

# The Novel Calpain Inhibitor SJA6017 Improves Functional Outcome after Delayed Administration in a Mouse Model of Diffuse Brain Injury

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## ABSTRACT

A principal mechanism of calcium-mediated neuronal injury is the activation of neutral proteases known as calpains. Proteolytic substrates for calpain include receptor and cytoskeletal proteins, signal transduction enzymes and transcription factors. Recently, calpain inhibitors have been shown to provide benefit in rat models of focal head injury and focal cerebral ischemia. The present study sought to investigate, in experiment 1, the time course of calpain-mediated cytoskeletal injury in a mouse model of diffuse head injury by measuring the 150- and 145-kDa  $\alpha$ -spectrin breakdown products (SBDP). Secondly, in experiment 2, we examined the effect of early (20 min postinjury) administration of the novel calpain inhibitor SJA6017 on functional outcome measured 24 h following injury and its effect on posttraumatic  $\alpha$ -spectrin degradation. Lastly, in experiment 3, we examined the effect of delayed (4 or 6 h postinjury) administration of SJA6017 on 24-h postinjury functional outcome. In experiment 1, isoflurane-anesthetized male CF-1 mice (18–22 g) were subjected to a 750 g-cm weight drop-induced injury and were sacrificed for SBDP analysis at postinjury times of 30 min, and 1, 2, 6, 24 and 48 h (plus sham). In experiments 2 and 3, mice were injured as described, and delivered a single tail vein injection of either SJA6017 (0.3, 1, or 3 mg/kg) or vehicle (administered immediately, 4 or 6 h postinjury [3 mg/kg]). Functional outcome was evaluated in both studies, and, in experiment 2, 24-h postinjury assessment of SBDPs was determined. Following injury, the level of SBDP 145 was significantly different from sham at 24 and 48 h in cortical and at 24 h in the hippocampal tissues and at 48 h in the striatum. Immediate postinjury administration of SJA6017 resulted in a dose-related improvement in 24-h functional outcome ( $p < 0.05$  at 3 mg/kg). Significance was maintained after a 4-h delay of the 3 mg/kg, but was lost after a 6-h delay. Despite improvement in functional outcome at 24 h, SJA6017 did not reduce spectrin breakdown in cortical or hippocampal tissues. These results support a role for calpain-mediated neuronal injury and the potential for a practical therapeutic window for calpain inhibition following traumatic brain injury. However, measurements of regional spectrin degradation may not be the most sensitive marker for determining the effects of calpain inhibition.

**Key words:** calpain activation; calpain-mediated spectrin proteolysis; neuroprotection; traumatic brain injury

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## INTRODUCTION

**D**ISRUPTION of intracellular calcium homeostasis is a key constituent in the pathophysiology of traumatic or ischemic brain injury. Following an injurious event, there is a massive posttraumatic calcium influx secondary to glutamate release and opening of glutamate receptor-associated and voltage-dependent calcium channels (for review, see McIntosh et al., 1997). Excessive intracellular calcium accumulation can lead to neuronal degeneration by (1) activation of various enzymes including proteases, kinases, phosphatases, and phospholipases (McIntosh et al., 1997; Siesjo et al., 1989; Verity, 1992), (2) induction of free radical release (Hall, 1998; Kontos, 1989), and (3) by mediating detrimental changes in gene expression (Bading et al., 1993; Rink et al., 1995). Evidence of increased calcium concentrations in models of traumatic brain injury (TBI) has been demonstrated by multiple laboratories (Fineman et al., 1993; Nadler et al., 1995; Nilsson et al., 1993; Shapira et al., 1989).

A principal mechanism of calcium-mediated neuronal injury is the activation of the neutral proteases known as calpains (Bartus, 1997; Kampfl et al., 1997). There are six distinct members of the calpain family, which can be divided into two groups—calpains that are tissue-specific and calpains that are ubiquitously expressed (Sorimachi et al., 1994). The two most recognized isoforms of calpain,  $\mu$ -calpain and m-calpain, require  $\mu$ M and mM concentrations of calcium, respectively, for activation *in vitro*. However, *in vivo* activation of both may occur at much lower calcium concentrations (Coolican et al., 1984; Nixon, 1989; Suzuki et al., 1990). Identified proteolytic substrates of calpain include receptor proteins (EGF, IP<sub>3</sub>, and estrogen receptors), calmodulin-binding proteins (G proteins, calcineurin), cytoskeletal proteins ( $\alpha$ -spectrin, microtubule-associated protein 2, neurofilaments, tau), signal transduction enzymes (phospholipase C, protein kinase C, tyrosine phosphatase IIb), and transcription factors (c-fos, c-jun, c-myc; for reviews, see Yuen et al., 1996; Kampfl et al., 1997).

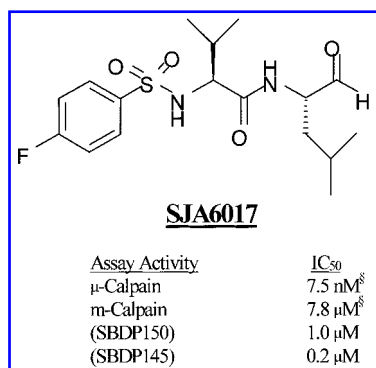
Perhaps the most studied target for calpain-mediated injury is the cytoskeleton. Indeed, several laboratories have shown that degradation of cytoskeletal proteins occurs in experimental models of stroke (Aronowski et al., 1999; Hong et al., 1994; Liebetrau et al., 1999; Roberts-Lewis et al., 1994), TBI (Buki et al., 1999; Kampfl et al., 1996; Newcomb et al., 1997; Posmantur et al., 1996; Saatman et al., 1996a), spinal cord injury (Banik et al., 1997; Braugher and Hall, 1984; Ray et al., 1999), and experimental allergic encephalomyelitis (EAE), a model of the demyelinating disease, multiple sclerosis (for review, see Shields and Banik, 1999). While damage to cytoskeletal proteins (e.g., neurofilaments, microtubule-as-

sociated protein,  $\alpha$ -spectrin) is evident within the first minutes following experimental TBI, the height of proteolytic activity appears to occur several hours after injury (Newcomb et al., 1997; Posmantur et al., 1994), and in the case of mild injury, occurs as late as several days (Saatman et al., 1998). This delayed and long sustained (Saatman et al., 1996a) degradation of the cytoskeleton may provide a practical therapeutic window for pharmacological calpain inhibition beyond the early moments after TBI.

Several pharmacological inhibitors of calpain have been identified including leupeptin, antipain, calpain inhibitor I and II, calpeptin, E64, AK295, MDL 28170, and PD150606 (an  $\alpha$ -mercaptoacrylic acid derivative; Wang et al., 1996; for reviews, see Wang and Yuen, 1994, 1998; Yuen and Wang, 1996, 1998). The approach of calpain inhibition as a neuroprotective strategy has been validated based on the reported efficacy of calpain inhibitor II (Posmantur et al., 1997a), AK295 (Bartus et al., 1994; Saatman et al., 1996a,b), and MDL 28170 (Li et al., 1998; Markgraf et al., 1998) in models of traumatic and ischemic brain injury. In the case of MDL 28170, it has been reported to reduce infarct size in rats subjected to temporary middle cerebral artery occlusion even when administered 6 h after onset of ischemia (Markgraf et al., 1998). However, the therapeutic window for calpain inhibition in TBI models has not been reported.

More recently, a new reversible peptide aldehyde inhibitor of calpain, SJA6017 (Fig. 1), has been introduced (Fukiage et al., 1997). SJA6017 is similar in many ways to others of its class (MDL 28170, calpain inhibitor I and II), yet, based on unpublished data, perhaps less cytotoxic than the MDL 28170 compound. For example, in a human neuroblastoma SH-SY5Y cell based assay which measures lactate dehydrogenase (LDH) release as a measure of cell toxicity (Posmantur et al., 1997b), the 50% lethal concentration (LC<sub>50</sub>) for SJA6017 was >500  $\mu$ M compared to 74  $\mu$ M for MDL 28170. Similar results were also seen in a rat cerebellar granular neuron based assay (Nath et al., 1998) where the LC<sub>50</sub> for SJA6017 was >500  $\mu$ M compared to 100  $\mu$ M for MDL 28170 (K.K.W. Wang, R. Nath, and M. Fields, unpublished data).

The presently reported study involved an examination of SJA6017 using a mouse model of moderately severe, diffuse head injury (Hall et al., 1995; Fig. 2). To our knowledge, this is the first time a calpain inhibitor has been studied in a model of diffuse traumatic brain injury, as well as the first to employ mice. Previous studies have been conducted in rat models of focal injury such as controlled cortical impact (Kampfl et al., 1996, 1997; Posmantur et al., 1997a) or in temporal fluid percussion (Saatman et al., 1996b; 2000). The design of our study



**FIG. 1.** Chemical structure of the calpain inhibitor SJA6017 and 50% inhibitory concentration (IC<sub>50</sub>) activity levels in relevant assays. IC<sub>50</sub> assay for SBDP 150 and SBDP 145 was done in triplicate using Molt-4 (human leukemic) cells that were preincubated 1 h with varying concentrations of SJA6017. Endogenous calpain was activated by addition of the calcium ionophore A23187, and, following incubation, the proteins were extracted and concentrations were determined. Protein samples were run on SDS/PAGE and transferred onto PVDF membranes, which were probed with anti- $\alpha$ -spectrin antibody (Chemicon), followed by a standard development protocol. Densitometric analysis of the 150- and 145-kDa bands were performed with the NIH program Image 1.5; mean values were plotted and the concentration of SJA6017 that reduced the intensity of the 150- and 145-kDa bands by 50% was determined. (<sup>§</sup>Assays referenced in Wang et al., 1996).

sought first to establish a time course of calpain-mediated cytoskeletal degradation by measuring  $\alpha$ -spectrin breakdown over the first 48 h postinjury.  $\alpha$ -Spectrin is a 280-kDa nonerythroid cytoskeletal protein that provides structural support to membranes. It is also a substrate for calpain, which can cleave  $\alpha$ -spectrin at tyrosine 1176 to yield a 150-kDa fragment (SBDP150) or at glycine 1230 to yield a 145-kDa fragment (SBDP145; Harris and Morrow, 1988). The second aim was to establish a dose-response analysis of early (20 min postinjury) administration of SJA6017 in terms of improvement in 24-h behavioral response. After definition of a dose that promoted improved functional outcome, the effect of SJA6017 on posttraumatic  $\alpha$ -spectrin degradation was examined. The third aim was to evaluate the therapeutic, posttraumatic, time window by examining the efficacy of delayed (4 or 6 h postinjury) administration of SJA6017.

## MATERIALS AND METHODS

All procedures were carried out in strict compliance with the Institutional Animal Care and Use Committee of Parke-Davis Pharmaceutical Research.

### Mouse Model of Diffuse Head Injury

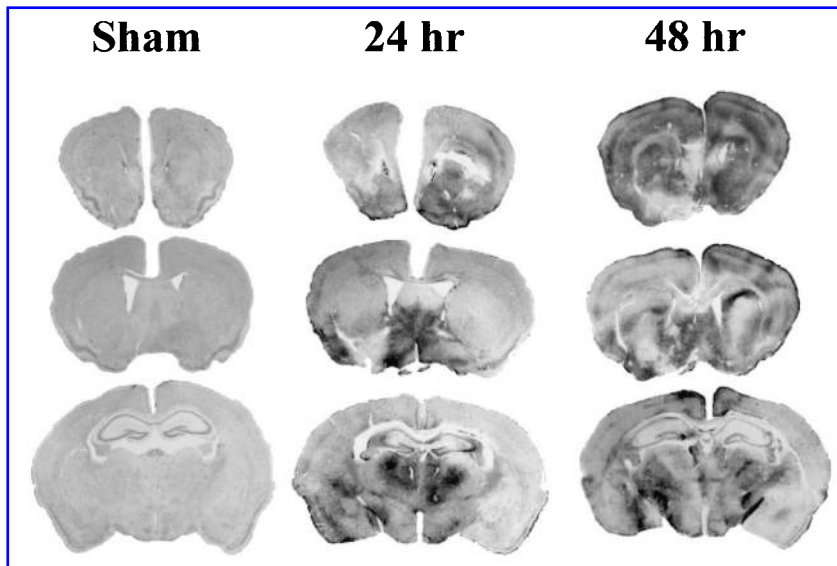
The mouse head injury method was first introduced by Hall et al. (1995) and was employed as described previously except for the addition of an anesthetic component. Male CF-1 mice (Charles River, Portage, MI), weighing 17–21 g, were fed *ad libitum* prior to injury. Mice were briefly anesthetized in a chamber containing 2.5% isoflurane (Anaquest) balanced with air and oxygen. Each mouse was grasped by the dorsal skin of the neck, and its head placed upon the base of the injury device. A round, flat, 6-mm diameter Teflon impounder was positioned firmly against the top of the head, centered between the ears and eyes, and a 50-g stainless steel weight was released at a height of 15 cm, producing a velocity calculated at 171.5 cm/sec. The resulting moderately severe injury creates a pattern of diffuse degeneration, which is diversely projected into the brain with variable intensity (Fig. 2). To prevent posttraumatic hypothermia, injured mice were placed in a Hova-Bator incubator (model 1583, Randall Burkey Co.) at 37°C until consciousness was regained (20–30 min).

### Experimental Design

Three separate experiments were performed. Experiment 1 measured  $\alpha$ -spectrin degradation in cortex, hippocampus and striatum over a 48-h postinjury time course. Experiment 2 examined the dose-response for the effect of immediate postinjury administration of SJA6017 on functional recovery and  $\alpha$ -spectrin degradation measured 24 h following injury. Experiment 3 looked at the effect of delayed administration of SJA6017 on functional recovery measured 24 h following injury.

### Western Immunoblotting of $\alpha$ -Spectrin Degradation Products

In experiment 1 and 2, spectrin degradation was quantified by Western immunoblots as previously described (Nath et al., 1996), and expressed as the ratio of 150- or 145-kDa spectrin fragment to intact spectrin. The mice were deeply anesthetized with isoflurane, killed and their brains immediately removed and briefly rinsed in chilled saline (0.9% sodium chloride for irrigation, USP). Tissues from each brain, representing cortex, striatum and hippocampus, were dissected on a chilled stage and then frozen in ice cold isopentane (–40°C) and stored at –80°C. Frozen brain tissue was powdered with pre-cooled mortar-pestle over dry ice. Approximately 50 mg of powdered tissue was resuspended in triton lysis buffer [1% triton, 20 mM TrisHCL, 150 mM NaCl, 5 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Boehringer Mannheim)]. The samples were placed on ice for 5 min with intermittent vortexing. The lysate was



**FIG. 2.** Sample time course of diffuse neuronal degeneration in male mice following diffuse head injury as measured by amino-cupric silver staining (shown in black), a marker for neuronal degeneration and debris (Fix et al., 1996; de Olmos et al., 1994; Switzer, 2000). Neurohistology was performed by NeuroScience Associates (Knoxville, TN). Brains were embedded together in a single block of geletin, freeze sectioned at  $35\ \mu\text{M}$ , and stained for degeneration using the amino cupric silver method of de Olmos et al., 1994) and counterstained with neutral red to reveal normal cell bodies. The resulting, moderately severe, injury creates a pattern of diffuse degeneration, which is diversely projected into the brain with variable intensity. The progression and degree of neuronal degeneration appears to occur over a number of days following TBI.

cleared by centrifugation at  $20,000\times g$  for 15 min at  $4^{\circ}\text{C}$ . Protein concentration in the cleared lysate was determined using a modified Lowry assay (Bio-Rad). Each lane was loaded with  $20\ \mu\text{g}$  of protein and run on SDS/PAGE [4–20% (w/v) acrylamide] with Tris/glycine running buffer system and then transferred to a nitrocellulose membrane using a semi-dry electrotransferring unit (Bio-Rad) at 20 mA for 2 h. The blots were probed with an anti- $\alpha$ -spectrin antibody (monoclonal, Affi Labs, U.K.) and developed in a linear range using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Densitometric analysis of Western blots was done using a color scanner (Umax UC630) and the NIH program, Image 1.5.

Western blot data are expressed as mean  $\pm$  SE. Injury-induced increases in the ratio of SBDP 150 or 145/intact spectrin were compared to control animals using a one-way ANOVA followed by a Bonferroni Multiple Comparisons test for selected pairs. A  $p$  value of 0.05 compared to sham was considered significant.

#### *SJA6017 Administration*

In experiment 2, beginning approximately 20 min post-TBI, 17–18 mice each were randomly assigned to one of four groups, each receiving a single tail-vein injection of SJA6017 at 0.3, 1, or 3 mg/kg or vehicle. SJA 6017 was

dissolved in 88% propylene glycol, 10% ethyl alcohol, and 2% benzyl alcohol at varying concentrations to accommodate a dosing volume of 0.05 mL per animal. In experiment 3, beginning 4 or 6 h post-TBI, 15 mice each were randomly assigned to one of two groups, each receiving a single tail-vein injection of either SJA 6017 (3 mg/kg; 60 mg/mL/kg; 0.05 mL per injection) or vehicle. SJA 6017 was prepared as described in experiment 2.

#### *Assessment of Functional Outcome*

In experiment 2 and 3, functional outcome was assessed by means of a previously described grip score (Hall, 1995) with modifications. Briefly, at 24 h post-TBI, the mice were suspended by their tails and their forepaws were placed in contact with a taut string stretched 40 cm above a padded surface. Once the forepaws were in contact, the tails were released and the mice were evaluated for (1) length of time they remained on the string (with a 30-sec maximum) and (2) their performance while on the string. Both measurements were tallied and then combined into a single grip score, with a total score of 6 representing the best measurable outcome (Table 1).

In experiment 2 and 3, data are expressed as mean  $\pm$  SE. Grip scores were compared, in study 1, by Kruskal Wallis nonparametric ANOVA and posthoc Mann-Whit-

**TABLE 1. GRIP SCORE SCALE FOR THE MEASUREMENT OF FUNCTIONAL OUTCOME IN MICE FOLLOWING TBI***Time score*

- 0 = unable to hold onto string
- 1 = hold onto string 1–10 sec
- 2 = holds onto string 11–20 sec
- 3 = holds onto string 21–30 sec

*Performance score*

- 0 = unable to hold onto string
- 1 = holds on, but unable to bring hind limb(s) to string
- 2 = holds on and draws hind limb(s) to string
- 3 = holds on and able to move along string

Time and performance scores for each mouse were combined into a single grip score with a combination of six representing highest measurable outcome.

ney test (Fig. 5A). An analysis of mice exhibiting the best measurable outcome score compared to those of partial scores was measured by  $\chi^2$  for trend (Fig. 5B). In experiment 3, grip scores were compared by a Mann-Whitney test (Fig. 7A), and an analysis of mice exhibiting the best measurable outcome score compared to those of partial scores was measured by Fisher's exact test (Fig. 7B).

**RESULTS***Time Course of  $\alpha$ -Spectrin Degradation Following Traumatic Brain Injury*

The time course of cortical  $\alpha$ -spectrin breakdown (generation of SBDP150 and SBDP145) in mice subjected to diffuse head injury is demonstrated by Western immunoblot in Figure 3. The SBDP150 and SBDP145 bands were densitometrically measured and then normalized to intact  $\alpha$ -spectrin (280 kD level), shown in Figure 4. Cortical levels of SBDP 145 were significantly different than sham at 24 and 48 h postinjury. Significant SBDP 145 increases in the striatum were present only at 24 h postinjury, while hippocampal levels of SBDP 145 reached significance by 48 h following injury. Although a similar trend towards time course increases in SBDP 150 for the three different brain regions can be appreciated, there were no significant differences in SBDP 150 levels in cortex, striatum, or hippocampus.

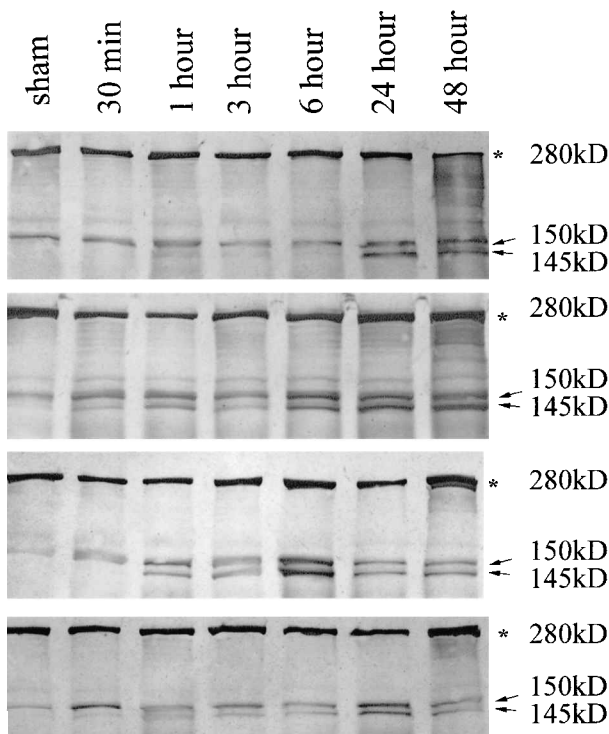
*Effect of Immediate Postinjury Administration of SJA6017 on Functional Outcome*

Figure 5A illustrates the dose-response effect of SJA6017 on functional outcome at 24 h following injury in mice that received an immediate, postinjury, tail-vein

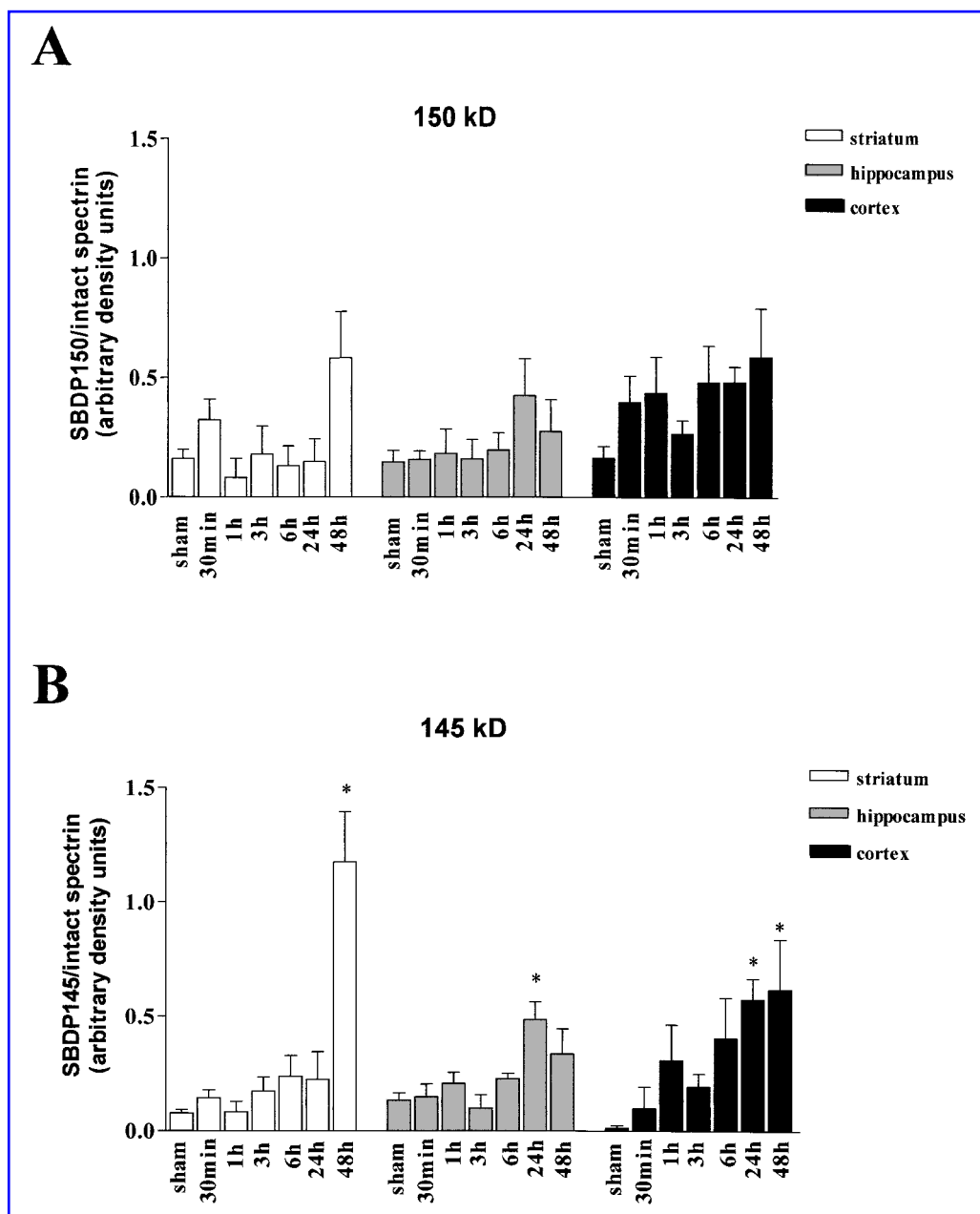
injection of SJA6017. Functional outcome was enhanced in a dose-dependent manner, with a significant improvement in grip score seen at the 3 mg/kg dose (nonparametric ANOVA Kruskal-Wallis,  $p = 0.0475$ ; Mann-Whitney test compared to sham,  $p < 0.05$ ). Similarly, drug-treated mice displayed a dose-dependent increase in the incidence of "best outcome" scores when compared to vehicle-treated mice ( $\chi^2$ ,  $p = 0.0024$ ; Fig. 5B).

*Effect of SJA6017 on Posttraumatic  $\alpha$ -Spectrin Degradation*

Cortical and hippocampal tissues were harvested for measurement of SBDPs at 24 h postinjury from the brains of mice receiving 3 mg/kg of SJA6017 in the dose response study (Fig. 5). Striatal tissues were not evaluated, based upon time course data indicating low SBDP levels 24 h following injury (Fig. 4). Figure 6 is a summary of Western immunoblots that were densitometrically measured and normalized to intact  $\alpha$ -spectrin (280-kD level). Despite a beneficial effect on functional outcome,

**Cortex (N=4)**

**FIG. 3.** Western immunoblots showing the time course of  $\alpha$ -spectrin breakdown products, SBDP150 and SBDP145, in the mouse cortex following TBI. Intact  $\alpha$ -spectrin is represented by the 280-kD band ( $n = 4$  mice/group).



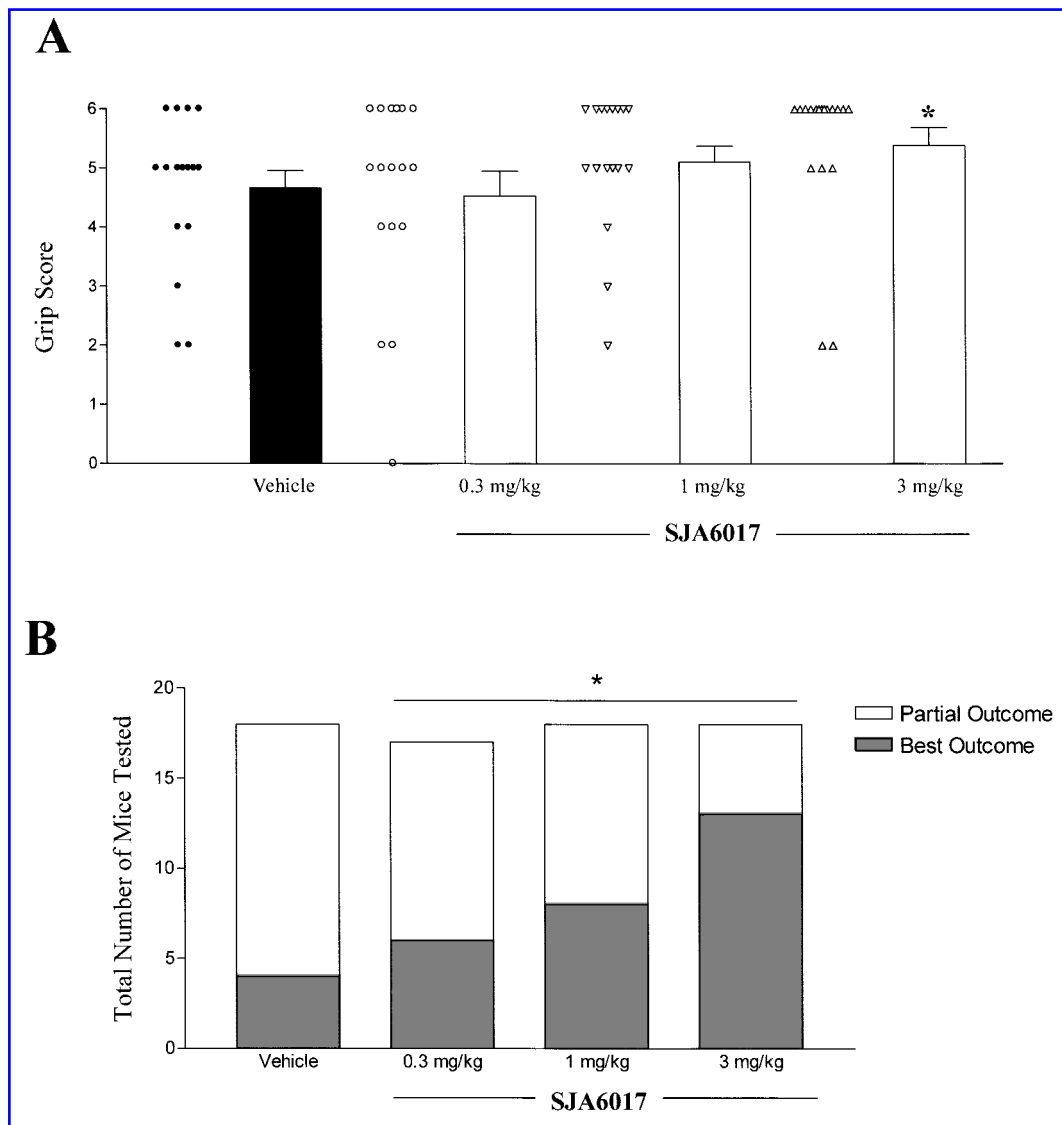
**FIG. 4.** Summary graph of the Western immunoblot time course of SBDP150 (**A**) and SBDP145 (**B**) in the mouse hippocampus, striatum, and cortex following TBI (mean  $\pm$  SE). Data is densitometrically measured and expressed as a ratio of SBDP/intact  $\alpha$ -spectrin (280 kD). \* $p < 0.05$  compared to sham ( $n = 4$  mice/group).

SJA6017 did not attenuate the levels of SBDPs in cortical or hippocampal tissue.

#### *Effect of Delayed Postinjury Administration of SJA6017 on Functional Outcome*

Twenty-four hours following head injury, mice that received a 4-h postinjury dose of 3 mg/kg SJA6017, had

significant improvement in functional outcome compared to vehicle-treated mice (Mann-Whitney test,  $p < 0.05$ ; Fig. 7A). The incidence of a head-injured mouse receiving a score of best outcome was 4.5 times greater in the drug treated mice than in time-matched, vehicle-treated mice (Fisher's Exact test,  $p < 0.01$ ; Fig. 7B). However, both of these effects were lost when SJA6017 was withheld until 6 h postinjury (Fig. 7A,B).



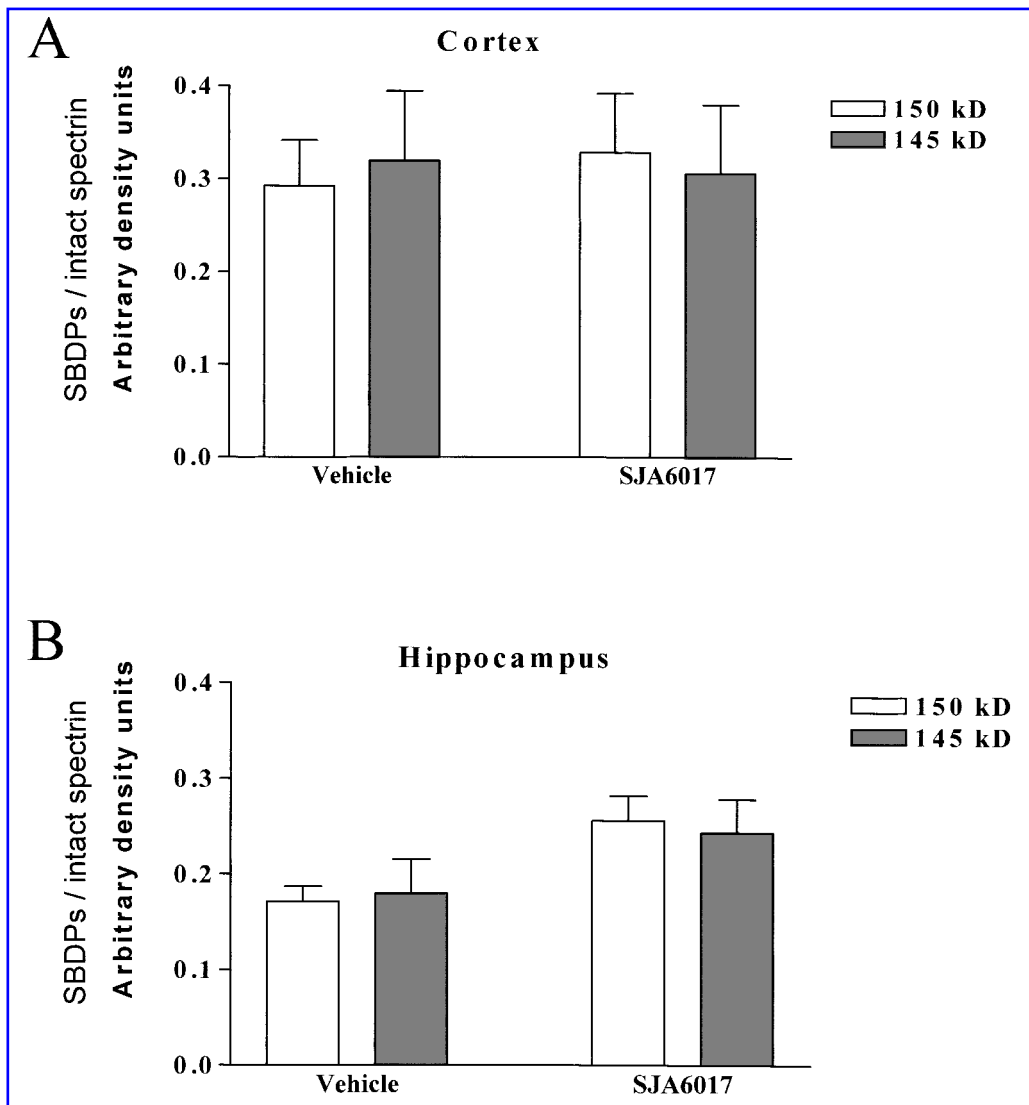
**FIG. 5.** (A) Dose response curve showing the ability of immediate postinjury administration of SJA6017 to improve functional outcome in mice 24 h following TBI (nonparametric ANOVA,  $p = 0.0475$ , mean  $\pm$  SE). \*Posthoc test,  $p < 0.05$  compared to sham ( $n = 17$ – $18$  mice/group). (B) Graph of a contingency table analysis indicating the number of mice in each treatment group exhibiting the best outcome score against those of partial scores ( $\chi^2$  for trend,  $p = 0.0024$ ).

## DISCUSSION

The approach of calpain inhibition as a therapeutic strategy for traumatic brain injury is based, in large part, upon the desire to impede the occurrence of delayed axonal injury resulting from the calcium-mediated cascade of events following an injurious insult to the brain (Buki et al., 1999; Povlishock et al., 1999). Calcium-mediated cytoskeletal damage can lead to disruption in axonal transport, structural collapse and subsequent retrograde neuronal cell death. However, calpain activation and cytoskeletal degradation also occurs in neuronal cell bodies and dendrites. In either event, antibodies recognizing

the breakdown products of ( $\alpha$ -spectrin, a substrate for calpain-mediated proteolysis, have been used as a tool to identify cytoskeletal damage associated with calpain activation (Kampfl et al., 1996; Posmantur et al., 1997a; Roberts-Lewis and Siman, 1993). In models of focal brain injury, these antibodies have been employed to establish the early presence of SBDP in areas of primary tissue injury and, at later postinjury time points, have also provided evidence for SBDP in areas of the brain distal from primary focal insult (Saatman et al., 1996a).

Utilizing a model of diffuse brain injury, we, too, report significant increases in SBDP, but only at time points beginning 24–48 h postinjury, likened to the later, sec-

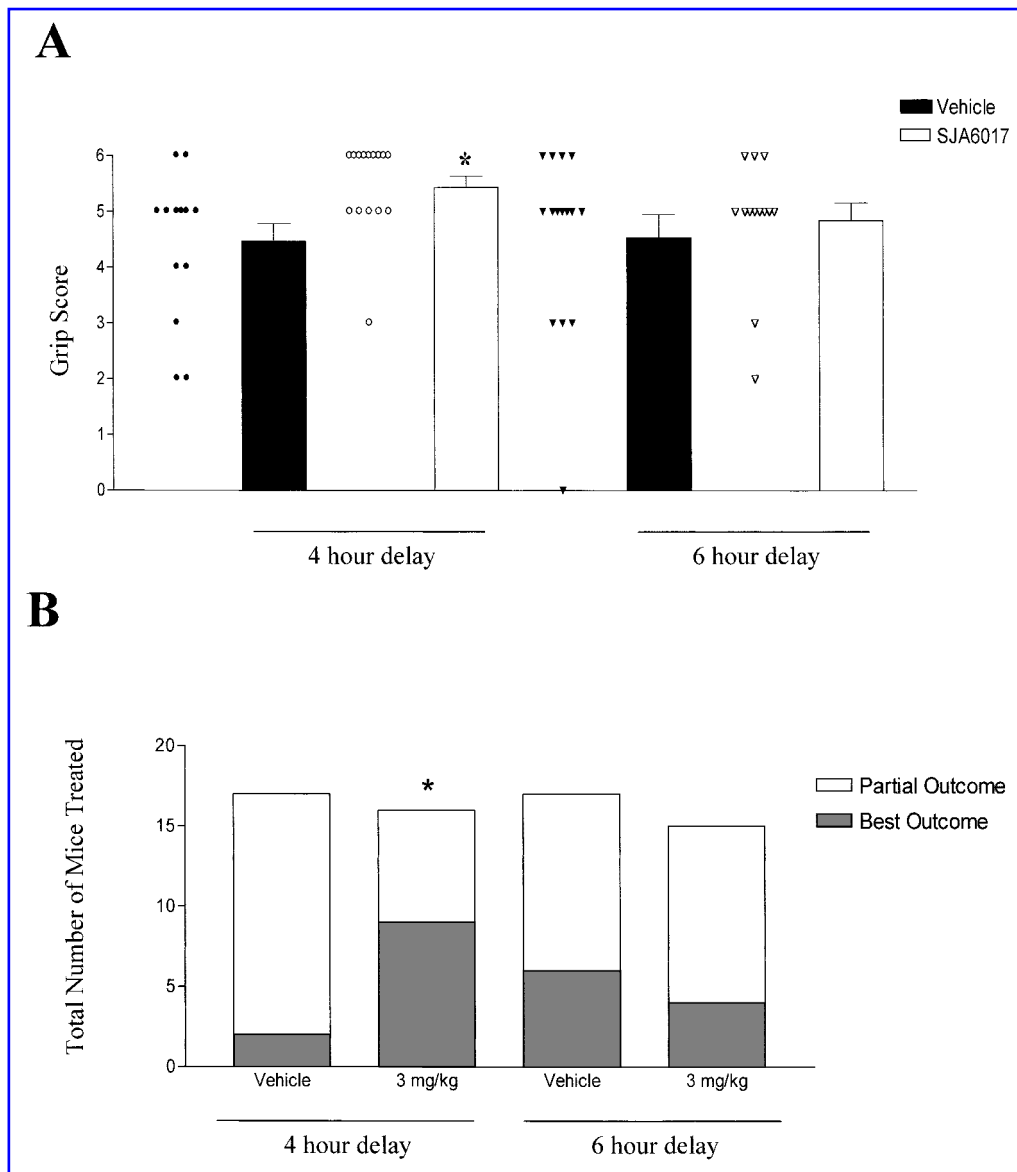


**FIG. 6.** Summary graph of Western immunoblots measuring SBDP150 and SBDP145 in TBI mouse cortex (A) and hippocampus (B) 24 h after receiving 3 mg/kg of SJA6017 immediately postinjury (mean  $\pm$  SE). Data is densitometrically measured and expressed as a ratio of SBDP/intact  $\alpha$ -spectrin (280 kD;  $n = 13$  mice/group).

ondary phase of calpain-mediated spectrin proteolysis seen in focal brain injury models. Povlishock and colleagues (Buki et al., 1999), employing a similar model of diffuse brain injury in rats, have provided compelling evidence (via ultrastructural, immunohistochemical examination with antibodies to SBDP and RMO-14, a marker for neurofilament compaction) for delayed, progressive calpain-mediated spectrin proteolysis. In the clinical setting, there is also evidence to support secondary axonal injury, which is believed to occur over a period of 12 or more hours (Blumbergs et al., 1995; Christman et al., 1994; Gentleman et al., 1993; Grady et al., 1993). McCracken and colleagues (McCracken et al.,

1999) have recently described SBDPs and neurofilament protein degradation in the corpus callosum of patients suffering blunt head injury at time points greater than 24 h following injury (compared to control subjects), furthering the suggestion that calpain-mediated damage to the cytoskeleton is a likely contributor to axonal injury in human head trauma. The reported efficacy of SJA6017 in our present study is believed to be the first demonstration of a calpain inhibitor having beneficial effects in a primarily diffuse injury paradigm. Significant improvement in functional outcome was demonstrated following immediate post-injury administration, as well as after a 4-h delay in treatment. This type of therapeutic





**FIG. 7.** (A) Graph showing improved functional outcome 24 h following TBI in mice receiving a 4-h delayed, postinjury, tail-vein injection of SJA6017 (3 mg/kg), but no improvement in outcome following a 6-h delayed administration of SJA6017 (mean  $\pm$  SE). \* $p < 0.05$  compared to time-matched vehicle control ( $n = 15$ – $16$  mice/group). (B) Graph of a contingency table analysis indicating the number of mice in each treatment group exhibiting the best outcome score against those of partial scores. \* $p < 0.05$  between the 4-h delayed vehicle and drug-treated groups (Fisher's exact test).

window has been demonstrated in a model of focal cerebral ischemia where 6-h delayed administration of the calpain inhibitor MDL 28170 was shown to be protective (Markgraf et al., 1998).

Interestingly, the beneficial behavioral effects of SJA6017, measured 24 h postinjury, were not accompanied by a quantifiable effect on  $\alpha$ -spectrin degradation in cortical and hippocampal regions.  $\alpha$ -Spectrin degradation was equal for both treated and untreated brain-injured mice, despite significantly different behavioral outcome

profiles for the same two treatment groups. One possible explanation for not seeing lower levels of SBDPs in the TBI mice receiving SJA6017 might be that the 24 h postinjury time point chosen may not be the most sensitive for seeing significant differences in SBDPs. It may be that postinjury time points of 48 h or later may provide better reflections of calpain inhibition. An additional issue to consider is the choice of brain regions in which SBDPs were measured. Because of the diffuse and uneven nature of neuronal degeneration in the cortex, hip-

pocampus and striatum in our model, the western immunoblot analysis of calpain-mediated cytoskeletal damage may not be sensitive enough to detect the early inhibition of  $\alpha$ -spectrin degradation when measured in large portions of these brain regions. In other words, drug effects on axonal cytoskeletal preservation may be simply diluted out.

The lack of effect of SJA6017 on SBDP inhibition at doses that promote functional improvement is not unprecedented. Saatman and colleagues have recently demonstrated a similar finding with the calpain inhibitor AK295 (Saatman et al., 2000). While AK295 was previously shown to attenuate motor and cognitive deficits following lateral fluid percussion brain injury in rats (Saatman et al., 1996b), in separately treated animals, there was no evidence that doses of AK295 which enhanced behavioral recovery could reduce calpain-mediated spectrin proteolysis or overt cortical damage (Saatman et al., 2000). These investigators have postulated that this lack of quantifiable SBDP effect, despite positive functional outcome, might be due to multiple explanations. First of all, calpain inhibition may be occurring in regions within the brain that were beyond the capacity of their present study. Secondly, because calpain plays a role in many intracellular events, it is quite possible that the behavioral efficacy noted might be a result of the protection of calpain proteolytic substrates other than the more commonly measured cytoskeletal break down products. These alternative targets for calpain include receptor and calcium-binding proteins, signal transduction enzymes and/or transcription factors (Kampf et al., 1997; Yuen et al., 1996).

As a final point, it should be noted that caspase 3 activation, in addition to that of calpain, can contribute to ( $\alpha$ -spectrin proteolysis, although caspase 3 activity only generates the 150-, but not the 145-kD band (Wang et al., 2000). Thus, in the case of the time-related increase in the 150-kD SBDP, caspase 3 and calpain may both play a degradative role. However, by 24-h, at which time our behavioral outcome assessment was made, the increase in the 145-kD SBDP is quantitatively much more convincing than that of the 150-kD fragment. Therefore, at least during the first 24 h postinjury, it seems likely that calpain activation is quantitatively more important than caspase 3. Moreover, SJA6017 does not inhibit caspase 3 or, for that matter, caspase 1 (i.e., interleukin-converting enzyme; a.k.a. ICE), and it does not possess any hypothermic effects (Senju Pharmaceuticals, Ltd., unpublished data). These facts, taken together, strongly support the conclusion that the beneficial effects of SJA6017 on 24-h behavioral outcome are, almost certainly, related to calpain inhibition, despite the lack of a demonstrable decrease in posttraumatic spectrin degradation at that time point.

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