Presenilin-1/γ-Secretase Controls Glutamate Release, Tyrosine Phosphorylation, and Surface Expression of N-Methyl-d-aspartate Receptor (NMDAR) Subunit GluN2B*

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Presenilin-1/γ-secretase is critical to neuronal function. Inhibition of presenilin-1/γ-secretase increases release of glutamate, stimulating NMDAR and calpain while decreasing phosphorylation and surface expression of neuronal GluN2B.

Abnormally high concentrations of extracellular glutamate in the brain may cause neuronal damage via excitotoxicity. Thus, tight regulation of glutamate release is critical to neuronal function and survival. Excitotoxicity is caused mainly by overactivation of the extrasynaptic NMDA receptor (NMDAR) and results in specific cellular changes, including calcium-induced activation of calpain proteases. Here, we report that presenilin-1 (PS1) null mouse cortical neuronal cultures have increased amounts of calpain-dependent spectrin breakdown products (SBDPs) compared with WT cultures. NMDAR antagonists blocked accumulation of SBDPs, suggesting abnormal activation of this receptor in PS1 null cultures. Importantly, an increase in SBDPs was detected in cultures of at least 7 days in vitro but not in younger cultures. Conditioned medium from PS1 null neuronal cultures at 8 days in vitro contained higher levels of glutamate than medium from WT cultures and stimulated production of SBDPs when added to WT cultures. Use of glutamate reuptake inhibitors indicated that accumulation of SBDPs is due to antagonism between synaptic NMDAR-activated sur- face expression of GluN2B subunit of NMDAR, indicating decreased amounts of GluN2B expression and phosphorylation of the GluN2B subunit of NMDAR in the absence of PS1. Inhibition of γ-secretase activity in WT neurons caused changes similar to those observed in PS1 null neurons. Together, these data indicate that the PS1/γ-secretase system regulates release of glutamate, tyrosine phosphorylation, and surface expression of GluN2B-containing NMDARs.

Presenilins (PSs)4 are the catalytic subunits of the γ-secretase complexes that mediate production of amyloid-β peptides, the precursors of amyloid depositions of Alzheimer disease brains. In addition, γ-secretase processes cell surface transmembrane proteins to produce cytosolic cell signaling peptides (1). PSs play important roles in neurodegenerative conditions such as familial Alzheimer disease (FAD) and, in addition to their role in γ-secretase proteolysis, have been shown to have γ-secretase-independent functions, including regulation of survival signaling, autophagy, calcium homeostasis, and long-term potentiation (2–5). It is still unclear, however, what function(s) of PSs are involved in the neurodegeneration of FAD.

Calpains are calcium-dependent cysteine proteases activated following calcium influx in response to stimulation of the NMDA receptor (NMDAR) by its agonists. Activated calpains cleave many substrates, including the cytoskeletal protein spectrin, to produce spectrin breakdown products (SBDPs) (6–9). Furthermore, activated calpains contribute to necrotic morphology of primary neuronal cultures, although it is unclear whether calpains contribute to excitotoxic neuronal cell death (10).

There is a long-standing paradox that NMDAR signaling can be associated with both neuronal survival and neuronal death (11). However, recent studies show that NMDARs are found in both synaptic and extrasynaptic locations of neuronal processes. Stimulation of synaptic receptors is believed to promote neuronal survival, whereas stimulation of extrasynaptic NMDARs facilitates neuronal death (12). Although the mechanisms of the toxic effects of extrasynaptic NMDARs are still under investigation, it is believed that neuronal death may be due to antagonism between synaptic NMDAR-activated survival pathways and pro-death events such as calpain activation stimulated by extrasynaptic NMDAR containing the GluN2B

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4 The abbreviations used are: PS, presenilin; FAD, familial Alzheimer disease; NMDAR, NMDA receptor; SBDP, spectrin breakdown product; dl-TBOA, dl-threo-β-benzoxaspartate; APP, amyloid precursor protein; DIV, days in vitro; RIPA, radioimmunoprecipitation assay.
subunit (11, 13, 14). Furthermore, accumulation of extracellular glutamate and excessive stimulation of glutamate receptors, including NMDAR, are associated with increased excitotoxicity and neuronal cell death in the CNS (15, 16).

Here, we report that PS1 null cortical neuronal cultures contained high amounts of SBDPs, indicating increased calpain activity in the absence of PS1, a defect rescued by NMDAR antagonists. We also show that PS1 null cultures displayed enhanced rates of glutamate release and contained increased concentrations of this neurotransmitter in their conditioned media. Furthermore, PS1 null neurons displayed decreased phosphorylation and surface expression of the GluN2B subunit of NMDAR. Importantly, treatment of WT neuronal cultures with γ-secretase inhibitors had similar outcomes as those observed in PS1 null cultures, suggesting that the PS1/γ-secretase system controls release of neuronal glutamate.

EXPERIMENTAL PROCEDURES

Materials—Anti-spectrin, anti-GluN2B, and anti-phospho-GluN2B Tyr-1472 antibodies were from Millipore. Anti-tubulin and anti-phosphotyro sine antibodies were from Santa Cruz Biotechnology, Inc. Anti-phospho-ERK (Thr-202/Thr-204) and anti-total ERK antibodies were from Cell Signaling (Danvers, MA). The γ-secretase inhibitor L685,458 was from Calbiochem. MK801, d-(−)-2-amino-5-phosphonopentoic acid, and di-threo-β-benzoxylaspartate (dL-TBOA) were from Tocris Bioscience. Memantine was from Sigma. Sulfo-NHS-SS-biotin, NeutrAvidin-agarose beads, and Protein G-Sepharose beads were from Thermo Scientific. The fluorometric glutamic acid assay kit was from AAT Bioquest, Inc. (Sunnyvale, CA). The R1 antibody against the C-terminal domain of anoylid precursor protein (APP) has been described (17).

Primary Neuronal Cultures—All animal experiments were carried out in accordance with the rules and regulations of the Mount Sinai School of Medicine (New York, NY). Mouse brain cortical neuronal cultures were prepared as described recently (7, 18). Briefly, the cortices and hippocampi of embryonic day 15.5 mouse brains were dissected out, treated with trypsin, and mechanically dissociated. Neurons were suspended in Neurobasal medium supplemented with B27 (Invitrogen) and plated on poly-D-lysine-coated dishes. Neurons were kept 3–10 days in vitro (DIV) as indicated in the figure legends. Under these conditions, post-mitotic neurons represent >98% of cultured cells (19). Lysates of embryonic mouse brain tissue were prepared in 1% SDS buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 1% SDS with protease and phosphatase inhibitors) as described (20).

Western Blot Analysis and Immunoprecipitation—Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) containing protease and phosphatase inhibitors as described (7, 18). Protein content was determined by the BCA protein assay (Pierce). Equal amounts of total protein were resolved on denaturing 6% SDS-polyacrylamide gel and transferred to PVDF membranes, and protein detection was by Western blotting with the indicated antibodies. For immunoprecipitation, solubilized proteins were incubated overnight at 4 °C with anti-GluN2B anti-body, and immune complexes were collected with protein G-Sepharose beads for 2 h at 4 °C. Bound proteins were washed three times with PBS and probed with antibodies as described (20, 21).

RESULTS

Calpain Is Activated in PS1−/− Neurons Due to High Levels of Glutamate Release—Mouse cortical neuronal cultures at 7 DIV prepared from PS1 null (PS1−/−) embryonic brains showed increased levels of cleaved SBDPs compared with neuronal cultures from WT mice (Fig. 1A, lanes 1 and 4). These products are known to be derived from the calpain-dependent cleavage of neuronal spectrin and are induced in response to NMDAR activation by its agonists such as glutamate (7). Indeed, NMDAR antagonists, including MK801 and d-(−)-2-amino-5-phosphonopentoic acid, reduced the levels of the SBDPs in PS1−/− neuronal cultures but had little effect in WT cultures (Fig. 1A, lanes 2, 3, 5, and 6), suggesting that NMDARs are activated in the absence of PS1. Calpeptin, a calpain inhibitor, inhibited the increase in SBDPs in PS1−/− neurons (Fig. 1B), providing further evidence that calpain activity mediates their accumulation in the absence of PS1. To examine whether the conditioned media of PS1−/− neuronal cultures contain factors that stimulate the NMDAR-dependent increase in SBDPs, the conditioned medium from PS1−/− cultures was used to replace the media of WT neuronal cultures. Fig. 1C shows that incubation of WT cultures with the media from PS1−/− cultures increased the production of SBDPs in WT neurons and that this increase was blocked by NMDAR antagonists. These results indicate that the conditioned media of PS1−/− neuronal cultures contain agonists that activate NMDAR and its downstream target calpain, thus increasing production of neuronal SBDPs.

Measurements of glutamate using a fluorometric glutamic acid assay (see “Experimental Procedures”) showed that its concentration in the conditioned media of PS1−/− cultures was ∼10 μM higher than its concentration in the media of WT cultures. To examine whether comparable concentrations of glutamate are sufficient to cause an increase in SBDPs in WT neurons similar to the increase detected in PS1−/− neurons, WT neuronal cultures were treated with glutamate concentrations ranging from 2 to 15 μM. Fig. 2A shows that treatment of WT cultures with 10 μM exogenous glutamate increased neuronal SBDPs to approximately the same levels detected in neurons treated with PS1−/− media. Together, these data indicate that the absence of neuronal PS1 causes a dysregulation in glutamate metabolism, increasing its concentration in culture media
PS/γ-Secretase Regulates Glu Release/GluN2B Phosphorylation

**FIGURE 1.** A, WT or PS1−/− mouse cortical neuronal cultures at 7 DIV were non-treated (NT) or treated overnight with NMDA antagonists MK801 (10 μM) and D(-)-2-amino-5-phosphonopentanoic acid (APV; 100 μM). Neuronal extracts were then prepared in RIPA buffer and probed with anti-spectrin and anti-tubulin antibodies. Calpain-dependent SBDPs were normalized to tubulin, and Student’s t test was performed (n = 3). *p < 0.05. B, PS1−/− neuronal cultures as described above were treated overnight with calpeptin (20 μM), and neuronal extracts were probed with anti-spectrin antibody. Calpain-dependent SBDPs were normalized to full-length (FL) spectrin, and Student’s t test was performed (n = 3). **p < 0.01. C, WT neuronal cultures at 7 DIV were incubated in conditioned medium collected from either WT (control [Ctrl]) or PS1−/− cultures. WT neurons in medium from PS1−/− cultures were also treated with 10 μM of MK801 (right lane). Lysates were prepared the next day and probed on Western blots with anti-spectrin antibody. The blot shown is representative of four independent experiments with similar results.

**FIGURE 2.** A, WT cortical neuronal cultures at 7 DIV were treated overnight with conditioned media from WT cultures (control [ctrl]), media from WT cultures plus different concentrations of glutamate (2–15 μM as indicated), or media from PS1−/− cultures. Lysates were then probed by Western blotting with anti-spectrin antibody. The blot shown is representative of four independent experiments with similar results. FL, full-length. B, WT, PS1+/−, or PS1−/− neuronal cultures were pretreated for 2 days with DL-TBOA, and media were collected at 7 DIV. The glutamate concentration was then determined using the fluorometric glutamic acid assay kit. Numbers indicate fold increase of glutamate concentration between connected conditions and represent the average of three independent measurements. Differences were statistically significant. Two-way ANOVA was performed (n = 3).

Concentration of glutamate in the medium of neuronal cultures is the net effect of glutamate release and reuptake. To examine whether glutamate accumulation in the medium of PS1−/− cultures reflects a decreased rate of glutamate reuptake in the absence of PS1 compared with WT neurons, we used DL-TBOA, which blocks reuptake of glutamate (22). This treatment is expected to decrease the difference in glutamate concentrations between the two cultures if a slower reuptake drives glutamate accumulation in PS1 null cultures. Contrary to this expectation, DL-TBOA increased the difference in glutamate concentration of the two cultures from a ratio of 2.0 to 2.6, indicating an increased rate of glutamate release in the absence of neuronal PS1. This conclusion is also supported by the relative increase in glutamate concentration caused by DL-TBOA in the medium of each culture. As shown in Fig. 2B, in WT cultures, this drug caused a 1.3-fold increase in glutamate, whereas in PS1−/− cultures, this ratio increased to 1.8, again indicating a higher rate of glutamate release in the absence of PS1. Together, our data show that the absence of PS1 results in abnormally high rates of glutamate release, suggesting that PS1 is necessary for the regulated release of this neurotransmitter. Importantly, heterozygous (PS1+/−) cultures behaved similarly to WT cultures (Fig. 2B), suggesting that the presence of one PS1 allele is sufficient for normal regulation of glutamate release.

Calpain-dependent SBDPs are elevated upon maturation of Synapses in PS1−/− Neuronal Culture—Kinetic studies showed that WT and PS1−/− cortical neuronal cultures at 1–5 DIV contained similar amounts of SBDPs. At 7 DIV, however, the amounts of SBDPs increased dramatically in PS1−/− neurons and activating NMDARs and downstream targets such as calpains.
**PS/γ-Secretase Regulates Glu Release/GluN2B Phosphorylation**

**FIGURE 3.** A, lysates from WT or PS1<sup>−/−</sup> neuronal cultures at different DIV were probed by Western blotting with anti-spectrin antibody. The blot shown is a representative sample of two independent experiments with similar results. B, WT or PS1<sup>−/−</sup> neuronal cultures were pretreated for 2 days with memantine (mem; 10 μM), and lysates prepared at 7 DIV were probed with anti-spectrin antibody. Calpain-dependent SBDPs were normalized to full-length (FL) spectrin. Student’s t test was performed (n = 3), *, p < 0.05. Ctrl, untreated control; ctrl, control.

Compared with WT neurons (Fig. 3A), suggesting that the onset of SBDP increase coincides with maturation and synapse formation of in vitro primary neuronal cultures (23). At this time, GluN2B subunit-containing NMDAR is expressed at extrasynaptic locations (24). In agreement with this suggestion, memantine, a drug known to inhibit extrasynaptic NMDAR at low concentration (25), blocked the accumulation of SBDPs in PS1<sup>−/−</sup> neurons (Fig. 3B), suggesting that the calpain cleavage of spectrin is induced by activated extrasynaptic NMDAR.

**Tyrosine Phosphorylation and Cell Surface Expression of GluN2B Are Decreased in PS1<sup>−/−</sup> Neurons and in Embryonic Brain Tissue**—Because extrasynaptic NMDAR contains mainly GluN2B subunits (26), we examined the effects of glutamate on tyrosine phosphorylation of this subunit, as this event has been reported to regulate cell surface localization of NMDARs (27). As shown in Fig. 4A, total GluN2B was decreased after glutamate treatment, an outcome consistent with previous literature that glutamate increases GluN2B degradation (28). However, normalization to total GluN2B revealed a significant decrease in tyrosine phosphorylation of GluN2B following glutamate treatment (Fig. 4B). We then asked whether the absence of PS1 function affects tyrosine phosphorylation of GluN2B. Fig. 5 (A and B) shows that, compared with WT neurons, tyrosine phosphorylation of this NMDAR subunit was clearly decreased in PS1<sup>−/−</sup> neurons. Consistent with these data, decreased tyrosine phosphorylation of GluN2B was also detected in PS1<sup>−/−</sup> embryonic mouse brains (Fig. 5, C and D). Because phosphorylation at tyrosine 1472 of GluN2B is important for the cell surface expression of NMDAR (29, 30), we asked whether Tyr-1472-phosphorylated GluN2B is decreased in PS1<sup>−/−</sup> neurons. As shown in Fig. 5 (E and F), there was less Tyr-1472-phosphorylated GluN2B in PS1<sup>−/−</sup> neuronal cultures compared with WT neurons. Furthermore, the decrease in Tyr-1472-phosphorylated GluN2B was reversed following treatment of PS1<sup>−/−</sup> neuronal cultures with the NMDAR antagonist MK801 (Fig. 5G). Importantly, surface protein biotinylation experiments showed that surface GluN2B was also decreased in PS1<sup>−/−</sup> neuronal cultures compared with WT controls (Fig. 5, H and I), indicating that in the absence of PS1, there is a decrease in GluN2B-containing cell surface NMDARs.

**DISCUSSION**

Neuronal calpain is a calcium-dependent cysteine protease that cleaves many substrates, including spectrin and GluN2B, a main component of extrasynaptic NMDAR (28). It has been reported that early stages of Alzheimer disease are characterized by chronic activation of calpain, and this may contribute to the neurodegeneration of Alzheimer disease (31). Here, we have presented evidence that PS1 null cortical neuronal cultures contain increased levels of calpain-dependent SBDPs, an indication that calpain proteases are activated in the absence of neuronal PS1. NMDAR antagonists decreased the amounts of
Thus stimulating downstream effectors, including calpains. Our data showed that conditioned media from PS1 null cultures stimulated production of SBDPs in WT neurons, indicating that these media contain factor(s) able to stimulate calpain. Indeed, the conditioned media of PS1 null cultures were found to contain increased concentrations of glutamate. Furthermore, treatment of WT cultures with exogenous glutamate at concentrations similar to those found in the media of PS1 null cultures increased the SBDPs to levels similar to those found in PS1 null neurons, and NMDAR antagonists blocked accumulation of SBDPs. Together, these data show that the increased levels of SBDPs are due to the activation of NMDAR and its downstream target calpain by elevated concentrations of glutamate in the media of PS1 null neuronal cultures.

Low concentrations of memantine selectively inhibit extrasynaptic NMDAR (25). At these concentrations, memantine reduced the amounts of SBDPs in PS1−/− neurons, suggesting that calpain activation is mediated by extrasynaptic NMDAR. This conclusion is further supported by our observation that the increase in SBDPs was manifested in cultures older than 7 DIV, when GluN2B-containing NMDAR appears at extrasynaptic sites (24, 32). Together, our data indicate that calpain is chronically activated in PS1−/− neurons due to increased release of glutamate and excessive activation of extrasynaptic NMDAR probably by spillover of glutamate to extrasynaptic sites. Interestingly, activation of extrasynaptic NMDARs may lead to cell damage and has been proposed to be involved in major diseases of the CNS (11).
Accumulation of glutamate in PS1−/− media could result from increased glutamate release and/or reduced glutamate reuptake. In addition, a recent report suggests that PS1 loss of function causes a decrease in cell surface expression of the neuronal glutamate transporter EAAT (excitatory amino acid transporter) (33). We used a EAAT blocker (DL-TBOA) to investigate whether enhanced glutamate in our PS1−/− media was due to increased release or impaired reuptake of glutamate. Our data showed that DL-TBOA did not attenuate the difference in glutamate concentration between WT and PS1−/− media. Thus, we conclude that increased glutamate concentration in conditioned media from PS1 null neurons is due to enhanced release of glutamate rather than decreased uptake and degradation of the neurotransmitter. This conclusion is in agreement with recent data that neurons lacking PS1 show increased frequency of spontaneous miniature excitatory synaptic currents, suggesting enhanced neurotransmitter release (34).

Several mechanisms may be involved in the regulation of glutamate release by PS1, and these are not necessarily mutually exclusive. First, PS1 regulates calcium (35), and loss of this function could lead to increased calcium levels in the presynaptic terminal, increasing the probability of glutamate release. Second, the PS1/γ-secretase system processes presynaptic proteins such as cadherins, ephrins, neurexins, and aldehydeins (1). Inhibition of this function may impair control of neurotransmitter release at presynaptic terminals. Third, there is evidence of a PS1 role in vesicular transport (36), some of which may involve glutamate transport.

NMDAR is a heterotetramer composed of two NR1 and either two GluN2A or GluN2B subunits. At mature neuronal cultures, most synaptic NMDAR contains GluN2A, whereas extrasynaptic NMDAR contains exclusively GluN2B subunits (26). Tyrosine phosphorylation of the cytoplasmic tail of GluN2B, including phosphorylation of tyrosine 1472, is known to regulate the cell surface expression and stability of extrasynaptic NMDAR (27, 29, 30, 37). Extrasynaptic NMDAR stimulates calpain, which in turn cleaves GluN2B, thus decreasing the amounts of functional receptor and its downstream signaling. In addition, activation of NMDAR stimulates dephosphorylation of tyrosine 1472, further destabilizing functional extrasynaptic NMDAR (38). Under conditions of increased glutamate, these changes reduce the amount of functional extrasynaptic NMDAR, thus attenuating potential cell damage resulting from excessive calcium influx and signaling (28, 39). We observed that brains of PS1 null mice have decreased levels of tyrosine phosphorylation of GluN2B, and similar changes were found in primary neuronal cultures of PS1−/− neurons. This decrease may be due to a dysregulation of glutamate release in the absence of PS1 and may represent a neuronal effort to control excitotoxic damage resulting from elevated glutamate and increased calcium influx.

Because PS1 has γ-secretase-dependent and -independent functions, we sought to determine whether enhanced glutamate release in the absence of PS1 is due to loss of γ-secretase activity. Treatment of WT neuronal cultures with the selective γ-secretase inhibitor L685,458 induced changes similar to those observed in PS1 null cultures, including increased glutamate secretion, increased calpain-dependent SBDPs, and decreased levels of Tyr-1472-phosphorylated GluN2B. We conclude that PS1 regulates glutamate concentration in a γ-secretase-dependent way. Our data suggest that the PS1/γ-secretase system regulates glutamate release, thus controlling extrasynaptic NMDAR activity and protecting neuronal cells from potentially harmful excitotoxicity.

Although glutamate receptors play key roles in neuronal functions, excessive activation of these receptors results in calcium overload and increased oxidative stress that may lead to neuronal cell death (40–42). Importantly, excitotoxicity, a term used to describe neuronal damage resulting from abnormally high levels of glutamate and calcium influx, has been proposed to play crucial roles in several neurodegenerative disorders of the CNS, including Alzheimer and Parkinson diseases and stroke. It has been reported (43) that cells respond to increased concentrations of extracellular glutamate by promoting degradation of the NMDAR subunit GluN2B. It is believed this is a cellular strategy to limit damage resulting from excessive activation of NMDAR and increased calcium influx. Thus, our data that γ-secretase activity controls glutamate release support the suggestion that the PS1/γ-secretase system may contribute to neuronal survival by regulating glutamate release and limiting excitotoxicity. There has been a long debate about loss- versus gain-of-function mechanisms caused by PS mutations. However, recent evidence shows that PS1 FAD mutations cause loss of PS function, including loss of γ-secretase cleavage activity at e-sites of substrates (44, 45). These mechanisms may involve allelic interference, where inactive products of FAD mutant alleles exert trans-dominant inhibition effects by interfering with the function of the wild-type allele (46, 47). Our result that PS1 controls release of neuronal glutamate raises the possibility that PS FAD mutants interfere with presynaptic release of this neurotransmitter.

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