Persistent activation of calpain is correlated with hippocampal neuronal death in pilocarpine-treated C57BL/6N mice

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Objective: Systemic administration of pilocarpine to rodents induces status epilepticus (SE), which is followed by hippocampal damage and spontaneous recurrent seizures. In the present study, we characterized hippocampal damage after pilocarpine-induced SE in C57BL/6N mice.

Methods: C57BL/6N mice were treated with pilocarpine to induce SE, and SE was interrupted after 1.5 hours (1.5-h SE) or 4.5 hours (4.5-h SE) with phenobarbital injection. Hippocampal neurons were examined by immunohistochemical staining for the neuronal marker NeuN. We also examined the expression of N-methyl-D-aspartate (NMDA) receptor subunits in the hippocampus of C57BL/6N mice after pilocarpine-induced SE.

Results: Hippocampal neuronal death in the CA1 region was extensive 4 days after 4.5-h SE, whereas neuronal death was minimal with 1.5-h SE. Previous studies have demonstrated that GluN2A and GluN2B subunits of NMDA receptors are calpain substrates. Interestingly, we found a significant amount of C-terminal truncated GluN2A and GluN2B subunits in hippocampal lysates 1, 4, and 18 days after 4.5-h SE. Truncated GluN2 subunits were also observed in the hippocampus at 4 days after 1.5-h SE, although these levels returned to normal by 18 days after SE.

Conclusion: Elevated calpain activity persists for several days in 4.5-h SE mice and is correlated with neuronal death in the hippocampal CA1 region.

Key words: epilepsy, pilocarpine, N-methyl-D-aspartate (NMDA) receptor, calpain, cell death

Abbreviations: NMDA, N-methyl-D-aspartate; SBDP, spectrin breakdown product; SE, status epilepticus; TLE, temporal lobe epilepsy

Introduction

Temporal lobe epilepsy (TLE) is one of the most common forms of epilepsy in humans and is often associated with the loss of pyramidal neurons in the CA1, CA3, and hilus regions of the hippocampus.1-3 The pilocarpine model of epilepsy in rodents reproduces several features of TLE, including pathology, behavioral abnormalities, and spontaneous recurrent seizures.4,6 Pilocarpine administration results in status epilepticus (SE), and after a latency period, spontaneous recurrent seizures occur. Pilocarpine-induced SE causes abnormal synaptic rearrangements and neuronal death in the hippocampus and other brain regions.

Calpain is a calcium-dependent protease found in both the cytosol and synaptic terminals of neurons.7,8 Hyperactivation of N-methyl-D-aspartate (NMDA) receptors under pathological conditions such as ischemia and seizures leads to excitotoxicity due to the excessive entry of calcium leading to the activation of calpain.9,10 In addition, the NMDA receptor subunits GluN2A and GluN2B, but not GluN1, are cleaved in their C-terminal regions by calpain.11,12 Previous studies have shown that calpain plays an important role in the death of hippocampal neurons after pilocarpine-induced SE.13,14 For example, hippocampal neuronal death in pilocarpine-treated rats was observed in the CA3 region at 6 hours after SE and was sustained for 7 days.13 However, these studies did not focus on neuronal loss in the CA1 region of the hippocampus.

While the rat pilocarpine model is well characterized, corresponding mouse models have not been examined in detail, as the effects of pilocarpine on mice differ by strain. For example, several mouse strains develop more...
severe seizures and exhibit higher seizure-induced mortality than do rats. Therefore, the relationship between calpain activity and neuronal death in mouse pilocarpine models has not been fully elucidated. In the present study, we used the mouse C57BL/6N strain, in which SE was inducible by pilocarpine, and examined the relationship between calpain activation and hippocampal neuronal death in the CA1 region after SE.

Materials and Methods

Antibodies

Anti-GluN1 C-terminal rabbit antibody (05-432), anti-GluN2A C-terminal rabbit antibody (05-901R), anti-GluN2B C-terminal rabbit antibody (06-600), and anti-NeuN mouse antibody (MAB377B) were purchased from Millipore (Billerica, MA, USA). The rabbit polyclonal antibody against the extracellular region of GluN2A was purchased from GeneTex (Zeeland, MI, USA). The rabbit polyclonal antibody against the extracellular region of GluN2B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-fodrin (α-spectrin) mouse antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-actin mouse antibody (Ab-5) was purchased from BD Biosciences (San Jose, CA, USA). And the anti-p35/p25 rabbit antibody was purchased from Cell Signaling Technology (Beverly, MA, USA).

Seizure experiments

All animal experiments complied with National Institutes of Health guidelines. The study protocol was approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine. Every effort was made to minimize animal suffering. Male C57BL/6NJcl mice (8 – 10 weeks old; body weight, 20 – 35 g; CREA Japan) were subjected to pilocarpine-induced SE. In order to avoid peripheral cholinergic effects, methylscopolamine (1 mg/kg intraperitoneally [IP]) was administered 30 minutes before injecting pilocarpine (300 mg/kg IP). Control mice received methylscopolamine but no pilocarpine. SE was defined as continuous limbic seizure activity and was interrupted after 1.5 hours or 4.5 hours by injection of phenobarbital (0.13 mg/kg IP and 0.04 mg/kg IP, respectively). Control mice also received phenobarbital injections.

Immunohistochemistry

Mice were anesthetized with diethyl ether and fixed by cardiac perfusion with 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffered saline (PBS) (pH 7.4) for 25 minutes. Brains were fixed by immersion in the same fixative, equilibrated in 30% sucrose in PBS, and paraffinized. Serial sagittal sections were cut on a cryostat at 5 μm thickness. Paraffin-embedded sections were deparaffinized with xylene, rehydrated by ethanol at graded decreasing concentrations, and finally washed with water. To stain with anti-NeuN antibodies, the sections were washed with tris buffered saline tween (TBST) (150 mM NaCl, 0.2% tween 20, 20 mM tris-HCl, pH 7.5), heated in a microwave oven in 10 mM sodium citrate buffer for 10 minutes, and treated with 2 M HCl at 37°C for 30 minutes. Following rinsing in PBS and TBST, the sections were blocked by 0.5% casein for 30 minutes at room temperature, and incubated with mouse monoclonal anti-NeuN antibody (1 : 500, Millipore) at 4°C overnight. After three washes with TBST, the sections were incubated with peroxidase-labeled polymer-conjugated goat anti-mouse IgG (Histofine Simple Stain MAX-PO, Nichirei, Tokyo) for 1 hour at room temperature, and the reaction was detected with diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA, USA). The sections were then counterstained with hematoxylin.

Immunoblot analysis

The mice were euthanized 1, 4, or 18 days after 1.5 hours or 4.5 hours of pilocarpine-induced SE, and the brains were quickly removed. The hippocampi were individually homogenized in 1 × sodium dodecyl sulfate (SDS) buffer and boiled for 5 minutes. Protein concentrations were determined using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Rockford, IL, USA). Samples were separated by 5% – 20% SDS-PAGE (polyacrylamide gel electrophoresis) under reducing conditions and electrophoretically transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 5% non-fat milk with TBST for 1 hour at room temperature and then incubated in 1% non-fat milk with TBST and a primary antibody overnight at 4°C. After three 10-minute washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature in 1% non-fat milk with TBST. After several washes with TBST, proteins were visualized with enhanced chemiluminescence (Pierce) using a luminescence image analyzer with an electronically cooled charge-coupled device camera (LAS-4000 mini; GE Healthcare, Milwaukee, WI, USA).

Statistical analysis

All data are expressed as mean ± SE. One-way analysis
of variance (ANOVA) followed by the Tukey-Kramer post-test was used to compare means across groups. \( P \leq 0.05 \) was considered statistically significant.

**Results**

*Pilocarpine-induced 4.5-h SE effectively elicits CA1 pyramidal neuronal death in adult C57BL/6N mice*

Systemic administration of pilocarpine, a potent muscarinic agonist, has been extensively used to model TLE in mice and rats. In adult mice, pilocarpine injection leads to SE, followed by a chronic period when most animals exhibit spontaneous convulsive seizures typically 5 to 7 days after injection. In the present study, male C57BL/6N mice were injected with pilocarpine to induce acute seizures. SE was terminated with phenobarbital at 1.5 hours (1.5-h SE) or 4.5 hours (4.5-h SE) after seizure onset. The C57BL/6N substrain was highly susceptible to the induction of SE and had a low mortality rate after pilocarpine administration.

To examine the loss of hippocampal neurons in epileptic mice after pilocarpine injection, brain sections were stained with antibodies to the neuronal marker, NeuN, and hematoxylin counterstaining (Figure 1). Two days after pilocarpine-induced 1.5-h and 4.5-h SE, no significant losses of hippocampal neurons occurred in the CA1, CA3, or dentate regions. In contrast, the CA1 region 4 days after 4.5-h SE, but not 1.5-h SE, showed extensive death of pyramidal neurons. We also observed an increase in hematoxylin-stained nuclei in a region adjacent to the CA1 in the 4.5-h SE mice. Since the hippocampi in the 4.5-h SE mice were strongly positive for GFAP (glial fibrillary acidic protein) in thickened processes, these cells might be reactive astrocytes, which is indicative of astrogliosis.

![Figure 1](image)

**Figure 1.** Neuronal death in the hippocampus 2 or 4 days after pilocarpine-induced SE

A-C. Hippocampus 2 or 4 days after 1.5-h SE. D-F. Hippocampus 2 or 4 days after 4.5-h SE. C, F. High magnification of the CA1 region. E, F. Severe neuronal death is seen in the CA1 region, as assessed by NeuN immunohistochemistry. A, D. Mice at 2 days after SE induction show similar numbers of neurons in the CA1 and CA3 regions of the hippocampus and the granule cell layer of the dentate gyrus.
Decrease of full-length GluN2A and GluN2B subunits, but not GluN1, in pilocarpine-induced 4.5-h SE

We next examined the expression of NMDA receptor subunits in the hippocampi of C57BL/6N mice at various time points after pilocarpine-induced 4.5-h SE by immunoblot analysis (Figure 2). Actin levels for the control group were unchanged across all time points following 4.5-h SE. GluN1 levels were slightly decreased 1, 4, and 18 days after SE (86.1 ± 12.1, 80.0 ± 14.4, and 64.0 ± 17.7%, respectively, of control levels, n = 4). In contrast, expression of full-length GluN2A and 2B was significantly decreased 1 day after 4.5-h SE (53.6 ± 5.0 and 40.4 ± 4.7%, respectively, of control levels, n = 4), with greater decrease observed 4 and 18 days after 4.5-h SE.

Figure 2. Immunoblot analysis of full-length NMDA receptor expression at 1, 4, and 18 days after pilocarpine-induced 4.5-h SE using C-terminal antibodies

A. Representative blots and B. quantitative analysis of immunoblots. The expression of GluN2A and 2B was notably decreased 1, 4, and 18 days after 4.5-h SE. Although the difference was not statistically significant, GluN1 was slightly decreased 1, 4, and 18 days after 4.5-h SE. Values are presented as mean ± SE (n = 4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. *P < 0.05; **P < 0.01 compared with control animals.
Figure 3. Immunoblot analysis of C-terminal truncated GluN2 subunit expression at 1, 4, and 18 days after pilocarpine-induced 4.5-h SE using N-terminal antibodies

A. Representative blots and B. quantitative analysis of immunoblots. Immunoblotting was performed using an N-terminal antibody that recognizes GluN2A and GluN2B (both 180 kDa). Expression of full-length GluN2A and GluN2B was decreased 1, 4, and 18 days after pilocarpine-induced 4.5-h SE. Conversely, the C-terminal truncated forms of GluN2A (105 kDa) and GluN2B (110 kDa) increased after pilocarpine-induced SE. Values are presented as mean ± SE (n = 4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. *P < 0.05 compared with control animals.

Figure 4. Hippocampal spectrin and p35 expression 1, 4, and 18 days after pilocarpine-induced 4.5-h SE

A, C. Representative blots and B, D. quantitative analysis of immunoblots. The intensity of the 150/145 kDa spectrin break down products (SBDPs) significantly increased at 1 and 4 days after 4.5-h SE, but there was no significant difference observed in the 120 kDa SBDP levels. Expression of p35 was decreased 1 and 4 days after pilocarpine-induced 4.5-h SE. Values are presented as mean ± SE (n = 4). Statistical significance was determined using one-way ANOVA followed by the Tukey-Kramer post hoc test. *P < 0.05; **P < 0.01 compared with control animals.
4.5-h SE (17.6 ± 7.4 and 26.0 ± 12.2%, 24.1 ± 5.7 and 21.3 ± 8.6%, respectively, of control levels, n = 4). These results suggested that the ratio of full-length GluN2 subunits to GluN1 was significantly decreased in the hippocampi of pilocarpine-treated mice, and that the C-terminal regions of GluN2A and GluN2B were likely cleaved by calpain. Indeed, previous studies have shown that pilocarpine-induced SE can lead to calpain activation and that activated calpain can mediate hippocampal neuronal death in rats.13,14 Consistent with this, the C-terminal regions of GluN2A and GluN2B are cleaved at specific sites by calpain both in vivo and in vitro.11,12,15

Continuously present N-terminal fragments of GluN2A and GluN2B in response to pilocarpine-induced 4.5-h SE

To determine whether calpain-mediated cleavage of GluN2A and GluN2B subunits occurs in response to pilocarpine-induced 4.5-h SE, we performed immunoblot analysis using antibodies specific for the N-terminal region of GluN2A and GluN2B (Figure 3). The N-terminal GluN2A antibody detected bands of 180 kDa (full-length) and 105 kDa (cleaved fragment), and the N-terminal GluN2B antibody detected bands of 180 kDa (full-length) and 110 kDa (cleaved fragment) in control mice. Truncated GluN2A (105 kDa) and GluN2B (110 kDa) rapidly increased 1 day after 4.5-h SE (230.4 ±

Figure 5. Expression of hippocampal NMDA receptor and spectrin 4 and 18 days after pilocarpine-induced 1.5-h SE

A. The expression of GluN2A and GluN2B was transiently decreased 4 days after 1.5-h SE. However, no differences in GluN2A and GluN2B levels were observed 18 days after 1.5-h SE. B. There were no significant differences in spectrin or SBDP levels 4 days after 1.5-h SE, although 150/145 kDa spectrin was transiently increased at this time point.

163
Proteolysis of calpain substrates, cleavage persist for up to 18 days after 4.5-h SE. The N-terminal fragments resulting from calpain-mediated hippocampi of pilocarpine-treated epileptic mice, and GluN2A and GluN2B subunits are cleaved in the control levels, n = 4). These results demonstrate that pilocarpine-induced 1.5-h SE, hippocampal neuronal loss was not significant, p25 levels were increased at 4 days after 4.5-h SE (150.2 ± 14.2% of control levels, n = 4), and p25 gradually increased (116.5 ± 24.3 of control levels, n = 4). 1 day after 4.5-h SE (233.5 ± 26.4 of control levels, n = 4), no significant changes were observed for 120 kDa SBDP. As another well-known calpain substrate, we also evaluated changes in calpain-mediated proteolysis of p35, an activator of Cdk5 by p25,18,19 Hyperactivation of Cdk5 by p25 has been implicated in neurodegeneration after ischemia and kainic acid-induced seizure.19,20 As shown in Figure 4, p35 rapidly decreased (48.4 ± 5.2 of control levels, n = 4), and p25 gradually increased (116.5 ± 24.5 of control levels, n = 4), 1 day after 4.5-h SE. The toxic p25 fragment reached maximal levels 4 days after 4.5-h SE (150.2 ± 14.5 of control levels, n = 4). Although not significant, p25 levels were increased at 4 days after 4.5-h SE, which is consistent with the induction phase of pilocarpine-induced CA1 neuronal death. These results suggest that p25 generation is implicated in hippocampal neuronal loss in response to pilocarpine-induced 4.5-h SE.

Transiently activated calpain after 1.5-h SE in pilocarpine-treated mice
We next determined whether calpain-mediated cleavage of GluN2A and GluN2B subunits occurs in response to pilocarpine-induced 1.5-h SE (Figure 5). After pilocarpine-induced 1.5-h SE, hippocampal neuronal loss was minimal in the CA1 region. Moreover, full-length GluN2A and 2B were significantly decreased 4 days after 1.5-h SE (52.5 ± 9.1 and 64.0 ± 6.9% of control levels, n = 6), but recovered by 18 days after 1.5-h SE (72.6 ± 13.0 and 77.5 ± 12.7% of control levels, n = 6). Calpain-mediated truncation of GluN2A and GluN2B subunits 4 days after 1.5-h SE was also evident, although the changes were not significant.

We next assessed α-spectrin cleavage to determine whether calpain is activated in the hippocampi of C57BL/6N mice after pilocarpine-induced 1.5-h SE. Truncation 150/145 kDa SBDPs increased 4 days after 1.5-h SE (142.1 ± 24.3 of control levels, n = 6) and gradually recovered by 18 days after 1.5-h SE (113.7 ± 7.4 of control levels, n = 6). These results indicate that calpain activation is a transient phenomenon after 1.5-h SE and does not lead to hippocampal neuron loss.

Discussion
C57BL/6N mice more amenable to pilocarpine-induced SE than C57BL/6J mice
Systemic pilocarpine administration is widely used to induce SE in rodents, leading to hippocampal damage and spontaneous recurrent seizures resembling TLE. Previous studies demonstrated that the susceptibility of mice and rats to pilocarpine-induced SE differ, as does subsequent hippocampal damage and behavior. The present study focused on assessing the effects of pilocarpine-induced SE on hippocampal damage in C57BL/6 mice. To this end, SE was induced by injecting pilocarpine into two inbred C57BL/6J substrains. Interestingly, mortality after pilocarpine injection was very high in the C57BL/6J substrain but not in the C57BL/6N substrain. Genome sequence comparisons of C57BL/6J and C57BL/6N strains revealed 34 SNPs and two indels that differ between the two strains, in addition to 15 structural variants with gene overlap. These differences may account for the difference in mortality between the two strains.21 A recent study demonstrated that a strain of C57BL/6N mice from a barrier (8) of a German vendor (Charles River, Germany) was much more sensitive to SE induction than C57BL/6N mice from four other barriers,22 suggesting that slight genomic differences between mouse strains can influence the susceptibility to SE induction.

Pilocarpine-induced calpain-mediated truncation of GluN2A and GluN2B
The NMDA receptor is a Ca2+-permeable glutamate receptor channel implicated in diverse neuronal functions...
ranging from synaptic plasticity to excitotoxicity. The intracellular C-terminal domains of GluN2 subunits are required for NMDA receptor localization and function, as demonstrated in genetically modified mice expressing C-terminal truncated receptors. Hyperactivation of NMDA receptors leads to calpain activation in various contexts, including ischemia, traumatic brain injury, and epilepsy. While some studies have shown that calpain cleaves the C-terminal regions of both GluN2A and GluN2B subunits after focal cerebral ischemia in rat brains, calpain specifically targeted the GluN2B subunit, but not the GluN2A subunit, in rat brains following kainic acid-induced seizure. Our findings add to this body of literature by demonstrating that calpain also cleaves NMDA receptor subunits in the hippocampi of pilocarpine-induced epileptic mice.

**Calpain-mediated cleavage of p35, a cdk5 activator, to p25 in response to pilocarpine-induced 4.5-h SE**

A deregulated increase in intracellular calcium caused by hyperactivation of glutamate receptors likely contributes to neuronal death via activation of calcium-activated proteases such as calpain or by indirectly activating apoptotic-related caspases. Although we observed increases in calpain-specific fragments of α-spectrin (150/145 kDa) 1 day after pilocarpine-induced SE, the caspase-3-specific fragment (120 kDa) was unchanged. This suggests that the neuronal death observed in the hippocampus of C57BL/6N mice after pilocarpine-induced 4.5-h SE is caspase-independent.

Cdk5 is a member of the family of cyclin-dependent kinases, which, unlike other CDKs, is involved in the regulation of postmitotic neuronal signals and requires association with its regulatory partner, p35, to become active. In situations of deregulated calcium homeostasis, calpain cleaves p35 into p25, a more potent and long-lasting Cdk5 activator. The p25 formation induces neuronal death and is associated with neuropathology. One study demonstrated that pilocarpine induced p25 generation by 3-fold after 25 minutes of SE in mice. Although not significant, p25 levels increased 4 days after 4.5-h SE, which is consistent with the induction phase of pilocarpine-induced CA1 neuronal death in C57BL/6N mice.

**Persistent activation of calpain leading to CA1 neuronal death in C57BL/6N mice**

Calpain is transiently activated in the hippocampi after pilocarpine-induced 1.5-h SE in C57BL/6N mice (Figure 5). Yet, this transient activation did not correlate with hippocampal neuronal loss. In contrast, calpain was constitutively active after pilocarpine-induced 4.5-h SE, and this persistent activation effectively elicited CA1 pyramidal neuronal death. Although calpain has been associated with numerous cellular functions, our results raise the possibility that persistent activation of calpain could lead to hippocampal neuronal loss under several pathological conditions.

**References**


