>UAS-mitoGFP* [*PINK1*^{B9}], *PINK1*^{B9}, *PGAM5*^I/*Y*; *MHC-GAL4>UAS-mitoGFP* [*PINK1*^{B9}, *PGAM5*^I]. Found at: doi:10.1371/journal.pgen.1001229.s003 (1.40 MB TIF)

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Author Contributions

Conceived and designed the experiments: Y Imai, T Kanao. Performed the experiments: Y Imai, T Kanao, T Sawada, Y Kobayashi, Y Moriwaki. Analyzed the data: Y Imai, T Kanao, T Sawada. Contributed reagents/materials/analysis tools: Y Moriwaki, Y Ishida, K Takeda, H Ichijo, B Lu, R Takahashi. Wrote the paper: Y Imai.

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N-cadherin Regulates p38 MAPK Signaling via Association with JNK-associated Leucine Zipper Protein

IMPLICATIONS FOR NEURODEGENERATION IN ALZHEIMER DISEASE^[5]

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Synaptic loss, which strongly correlates with the decline of cognitive function, is one of the pathological hallmarks of Alzheimer disease. N-cadherin is a cell adhesion molecule essential for synaptic contact and is involved in the intracellular signaling pathway at the synapse. Here we report that the functional disruption of N-cadherin-mediated cell contact activated p38 MAPK in murine primary neurons, followed by neuronal death. We further observed that treatment with A β ₄₂ decreased cellular N-cadherin expression through NMDA receptors accompanied by increased phosphorylation of both p38 MAPK and Tau in murine primary neurons. Moreover, expression levels of phosphorylated p38 MAPK were negatively correlated with that of N-cadherin in human brains. Proteomic analysis of human brains identified a novel interaction between N-cadherin and JNK-associated leucine zipper protein (JLP), a scaffolding protein involved in the p38 MAPK signaling pathway. We demonstrated that N-cadherin expression had an inhibitory effect on JLP-mediated p38 MAPK signal activation by decreasing the interaction between JLP and p38 MAPK in COS7 cells. Also, this study demonstrated a novel physical and functional association between N-cadherin and p38 MAPK and suggested neuroprotective roles of cadherin-based synaptic contact. The dissociation of N-cadherin-mediated synaptic contact by A β may underlie the pathological basis of neurodegeneration such as neuronal death, synaptic loss, and Tau phosphorylation in Alzheimer disease brain.

Alzheimer disease (AD)² is pathologically characterized by the presence of amyloid β -peptide (A β) and neurofibrillary tangles in the neocortex and hippocampus. Insoluble A β

fibrillar aggregates found in senile plaques have long been considered to cause the neurodegeneration of AD. On the other hand, synaptic loss is another pathological hallmark of AD, which strongly correlates with the severity of cognitive impairment better than senile plaques or neurofibrillary tangles (1). Interestingly, recent studies from AD mouse models have shown that learning impairment and synaptic dysfunction become apparent before the formation of plaques, suggesting the hypothesis that soluble A β causes “synaptic failure” before plaques develop and neuron death occurs (2). Converging lines of evidence suggest that natural soluble A β oligomers trigger synaptic loss (3). Thus, in addition to the investigation of molecular mechanisms, which develop senile plaques and neurofibrillary tangles, research focusing on synaptic dysfunction is important to clarify the earliest pathology in AD.

Presenilin (PS) 1/2 is the essential catalytic component of γ -secretase proteolytic complex (4, 5), which is responsible for the final cleavage of amyloid precursor protein to generate A β peptides. Mutations in PS1 have been known as the most common cause of autosomal dominant familial Alzheimer disease (6–8). Interestingly, PS1 binds to N-cadherin, which is an essential molecule for synaptic contact and is abundantly localized in hippocampal synapses (9). The cytoplasmic domain of cadherin associates with the actin cytoskeleton via β -catenin and regulates synaptic contact, synaptogenesis, and dendritic spine morphology (10, 11). In addition to the structural role as an adhesive molecule, N-cadherin plays important roles in intracellular signaling pathways such as β -catenin or Wnt signaling. Also, N-cadherin-based cell-cell adhesion activates PI3K/Akt cell survival signaling by recruiting PI3K into the N-cadherin adhesion complex (12). Further, PS1 facilitates this process by promoting cadherin/PI3K association (13). As a consequence, PS1/N-cadherin interaction at the synapse seems to be neuroprotective by facilitating the PI3K/Akt survival signaling. Recently, we demonstrated that N-cadherin promotes the cell surface expression of PS1/ γ -secretase, thereby activating the PI3K/Akt/GSK3 β signaling pathway (14) and that N-cadherin-mediated synaptic adhesion modulates A β secretion as well as A β _{42/40} ratio via PS1/

[□] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S5.

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² The abbreviations used are: AD, Alzheimer disease; A β , amyloid β ; JLP, JNK-associated leucine zipper protein; PS, presenilin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANOVA, analysis of variance.

N-cadherin Regulates p38 Signaling via JLP

N-cadherin interactions (15). Therefore, it is plausible that N-cadherin functions not only as a synaptic adhesion molecule but also as a modulator of AD pathology by affecting A β production and PI3K/Akt signaling.

On the other hand, increased p38 MAPK activity is associated with the neuropathology of AD (16). For example, both p38 MAPK and its upstream kinase MKK6 are activated in AD brain tissue as demonstrated by immunohistochemistry (17, 18). Activation of the p38 MAPK signaling is also reported in an AD-relevant animal model (19). Moreover, a previous report demonstrated that A β employs synaptic depression to drive endocytosis of synaptic AMPA receptor by activating p38 MAPK (20). Among the MAPK family members, p38 MAPK is activated by numerous unique signals such as environmental stressors and toxins, cellular injury, growth factors, and inflammatory cytokine leading to various neuronal cell fates including apoptosis, differentiation, and proliferation. However, the relationship between p38 MAPK and N-cadherin has not been elucidated in AD pathology.

Based on the above observations, we hypothesized that disruption of N-cadherin-based cell-cell contact may up-regulate the p38 MAPK signaling, leading to the neurodegeneration of AD. We demonstrated an inverse correlation between the expression levels of phosphorylated (and thus activated) p38 MAPK and those of N-cadherin in human brains. Moreover, we showed that the disruption of N-cadherin-based contact leads to an activation of p38 MAPK signaling in murine primary neurons, followed by neuronal death. Furthermore, we performed proteomic analysis using human brains and identified JNK-associated leucine zipper protein (JLP) as a novel interacting protein of N-cadherin, thus demonstrating a new signaling pathway from N-cadherin to p38 MAPK through the association with JLP, which might be compromised in AD pathogenesis.

EXPERIMENTAL PROCEDURES

Human Material and Proteomics—Human brain tissues were provided by the Tokyo Institute of Psychiatry. Brains from non-AD and AD patients were dissolved in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, and 0.5% deoxycholate) supplemented with protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor mixture (Sigma). Each sample was then centrifuged at 14,000 \times *g* for 20 min at 4 °C, and the supernatants were collected to obtain soluble proteins. Protein concentration was determined using the Bradford assay. Equal amounts of protein were subjected to SDS-PAGE followed by Western blot. For proteomic analysis, equal amounts of aliquots were treated with protein G-Sepharose (GE Healthcare) for 1 h at 4 °C. After removing protein G-Sepharose by centrifugation at 2,000 \times *g* for 5 min, anti-N-cadherin antibody (BD Biosciences) was added to the supernatants. Each sample was rotated for 2 h at 4 °C and then treated with protein G-Sepharose for 1 h at 4 °C. The immunoprecipitates were washed with radioimmune precipitation assay buffer five times and resuspended in 2 \times sample buffer (125 mM Tris-HCl, pH 6.8, 4.3% SDS, 30% glycerol, 10% 2-mercaptoethanol, and 0.01%

bromophenol blue). After boiling for 4 min, the supernatants were subjected to SDS-PAGE. To visualize proteins, the gels were stained with silver nitrate using PlusOne silver staining kit protein (GE Healthcare). The protein bands were excised and subjected to in gel trypsinization, and molecular mass analysis of the tryptic peptides was performed by MALDI-TOF/MS with an Ultraflex MALDI-TOF/TOF system (Bruker Daltonics, Billerica, MA).

Cells, Plasmids, and Transfection—HEK293 and COS7 cells were maintained in DMEM (Sigma) containing 10% FBS (Invitrogen) and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ incubator. SH-SY5Y cells, which are derived from human neuroblastoma cell lines, were maintained in Opti-MEM[®] (Invitrogen) containing 10% FBS. Primary neurons were obtained from the cerebral cortices of fetal mice (14–16 days of gestation) and cultured in neurobasal medium supplemented with B-27 (Invitrogen). Expression plasmids encoding S-tagged JLP and its mutant derivatives were kind gifts from Dr. Reddy (Temple University) (21). FLAG-tagged p38 MAPK and FLAG-tagged MKK4 (SEK1) were described previously (22). HA-tagged MEKK3 (Addgene plasmid 12186) was provided by Dr. Johnson (National Jewish Center for Immunology and Respiratory Medicine) (23). HA-tagged N-cadherin was described elsewhere (14). Transfection of either HEK293 or COS7 cells was carried out using Transfectin reagent (Bio-Rad) according to the manufacturer's protocol.

Antibodies and Reagents—The following antibodies were used in the study: mouse monoclonal antibody to N-cadherin (BD Biosciences), rabbit polyclonal antibody to JLP (Abcam), rabbit polyclonal antibody to p38 and phospho-p38 (Cell Signaling Technology), rabbit polyclonal antibody to S-probe (Santa Cruz Biotechnology), monoclonal and rabbit polyclonal anti-HA antibodies, mouse monoclonal anti-N-cadherin N terminus antibody (N-cadherin neutralizing antibody, GC-4), anti- β -actin antibody, anti-FLAG-M2 antibody, control normal mouse IgG (Sigma), mouse monoclonal antibody to PHF-Tau (AT8) (Pierce), and Alexa Fluor 546 goat anti-rabbit IgG conjugate and Alexa Fluor 488 goat anti-mouse IgG conjugate (Molecular Probe). ADH-1 was a kind gift from Dr. Gupta (Adherex Technologies Inc.). Synthetic A β ₄₂ peptides were obtained from Peptide Institute Inc. SB203580 was purchased from Calbiochem. S-protein-agarose beads were from Novagen.

Western Blot, Immunoprecipitation, Pull-down Assay, MTT Assay, and Cell Treatment by Reagents—Preparation of protein samples, Western blot, and immunoprecipitation were carried out as described elsewhere (14). Pull-down assay using S-protein-agarose beads (Novagen) was carried out as described elsewhere (21). MTT assay was performed using the MTT cell proliferation assay kit (Cayman) according to the manufacturer's instructions. For inhibition of N-cadherin-mediated cell-cell contact, the cells were treated either with ADH-1 as indicated or with N-cadherin-neutralizing antibody (GC-4) as described elsewhere (15).

Immunostaining—The samples for immunostaining were prepared as described elsewhere (15). After fixation, the samples were examined using a laser scanning confocal microscope, LSM 510 META (Zeiss).

TABLE 1

Characteristics of human cases

Clinical and histopathological information on brain samples used for analysis in Fig. 1A. We analyzed five AD patient brains confirmed by neuropathology and five control subjects without neurological complications. There is no statistical difference in age between AD and control cases. NA, not available. NFT, neurofibrillary tangle.

Case	Age	Sex	Post-mortem interval	Clinical diagnosis	Pathological findings
	years		h		
Non-AD					
Case 1	60	Male	NA	Alcoholism	Plaque(-), NFT stage I
Case 2	80	Female	NA	Abdominal aortic aneurysm rupture	Plaque(-), NFT stage II
Case 3	77	Male	7.5	Liver cirrhosis	Plaque(-), NFT stage II
Case 4	66	Male	11	Rectal cancer	Plaque(-), NFT stage I
Case 5	48	Male	10	Familial idiopathic basal ganglia calcification	Plaque(-), tangle(-)
AD					
Case 1	75	Male	12	Alzheimer disease	Braak stage C, VI
Case 2	68	Female	9	Alzheimer disease	Braak stage C, VI
Case 3	75	Male	17.5	Alzheimer disease	Braak stage C, VI
Case 4	81	Female	61	Dementia with Levy bodies	Alzheimer disease Braak stage C, VI
Case 5	56	Male	18	Alzheimer disease	Braak stage C, VI

Statistical Analysis—Signals on films were quantified with National Institutes of Health Image software (National Institutes of Health). Comparisons were performed using a Mann-Whitney *U* test or a Student's *t* test. For comparison of multiparametric analysis, one-way ANOVA, followed by the post hoc analysis by Fisher's protected least significant difference (PLSD) was used. Pearson's correlation coefficients and significance were defined by STATVIEW software. The data are expressed as the means \pm S.E., and statistical significance was assessed at $p < 0.05$.

RESULTS

The Expression Levels of Phosphorylated p38 MAPK Were Negatively Correlated with N-cadherin Expression Levels in Human Brains—N-cadherin is an essential adhesion molecule for forming synapses, and synaptic loss is one of pathological hallmarks of AD. Because previous reports showed that synaptic proteins such as synaptophysin or PSD-95 were reduced in AD (24, 25), we hypothesized that N-cadherin expression is also decreased in the brains of AD patients. First, the expression levels of N-cadherin were analyzed in human brain tissues from temporal cortices of AD patients and age-matched non-AD controls (Table 1). As expected, Western blot analysis using anti-N-cadherin antibody showed that expression levels of N-cadherin were decreased in AD brains compared with non-AD controls (Fig. 1A). Quantitative analysis showed that the ratio of N-cadherin/ β -actin was significantly decreased in AD brains compared with that in non-AD controls (Fig. 1B, $p < 0.05$). To investigate whether the phosphorylation of p38 MAPK was enhanced in AD brains, we subsequently examined the expression levels of both phosphorylated and total p38 MAPK in the same tissues of human brains by Western blot (Fig. 1A). Consistent with previous reports (18–20), quantitative analysis showed a significant increase in the ratio of phospho/total p38 MAPK in AD brains, compared with that in non-AD controls (Fig. 1C, $p < 0.01$). Moreover, when we plotted the ratio of phospho/total p38 MAPK against that of N-cadherin/ β -actin, we found that the phospho/total p38 MAPK ratio negatively correlated with N-cadherin/ β -actin ratio (Fig. 1D, $r = -0.774$, $p < 0.01$). These results suggested a negative correlation between phosphorylated p38 MAPK and N-cadherin expressions in human brain.

ADH-1 (N-cadherin Antagonist) Induced Neuronal Cell Death by Activating p38 MAPK in Murine Primary Neurons—

To elucidate the link between the reduced level of N-cadherin and p38 MAPK activation in AD brains, we analyzed whether the inhibition of N-cadherin-based synaptic contact could lead to p38 MAPK activation in neuronal cells. The N-terminal extracellular domain of N-cadherin harbors the homophilic cell adhesion recognition sequence, His-Ala-Val (HAV). It has been established that ADH-1, which mimics the natural HAV sequence of N-cadherin, can specifically disrupt N-cadherin-mediated cell adhesion (26). Therefore, we used it as a specific N-cadherin antagonist in the present experiment. First, murine primary neurons were treated with different concentrations of ADH-1 to disrupt the N-cadherin-based synaptic contact, followed by analysis of p38 MAPK activation by Western blot. Interestingly, exposure of murine primary neurons to ADH-1 for 24 h enhanced the phosphorylation of p38 MAPK and Tau in a concentration-dependent manner (Fig. 2A and supplemental Fig. S1). To confirm the effect of N-cadherin inhibition on p38 MAPK signaling and Tau phosphorylation in an alternative way, we applied N-cadherin-neutralizing antibody to murine primary neurons for 6 h followed by Western blot, and the same result was obtained as ADH-1 (supplemental Fig. S2). Next, we asked whether activation of p38 MAPK after inhibition of N-cadherin-based synaptic contact could lead to neuronal death. To answer this question, we examined neuronal cell viability after ADH-1 treatment using MTT assay. We observed significant decreases in neuronal cell viability after treatment with ADH-1 ($n = 4$, $p < 0.001$) (Fig. 2B). To evaluate whether p38 MAPK plays an important role in ADH-1-induced neuronal death, we used SB203580, a well characterized p38 MAPK-specific inhibitor. ADH-1 application with or without SB203580 revealed that neuronal death induced by ADH-1 was significantly attenuated by co-treatment with SB203580 ($n = 4$, $p < 0.001$) (Fig. 2C). Thus, the increased level of p38 MAPK activation is responsible for the ADH-1-induced neuronal death. To prove the effect of ADH-1 on the structural integrity of N-cadherin-mediated cell contact and p38 MAPK activation, ADH-1 (0.5 mg/ml) was added to confluent SH-SY5Y cells for 24 h. As expected, confocal microscopic analysis showed that

N-cadherin Regulates p38 Signaling via JLP

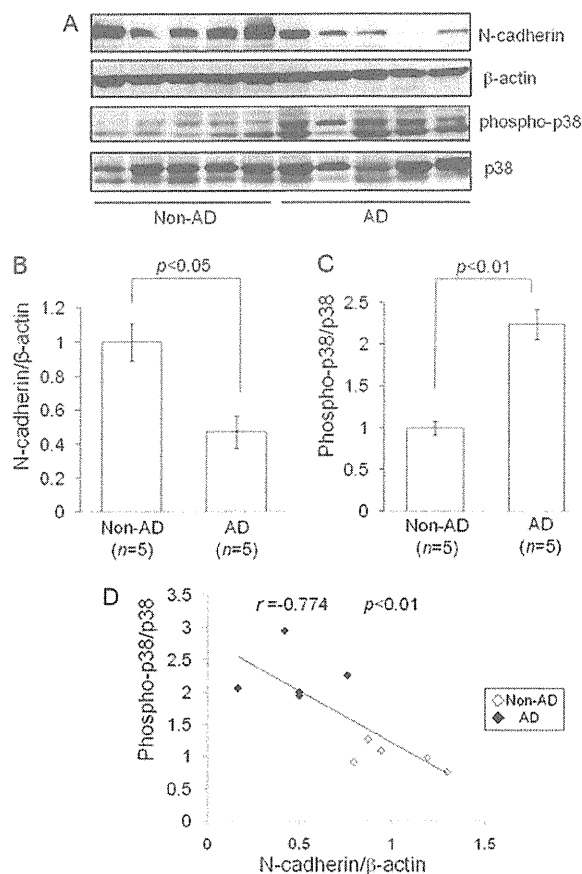


FIGURE 1. Increased phospho-p38 MAPK expressions were negatively correlated with decreased N-cadherin expressions in human brains. *A*, brain homogenates of temporal cortex from AD patients (AD, $n = 5$) and age-matched non-AD controls (Non-AD, $n = 5$) were analyzed by Western blot using anti-N-cadherin, β -actin, phospho-p38, and p38 MAPK antibodies. *B*, the band densities of N-cadherin and control β -actin were quantified by National Institutes of Health Image. The ratio of N-cadherin to β -actin (N-cadherin/ β -actin) was calculated and analyzed by Mann-Whitney's *U* test. The N-cadherin/ β -actin ratio was significantly decreased in the brains of AD patients compared with that of non-AD controls ($p < 0.05$). *C*, the band densities of phospho-p38 and p38 MAPK were quantified by National Institutes of Health Image. The phospho/total p38 MAPK ratio was calculated and analyzed by Mann-Whitney's *U* test. The phospho/total p38 MAPK ratio was significantly increased in the brains of AD patients compared with that of non-AD controls ($p < 0.01$). *D*, significant correlation was established in comparison between the phospho/total p38 MAPK ratio and the N-cadherin/ β -actin ratio by Pearson's correlation co-efficients. The phospho/total p38 MAPK ratio was negatively correlated with the N-cadherin/ β -actin ratio in human brain samples. ($r = -0.774$, $p < 0.01$).

the treatment with ADH-1 resulted in the significant decrease of N-cadherin immunoreactivity at the sites of cell-cell contact (Fig. 2D, left panels), indicating the disruption of N-cadherin-mediated cell contact. Consistent with the above result, enhanced immunoreactivity of phospho-p38 MAPK was observed in cells treated with ADH-1, compared with non-treated cells (Fig. 2D, middle panels). No immunoreactivities of both N-cadherin and phospho-p38 MAPK were observed in the absence of primary antibodies (supplemental Fig. S3).

$A\beta_{42}$ Decreased N-cadherin Expression through NMDA Receptors in Murine Primary Neurons—A number of recent studies have found that $A\beta$ is synaptotoxic (20, 27). Therefore, we next examined whether $A\beta$ could down-regulate N-

cadherin expression in neurons. To assess the effect of $A\beta$ on N-cadherin expressions, murine primary neurons were treated with synthetic $A\beta_{42}$ peptides (100 nM) for 48 h and subjected to Western blot. As shown in Fig. 3A, $A\beta_{42}$ treatment decreased N-cadherin expression in neurons. Previous reports have demonstrated that p38 MAPK is activated by the fibrillar $A\beta$ (28) and that p38 MAPK can phosphorylate Tau at Ser-202/Thr-205 (29). Thus, we examined whether synthetic $A\beta_{42}$ peptides could trigger p38 MAPK activation and Tau phosphorylation at Ser-202/Thr-205 in our system. Consistent with the previous reports, we observed increased levels of phosphorylated p38 MAPK and phosphorylated Tau in the $A\beta_{42}$ -treated cell preparations as compared with nontreated ones by Western blot ($n = 3$, $p < 0.01$) (Fig. 3, A and B). Together, this experiment indicated that $A\beta_{42}$ treatment decreases the levels of N-cadherin, activates p38 MAPK, and phosphorylates Tau in neuronal cells. Previous reports demonstrated that $A\beta$ treatment induces excessive excitation of glutamate receptors to cause excitotoxicity (30, 31). Thus, we hypothesized that the decreased level of N-cadherin was induced by the $A\beta$ -mediated excitotoxicity. To test this, murine primary neurons were pretreated (30 min) with the NMDA receptor antagonist MK-801 (10 μ M) before being exposed to $A\beta_{42}$. Subsequent Western blot analysis of cell lysates revealed that MK-801 inhibited $A\beta_{42}$ -induced reduction of N-cadherin levels ($n = 3$, $p < 0.05$) (Fig. 3C).

N-cadherin Associates with JLP Both in Human Brains and in Murine Primary Neurons—To clarify the mechanistic link between the disruption of N-cadherin-based contact and p38 MAPK activation, we set out to identify the proteins that associate with N-cadherin in human brain samples. For this, lysates of temporal cortices from brains of AD and non-AD patients were immunoprecipitated with anti-N-cadherin antibody, and the immunoprecipitates were subsequently subjected to SDS-PAGE. The separated proteins were visualized by silver staining, demonstrating a protein band of ~ 180 kDa in the lysates of brains of both AD patients and non-AD controls (supplemental Fig. S4). The protein band was identified by mass spectrometry as JLP, a scaffold protein that has been known to mediate the interaction between p38 MAPK and its upstream kinases (21). To confirm the association between N-cadherin and JLP, we transiently transfected HA-tagged N-cadherin and/or FLAG-tagged JLP expression constructs into HEK293 cells. Equal amounts of cell lysates obtained from each transfected cell were immunoprecipitated with anti-HA antibody and then immunoblotted with anti-FLAG antibody (Fig. 4A). A protein band of ~ 180 kDa was visualized with anti-FLAG antibody in the immunoprecipitate using anti-HA antibody obtained from the double-transfected cells (Fig. 4A, third lane), clearly indicating that N-cadherin associates with JLP. To further verify this association, the lysates derived from HEK293 cells transiently transfected with HA-tagged N-cadherin and/or FLAG-tagged JLP plasmids were immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4B, HA-tagged immunoreactivity was observed in the immunoprecipitate with anti-FLAG antibody, confirming the association of N-cadherin with JLP. Finally, to demonstrate the endogenous association between N-cadherin and JLP in

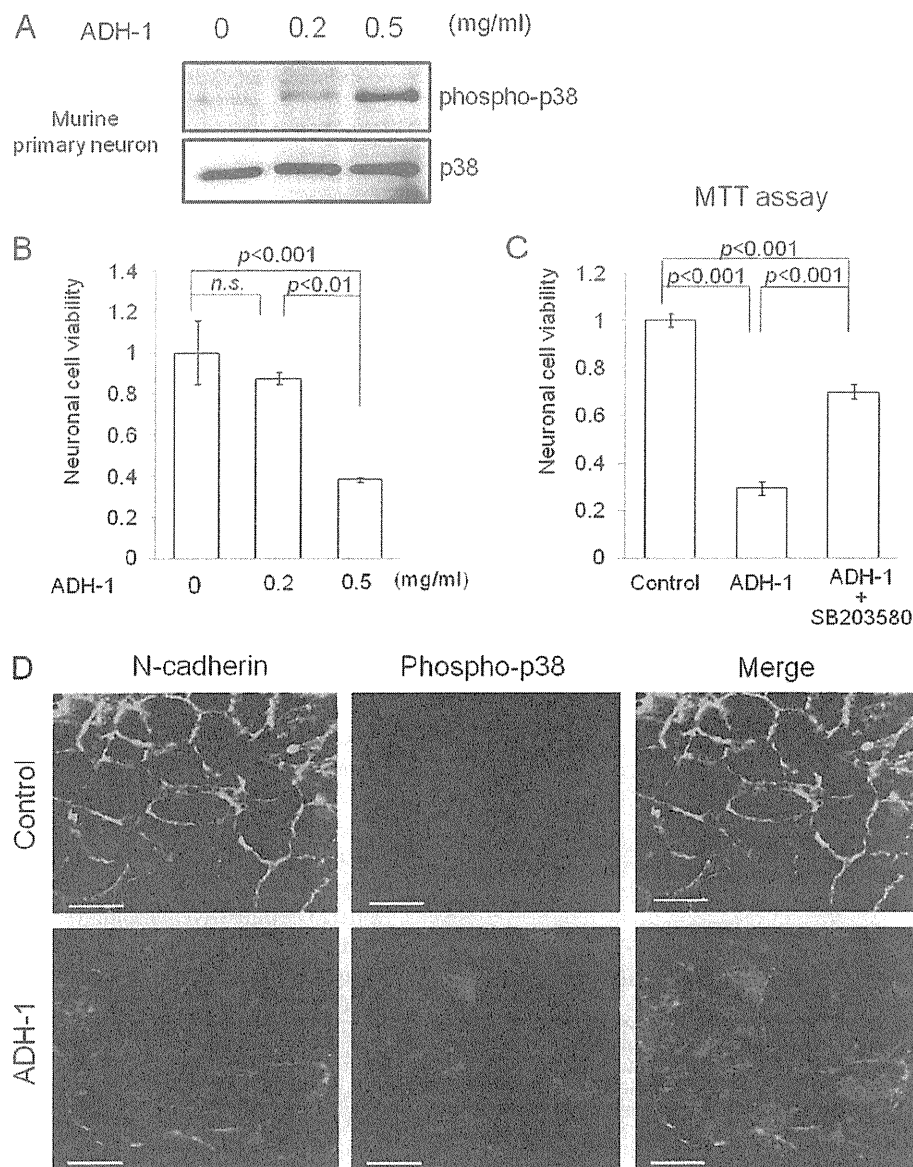


FIGURE 2. ADH-1 induced neuronal cell death by activating p38 MAPK in murine primary neurons. *A*, ADH-1, N-cadherin antagonist, was applied to murine primary neurons at different concentrations as shown. 24 h after treatment, the lysates were immunoblotted with anti-phospho-p38 and p38 MAPK antibodies. ADH-1 increased phosphorylation of p38 MAPK. *B*, neuronal cell death induced by ADH-1 was evaluated by MTT assay ($n = 5$, $p < 0.001$). *C*, murine primary neurons were treated with ADH-1 (0.5 mg/ml) for 24 h with or without 30 min of pretreatment with 10 μ M SB203580, a specific p38 MAPK inhibitor, and the cell death was examined by MTT assay ($n = 4$, $p < 0.001$). SB203580 attenuated neuronal cell death induced by ADH-1 ($n = 4$, $p < 0.001$). *D*, ADH-1 (0.5 mg/ml) was added to confluent SH-SY5Y cells for 24 h followed by the immunostaining, using anti-N-cadherin and anti-phospho-p38 antibodies. Treatment with ADH-1 perturbed N-cadherin immunoreactivity, indicating a partial loss of N-cadherin from cell-cell junctions. Scale bar, 10 μ m.

neurons, murine primary neurons were lysed, immunoprecipitated with anti-N-cadherin antibody, and then subjected to Western blot with anti-JLP antibody (Fig. 4C). The result showed endogenous association between N-cadherin and JLP in murine primary neurons, as well as in human brains.

N-cadherin/JLP Association Is Mediated by the Region Spanning Amino Acids 160–209 and Leucine Zipper II Domain of JLP—To determine the N-cadherin-binding domains of JLP, a series of S-tagged JLP mutants truncated at the C terminus was transfected into COS7 cells (Fig. 5A). Immunoprecipitation with anti-N-cadherin antibody showed that all of the S-tagged C-terminal deletion mutants of JLP were associated with N-cadherin (Fig. 5B), indicating that the JLP N

terminus plays an important role in the association with N-cadherin. To further characterize the sequences of JLP involved in this association, various N-terminally truncated JLP mutants tagged with the S-sequence were prepared as indicated (Fig. 5C). COS7 cells were transiently transfected with these deletion mutants, and the lysate derived from each transfected cell was subjected to immunoprecipitation assay with anti-N-cadherin antibody. We observed that the JLP fragment corresponding to the region of amino acids 160–463 was strongly associated with N-cadherin, whereas that corresponding to the region of amino acids 160–398 showed a weaker association with N-cadherin (Fig. 5D). In contrast, a shorter fragment corresponding to the region of amino

N-cadherin Regulates p38 Signaling via JLP

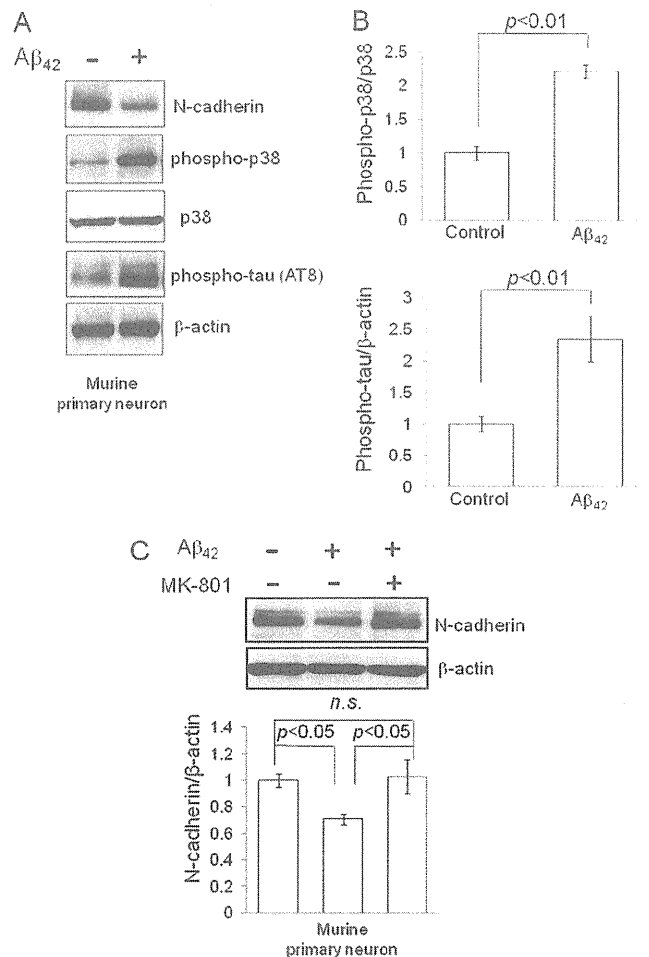


FIGURE 3. Aβ₄₂ decreased N-cadherin expressions through NMDA receptors in murine primary neurons. *A*, murine primary neurons were treated with or without 100 nM synthetic Aβ₄₂ peptides for 48 h, and the lysates were immunoblotted with anti-N-cadherin, phospho-p38, p38 MAPK, phospho-Tau (AT8), and β-actin antibodies, successively. Aβ₄₂ treatment reduced N-cadherin expressions and induced phosphorylation of p38 MAPK and Tau in murine primary neurons. *B*, the band densities of phospho-p38, p38 MAPK, phospho-Tau (AT8), and β-actin were quantified by National Institutes of Health Image. The ratios of phospho/total p38 MAPK and phospho-Tau (AT8)/β-actin were calculated and analyzed by Student's *t* test ($n = 3$, $p < 0.01$). *C*, murine primary neurons were pretreated with 10 μM MK-801, NMDA receptor antagonist for 30 min followed by 100 nM synthetic Aβ₄₂ peptides for 48 h. The lysates were evaluated by Western blot using anti-N-cadherin and β-actin antibodies. The band densities of N-cadherin and β-actin were quantified by National Institutes of Health Image. The N-cadherin/β-actin ratio was calculated and analyzed by one-way ANOVA. NMDA receptor antagonist MK-801 prevented the Aβ₄₂-induced decrease in N-cadherin levels ($n = 3$, $p < 0.05$).

acids 210–398, lacking amino acids 160–209 and leucine zipper II domain of amino acids 398–463, failed to co-immunoprecipitate with N-cadherin. These results implicated that both the region containing amino acids 160–209 and leucine zipper II domain in JLP are essential for its association with N-cadherin.

N-cadherin Expression Had a Negative Effect on p38 MAPK Activation by Inhibiting JLP/p38 MAPK Association—We next focused on investigating the functional role of the molecular association between N-cadherin and JLP. Previously, JLP has been reported to act as a scaffolding protein to bring p38

MAPK together with their upstream kinases MKK4 and MEKK3 (21), thereby activating the p38 MAPK pathway. As demonstrated above, we found that one of the N-cadherin-binding domains is the region spanning amino acids 160–209 of JLP. Interestingly, the same JLP domain has been identified to be involved in the association with p38 MAPK (21). Therefore, we hypothesized that N-cadherin could regulate the p38 MAPK signaling pathway via modulating JLP-p38 MAPK association. To prove this hypothesis, we investigated the effect of N-cadherin expression on the p38 MAPK signaling pathway in COS7 cells. FLAG-tagged p38 MAPK was transiently co-transfected into COS7 cells together with FLAG-tagged MKK4, S-tagged JLP, and HA-tagged N-cadherin as designated in Fig. 6A. The cell lysates were analyzed by immunoblotting with anti-phospho-p38 MAPK or p38 MAPK antibodies, respectively (Fig. 6A). Consistent with the previous report, the phosphorylation of p38 MAPK was enhanced by the co-expression of JLP and MKK4, upstream kinase for p38 MAPK ($n = 3$, $p < 0.05$). Interestingly, we found that this activation of p38 MAPK induced by the co-expression of MKK4 and JLP was significantly suppressed by the co-expression of N-cadherin ($n = 3$, $p < 0.05$). Alternatively, we co-transfected HA-tagged MEKK3, another upstream kinase of p38 MAPK, into COS7 cells. Similarly, we observed that the enhancement of phosphorylated p38 MAPK under the co-expression of both MEKK3 and JLP was significantly suppressed by the co-expression of N-cadherin (Fig. 6B, $n = 3$, $p < 0.05$). Moreover, when we pull down JLP with S-agarose, we found a reduced interaction between JLP and p38 MAPK in the presence of N-cadherin (Fig. 6), indicating that N-cadherin competitively inhibits the binding of p38 MAPK to JLP. Indeed, co-transfection of FLAG-tagged p38 MAPK, FLAG-tagged MKK4, and JLP fragment corresponding to the region of amino acids 160–463, which could associate with N-cadherin into COS7 cells, showed the increased phosphorylation of p38 MAPK (supplemental Fig. S5). This result implies that this short fragment of JLP may compete with the endogenous JLP for the N-cadherin binding, resulting in the release of JLP from the N-cadherin and subsequent p38 MAPK activation. Taken together, these results suggested that N-cadherin expression had an inhibitory effect on the p38 MAPK signaling pathway via inhibition of JLP-p38 MAPK association.

DISCUSSION

The molecular mechanism of synaptic adhesion and cell viability pathway of p38 MAPK is not well understood yet. Our present study suggested a potentially new signaling pathway contributing to the aberrant activation of p38 MAPK in AD. We showed that ADH-1 caused a significant increase in p38 MAPK activation and Tau phosphorylation with a subsequent decrease in neuronal viability (Fig. 2, A and B, and supplemental Fig. S1). In alternative ways, application of N-cadherin-neutralizing antibody also activated p38 MAPK and increased phosphorylation of Tau in murine primary neurons (supplemental Fig. S2), suggesting that the disruption of N-cadherin-based cell adhesion could lead to p38 MAPK activation and Tau phosphorylation. Importantly, we demonstrated that the inhibition of the p38 MAPK pathway with specific

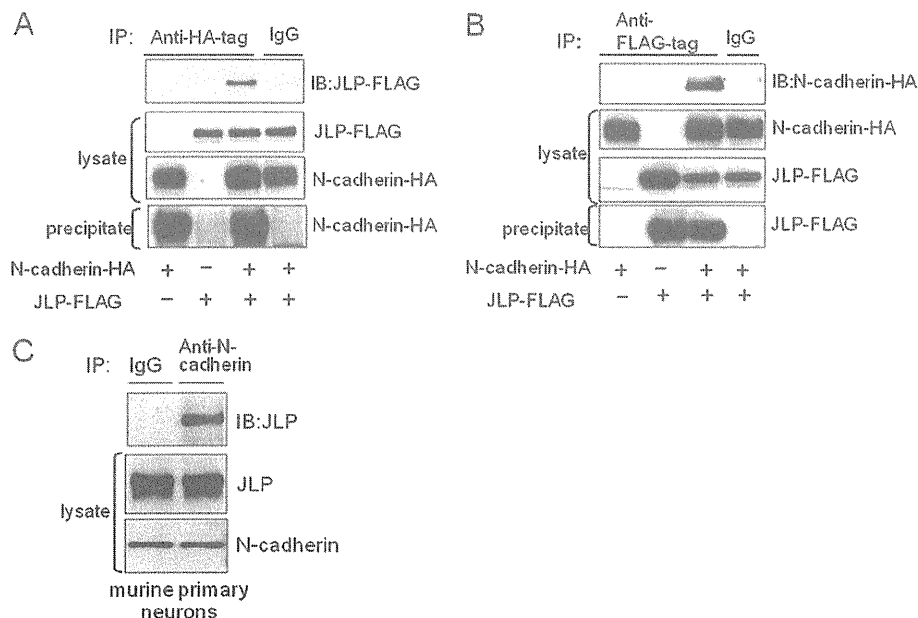


FIGURE 4. The association of N-cadherin with JLP in HEK293 cells and neuronal cells. *A*, lysates of HEK293 cells, transiently transfected with HA-tagged N-cadherin and/or FLAG-tagged JLP expressing vectors, were immunoprecipitated (IP) with anti-HA antibody (lanes 1–3) or normal IgG (lane 4). The immunoprecipitates and the lysates were analyzed by immunoblotting (IB) with the specific antibodies against the FLAG tag and HA tag. *B*, lysates of HEK293 cells, transiently transfected with HA-tagged N-cadherin and/or FLAG-tagged JLP expressing vectors were immunoprecipitated with anti-FLAG antibody (lanes 1–3) or normal IgG (lane 4). The immunoprecipitates and the lysates were analyzed by Western blot with the specific antibodies against the HA tag and FLAG tag. *C*, the endogenous association between N-cadherin and JLP in murine primary neurons was analyzed. The neurons were lysed and immunoprecipitated with either anti-N-cadherin antibody or normal IgG. The immunoprecipitates and the lysates were immunoblotted with the specific antibodies against JLP and N-cadherin.

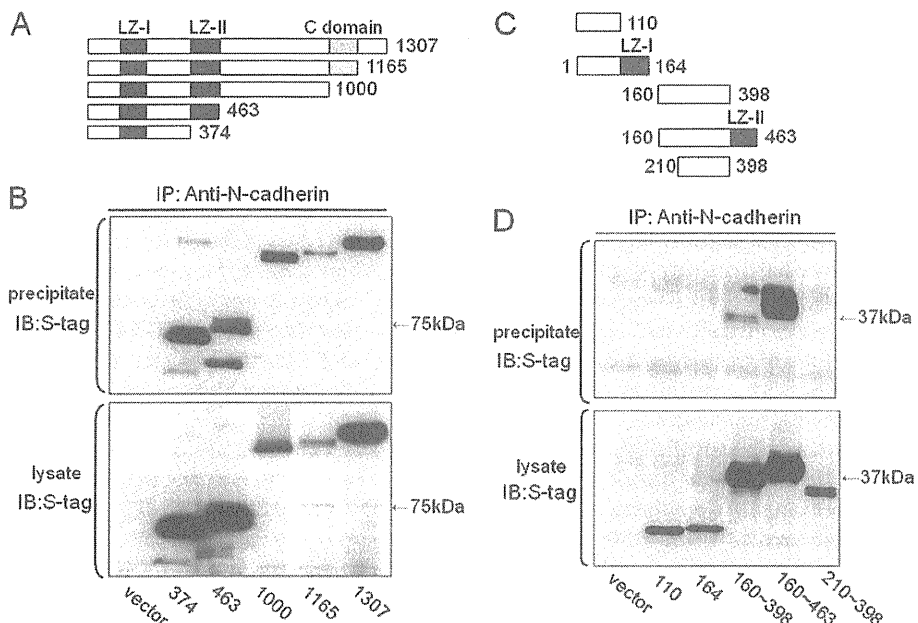


FIGURE 5. Deletion mapping of N-cadherin-interacting domain of JLP. *A*, schematic diagram of the C-terminally truncated S-tagged JLP constructs used for analysis of the JLP-N-cadherin interaction. The sizes of these deletion mutants were 374 (amino acids 1–374), 467 (amino acids 1–467), 1000 (amino acids 1–1000), 1165 (amino acids 1–1165), and 1307 amino acids (full length, residues 1–1307), respectively. *B*, COS7 cells were transiently transfected with the WT or C-terminally truncated mutants of S-tagged JLP. The lysates were immunoprecipitated (IP) with anti-N-cadherin antibody. The immunoprecipitates and the lysates were subjected to Western blot analysis with the specific antibodies against S-tag. All of these deletion mutants of JLP were co-immunoprecipitated with N-cadherin. *C*, schematic diagram representing shorter constructs of C-terminally truncated S-tagged JLP. N-terminally truncated short constructs were also prepared as shown in Fig. 5C. *D*, COS7 cells were transiently transfected with these different mutants of S-tagged JLP. The lysates were immunoprecipitated with anti-N-cadherin antibody, and the immunoprecipitates were immunoblotted (IB) with anti-S-tag antibody. The mutants consisting of amino acids 160–398 and 160–463 of JLP were co-immunoprecipitated with N-cadherin, whereas the mutant consisting of amino acids 210–398 of JLP was not co-immunoprecipitated with N-cadherin.

p38 MAPK inhibitor (SB203580) attenuated ADH-1-induced neurotoxicity (Fig. 2C), indicating an important role of p38 MAPK in the cell death caused by the inhibition of N-cad-

herin-based cell adhesion. However, because the application of SB203580 did not block ADH-1-induced cell death completely, we assume that other alterations in cellular signaling

N-cadherin Regulates p38 Signaling via JLP

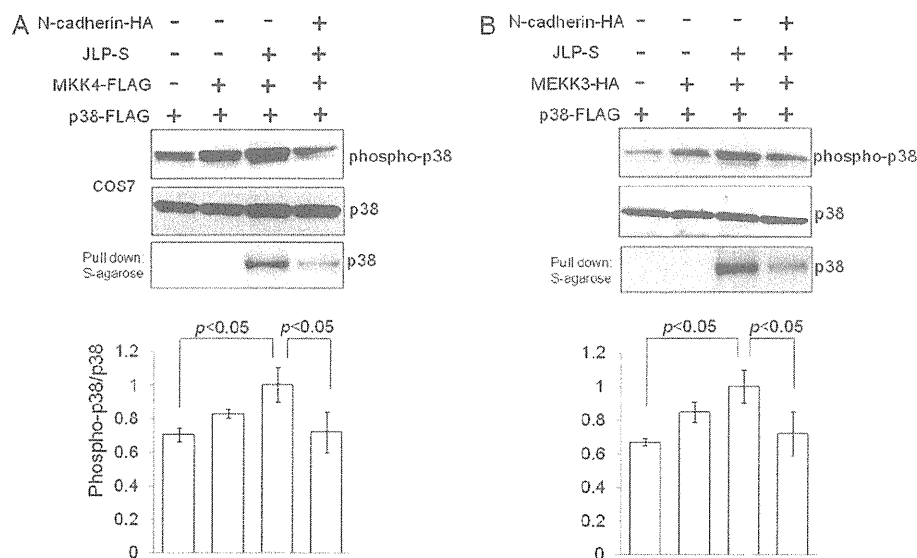


FIGURE 6. N-cadherin expression inhibited JLP-associated p38 MAPK pathway. *A*, COS7 cells were co-transfected with FLAG-tagged p38 α , FLAG-tagged MKK4, S-tagged JLP, and HA-tagged N-cadherin. 24 h after transfection, the lysates were precipitated with S-protein-agarose. The lysates and precipitates were subjected to Western blot analysis with anti-phospho-p38 or p38 MAPK antibodies. One representative immunoblot result is shown. The band densities of phospho-p38 and p38 MAPK were quantified by National Institutes of Health Image. The phospho/total p38 MAPK ratio was calculated and analyzed by one-way ANOVA. N-cadherin expression significantly inhibited JLP/MKK4-mediated phosphorylation of p38 MAPK compared with that without expression of N-cadherin ($n = 3$, $p < 0.05$). Pull-down assay using S-protein-agarose showed that N-cadherin expression decreased the interaction between JLP and p38 MAPK. *B*, COS7 cells were co-transfected with FLAG-tagged p38 α , HA-tagged MEKK3, S-tagged JLP, and HA-tagged N-cadherin. 24 h after transfection, the lysates were subjected to Western blot analysis with anti-phospho-p38 or p38 MAPK antibodies. One representative immunoblot result is shown. The band densities of phospho-p38 and p38 were quantified by National Institutes of Health Image. The ratio of phospho/total p38 MAPK was calculated and analyzed by one-way ANOVA. N-cadherin expression inhibited JLP/MEKK3-mediated phosphorylation of p38 MAPK significantly compared with that without expression of N-cadherin ($n = 3$, $p < 0.05$). Pull-down assay using S-protein-agarose showed that N-cadherin expression inhibited the interaction between JLP and p38 MAPK.

might be involved in neuronal death in addition to activation of p38 MAPK signaling. Notably, previous studies have shown that N-cadherin-mediated cell adhesion leads to the recruitment of PI3K into the N-cadherin adhesion complex followed by activation of Akt, which is an important regulator of anti-apoptotic pathways (12). Thus, down-regulation of PI3K/Akt signaling could also be participating in the ADH-1-induced cell death. Collectively, these results suggest that N-cadherin-mediated synaptic contact might contribute to neuroprotective signaling. Disruption of this synaptic contact might lead to neuronal cell apoptosis by perturbing these signalings.

Concerning a potential molecular link between N-cadherin and p38 MAPK signaling, we identified JLP as a novel N-cadherin-interacting protein in human brains by proteomic analysis. JLP is a member of the JIP (JNK-interacting protein) family, which provides a scaffolding function for the JNK/p38 MAPK signaling module. JLP is encoded by the *JIP4* gene, which generates three distinct splice variants, namely, JLP, JIP4, and SPAG9 (32). Specifically, JLP was identified as a scaffold protein involved in the p38 MAPK signaling pathway to bring p38 MAPK together with its upstream kinases (21). We also demonstrated that both the leucine zipper II domain and the region containing amino acids 160–209 of JLP were required for the association with N-cadherin, the same region for the interaction with p38 MAPK, which may explain the competitive regulation by N-cadherin. Moreover, our data showed that N-cadherin overexpression interfered with the physical interaction between JLP and p38 MAPK, thereby inhibiting the JLP-mediated activation of p38 MAPK signal-

ing. Thus, it is plausible that perturbation of N-cadherin-based cell adhesion and/or reduced expression of N-cadherin could lead to aberrant activation of p38 MAPK via facilitating JLP-mediated p38 MAPK signaling.

Several recent reports have shown that cysteine dioxygenase type I associates with both N-cadherin and JLP in myoblasts (33, 34). However, our observation demonstrated an opposite result to the recent report showing that N-cadherin ligation activates p38 MAPK in a cysteine dioxygenase type I- and JLP-dependent manner during myoblast differentiation (35). Although it was unclear whether cysteine dioxygenase type I is involved in the p38 MAPK activation induced by the inhibition of N-cadherin-based cell adhesion in our study, we speculate that the discrepancy between our study and previous reports could be attributed to the differences in cell type specificity and the cellular context examined (*i.e.* myoblast differentiation *versus* neuronal degeneration). Variable roles of JLP and N-cadherin according to the differential cellular context have been demonstrated in previous studies. For example, JLP has been reported to negatively regulate NGF-induced neurite outgrowth via JNK inhibition during neuronal differentiation (36) as opposed to the previous report demonstrating that N-cadherin is involved in NGF-induced neurite outgrowth (37). Thus, the association between N-cadherin and JLP may be involved in differential cell fate by modulating signaling pathways including p38 MAPK and JNK according to the cellular context.

With respect to AD pathophysiology, the effect of A β on N-cadherin expression has been unclear. In this study, we

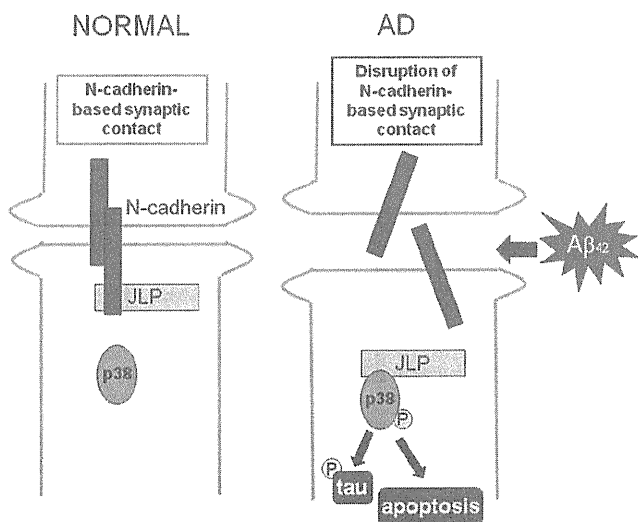


FIGURE 7. Hypothetical model of AD pathogenesis caused by the disruption of N-cadherin-based synaptic contact. *Left panel*, in the basal state at the synapse, N-cadherin-based synaptic adhesion stabilizes JLP, which suppresses neurotoxic p38 MAPK activation. *Right panel*, in AD, when N-cadherin-based synaptic contact is disrupted by $A\beta_{42}$, $A\beta_{42}$ could decrease N-cadherin expressions resulting in aberrant p38 MAPK activation followed by the subsequent Tau phosphorylation and neuronal death.

demonstrated that treatment with $A\beta_{42}$ decreased endogenous N-cadherin expression in murine primary neurons, whereas the aberrant phosphorylation of both p38 MAPK and p38 MAPK-sensitive Tau Ser-202/Thr-205 epitopes were simultaneously observed, as previously reported (29, 38). This result raises the possibility that $A\beta_{42}$ could interfere with N-cadherin-mediated synaptic contact, resulting in p38 MAPK activation. Recently, converging lines of evidence suggest that natural soluble $A\beta$ oligomers trigger synaptic loss (3, 20). Therefore, it is plausible that synaptic dissociation caused by $A\beta$ activates the p38 MAPK signaling pathway, leading to neuronal death as well as Tau phosphorylation. Interestingly, it has been shown that electroconvulsive and other excitatory stimuli induce arcadin, a protocadherin, to promote activation of p38 MAPK and the endocytosis of N-cadherin at the synapse (39). Indeed, the neurodegeneration caused by $A\beta$ has long been related to the excessive activation of glutamate receptors, namely, excitotoxicity (30). Moreover, it is demonstrated that even physiological levels of $A\beta$ can enhance glutamate excitotoxicity (31). Importantly, we showed that $A\beta_{42}$ -induced reduction of N-cadherin levels was NMDA receptor-dependent (Fig. 3B). Thus, it is possible that $A\beta$ enhances excitotoxicity to promote the endocytosis of N-cadherin at the synapse, presumably followed by its degeneration by endosome/lysosome pathway. Alternatively, another group previously reported calpain-mediated degradation of N-cadherin after NMDA receptor stimulation (40). In either scenario, a decrease in N-cadherin expression could accelerate the JLP-mediated p38 MAPK activation, resulting in synaptic loss, increased phosphorylated Tau, and neuronal death in AD pathology. Overall, we suggest the possibility that JLP is a key molecule linking synaptic adhesion to p38 MAPK signaling involved in Tau phosphorylation and neuronal death associated with AD (Fig. 7).

In summary, our study suggested the physical and functional association between N-cadherin and p38 MAPK via JLP. We also demonstrated that perturbation of N-cadherin-mediated synaptic contact activated p38 MAPK pathway and increased Tau phosphorylation, leading to neuronal death. From observations obtained from the present study, we would like to extend our view of JLP function further and suggest that JLP is involved in the N-cadherin/p38 MAPK signaling pathway from synaptic adhesion to neurodegeneration. Because there were no reports focusing on the effect of $A\beta$ on synaptic adhesion, this is the first report proposing an attractive possibility that $A\beta_{42}$ may dissociate N-cadherin-mediated synaptic contact to trigger p38 MAPK activation, followed by neurodegeneration such as synaptic loss, Tau phosphorylation, and neuronal death in AD. Future study in this field could lead to a better understanding of AD pathophysiology.

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N-cadherin Regulates p38 Signaling via JLP

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Chemical Library Screening Identifies a Small Molecule That Downregulates SOD1 Transcription for Drugs to Treat Amyotrophic Lateral Sclerosis

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Familial amyotrophic lateral sclerosis (fALS) accounts for 10% of ALS cases, and about 25% of fALS cases are due to mutations in superoxide dismutase 1 (SOD1). Mutant SOD1-mediated ALS is caused by a gain of toxic function of the mutant protein, and the SOD1 level in nonneuronal neighbors, including astrocytes, determines the progression of ALS (non-cell-autonomous toxicity). Therefore, the authors hypothesized that small molecules that reduce SOD1 protein levels in astrocytes might slow the progression of mutant SOD1-mediated ALS. They developed and optimized a cell-based, high-throughput assay to identify low molecular weight compounds that decrease SOD1 expression transcriptionally in human astrocyte-derived cells. Screening of a chemical library of 9600 compounds with the assay identified two hit compounds that selectively and partially downregulate SOD1 expression in a dose-dependent manner, without any detectable cellular toxicity. Western blot analysis showed that one hit compound significantly decreased the level of endogenous SOD1 protein in H4 cells, with no reduction in expression of β -actin. The assay developed here provides a powerful strategy for discovering novel lead molecules for treating familial SOD1-mediated ALS. (*Journal of Biomolecular Screening* 2011;16:405-414)

Key words: amyotrophic lateral sclerosis, superoxide dismutase 1, high-throughput screening, cell-based assay

INTRODUCTION

AMYOTROPHIC LATERALS SCLEROSIS (ALS) IS A DEVASTATING neurodegenerative disease that selectively involves motor neurons in the brain and spinal cord. ALS leads to muscle weakness, paralysis, and respiratory failure within 5 years of onset. Familial ALS (fALS) accounts for about 10% of all ALS cases, and approximately 25% of fALS cases are due to mutations in superoxide dismutase [Cu-Zn] (SOD1).¹

Some evidence suggests that mutant SOD1 protein has neurotoxic properties and leads to ALS via a gain of toxic function.

Mice carrying a high copy number of the mutant SOD1 gene suffer more severe muscle weakness and death than mice carrying a low copy number.² SOD1 knockout mice do not develop the motor neuron disease phenotype at all.³ In rats, only strains with the highest level of mutant SOD1 expression develop an ALS phenotype.⁴

Previous studies reported that the SOD1 level in neurons and in nonneuronal neighbors, including astrocytes and microglia, determines the onset and progression of motor neuron disease.^{5,6} Therefore, we hypothesized that reduction of SOD1 expression in astrocytes might ameliorate mutant SOD1-mediated ALS. This hypothesis is supported by prolonged survival of ALS model mice, following application of RNA interference or antisense oligonucleotide, which reduced SOD1 protein levels.^{7,8} Furthermore, inactivation of a mutant allele reversed the phenotypes in other neurodegenerative disease models, such as Huntington disease and Alzheimer disease, even after onset.^{9,10} The present study developed and optimized a high-throughput screening (HTS) system to identify compounds that downregulate the transcription of SOD1.

MATERIALS AND METHODS

Generation of a SOD1 promoter-luciferase reporter cell line

We used the SOD1 genomic promoter, including 5' and 3' untranslated regions (UTR), in our construct to generate SOD1

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