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ALLOPREGNANOLONE AND PROGESTERONE DECREASE CELL DEATH AND COGNITIVE DEFICITS AFTER A CONTUSION OF THE RAT PRE-FRONTAL CORTEX

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Abstract—We compared the effects of three different doses of allopregnanolone (4, 8 or 16 mg/kg), a metabolite of progesterone, to progesterone (16 mg/kg) in adult rats with controlled cortical impact to the pre-frontal cortex. Injections were given 1 h, 6 h and every day for 5 consecutive days after the injury. One day after injury, both progesterone-treated (16 mg/kg) and allopregnanolone (8 or 16 mg/kg)-treated rats showed less caspase-3 activity, and rats treated with allopregnanolone (16 mg/kg) showed less DNA fragmentation in the lesion area, indicating reduced apoptosis. Nineteen days after the injury, rats treated with progesterone and allopregnanolone (8 or 16 mg/kg) showed no difference in necrotic cavity size but had less cell loss in the medio-dorsal nucleus of the thalamus and less learning and memory impairments compared with the injured vehicle-treated rats. On that same day the injured rats treated with progesterone showed more weight gain compared with the injured rats treated with the vehicle. These results can be taken to show that progesterone and allopregnanolone have similar neuroprotective effects after traumatic brain injury, but allopregnanolone appears to be more potent than progesterone in facilitating CNS repair.

Key words: traumatic brain injury, neurosteroids, apoptosis, neuroprotection, caspase-3, memory deficits.

Traumatic brain injury (TBI) is one of the leading causes of death in people less than 44 years of age. In the United States alone, two million individuals suffer from TBI each year and 5.3 million are seriously disabled because of their injuries. Despite these statistics, there is still no effective clinical treatment to counteract the secondary neurodegenerative processes caused by TBI. In less than 10 years, over 300 articles have reported that apoptotic death occurs after brain injury (Shah et al., 1997; Sullivan et al., 2002; Wennersten et al., 2003). The first article reporting apoptotic as well as necrotic cell death after a TBI was published 8 years ago in the rat (Rink et al., 1995). Apoptotic DNA fragmentation generally occurs in two phases: the first phase occurs hours after an injury, reaching a peak at 10 h and lasting over 72 h (Pravdenkova et al., 1996) the second phase peaks at 4–7 days and lasts for about 2 weeks (Sinson et al., 1997), depending on the region injured and the injury model used.

Anti-apoptotic agents such as neurotrophins or inhibitors of caspases have been shown to reduce cognitive deficits observed after brain injury, suggesting that apoptotic cell loss contributes to these cognitive deficits (Sinson et al., 1997). The increase of caspase-3 expression after a brain injury has also been documented (Clark et al., 1999; Yakovlev et al., 2001). Inhibitors of caspase-3 are able to decrease the apoptotic process after TBI (Yakovlev et al., 1997, Yang et al., 2002). Caspase-3 involvement in this process leads to cleavage of DNA repair enzymes (e.g. poly-ADP-ribose polymerase; Endres et al., 1997; Liu et al., 2000) and activation of endonuclease enzymes (Clark et al., 2000).

Over the last decade, steroid hormones have been shown to influence the survival of neurons, the growth of neurites and the formation of synaptic connections from early development to the plastic changes seen in the adult nervous system (Garcia-Segura et al., 1999; McEwen, 1999). In particular, after injury or disease, steroids exert protective effects on neurons and glial cells and promote neuroregenerative processes (De Nicola, 1993; Compagnone et al., 1995; Schumacher et al., 2000).

There are also gender differences in brain injury outcomes produced by hormonal states. For example, it has been shown that, after damage to the frontal cortex, female rats have less edema and fewer functional deficits than males (Attella et al., 1987; Roof et al., 1993; Alkayed et al., 1998; Roof and Stein, 1999). Progesterone (PROG), a steroid synthesized in the ovaries but also in the brain, can have a variety of neuroprotective effects in the central or peripheral nervous system. Over the last few years, PROG has been reported to protect against glutamate toxicity in cultures of spinal cord neurons (Ogata et al., 1993), to down-regulate reactive gliosis and astrocyte proliferation after a penetrating brain injury in rats (Garcia-Estrada et al., 1993), and to increase axon myelination after a cryolesion of the sciatic nerve in male mice (Koenig et al., 1995). PROG also reduces neuronal cell loss (Roof et al., 1994; Shear et al., 2002), restores neuronal membrane potential, increases ion transport and nutrient uptake through its stimulatory effects on Na, K-ATPase subunits (Gonzalez

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Abbreviations: ALLOP, allopregnanolone; ANOVA, analysis of variance; CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; MDN, mediodorsal nucleus of the thalamus; MWM, Morris water maze; NbM, nucleus basalis magnocellularis; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PF, paraformaldehyde in phosphate-buffered saline; PROG, progesterone; TBI, traumatic brain injury; TUNEL, TαT-mediated x-dUTP nick end labeling.

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Deniselle et al., 2002), and accelerates reparative responses to injury by increasing the expression of growth-associated protein (GAP-43) after an acute spinal cord transection injury in rats (Labombarda et al., 2002).

The word "neurosteroid" was first introduced in 1985 when steroids like PROG and their production enzymes were detected in different parts of the brain (Corpechot et al., 1985; Jung-Testas et al., 1989, 1999; Baulieu and Robel, 1990). The mechanism of action of neurosteroids involves two kinds of responses: a rapid one through membrane or surface effects, and a slower one mediated by cytosolic receptors activating traditional genomic expression.

The fast steroid responses involve the modulation of neurotransmitter receptor activity, in particular the type A of GABA, the *N*-methyl-D-aspartate (NMDA) and the δ1 receptors, leading to a decrease of excitotoxicity. Our laboratory has previously shown that, following TBI in both male and female rats, PROG injections reduce brain edema (Roof et al., 1996), reduce secondary neuronal loss (Roof et al., 1994), reduce lipid peroxidation (Roof et al., 1997), increase blood–brain barrier integrity (Duvdevani et al., 1995) and enhance cognitive recovery (Roof et al., 1994). We also found that better cognitive and sensory recovery is obtained after an injury to the medial pre-frontal cortex, when 5 days instead of 3 days of post-injury PROG injections are provided (Shear et al., 2002). As a result of these and related findings, PROG is currently being tested in a phase II clinical trial in humans with moderate to severe, blunt TBI.

Allopregnanolone (ALLOP), a PROG metabolite, is also known to be a potent endogenous positive modulator of central nervous system (CNS) receptor functions (Paul and Purdy, 1992; Orchinik et al., 1994). We recently reported that injections of ALLOP at 4 mg/kg reduce memory deficits and stem the loss of cholinergic neurons in rats given bilateral damage to the pre-frontal cortex. In this latter experiment, animals began testing 7 days after the last injection of the hormone. ALLOP, as a metabolite of PROG in the brain, could be the molecule through which PROG acts to induce its neuroprotective effects.

In order to clarify this question, and to understand better how PROG and ALLOP act to protect brain neurons and improve behavioral deficits after a TBI, we compared the effects of these hormones in male rats with injury to the pre-frontal cortex. We examined the effects of these treatments on cognitive performance, and correlated, in the same animals, the functional outcome to necrotic cavity size, spared cells and weight loss.

EXPERIMENTAL PROCEDURES

Animals

A total of 48 adult male Sprague-Dawley rats weighing approximately 280–350 g served as subjects. All procedures involving animals conformed to guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences, 1996) and were approved by the Emory University Institutional Animal Care and Use Committee (IACUC of Emory University protocol 101–99). Approved protocols meeting NIH guidelines require that investigators take all necessary steps to minimize the number of animals needed for statistical analyses and to minimize any unnecessary pain and discomfort caused by the experimental procedures. The rats were handled for at least 5 days before surgery, and were individually housed. Food and water were provided *ad libitum* throughout the experiment and the animals lived in a reversed 12 h light/dark cycle controlled environment.

Surgery

Rats were placed under deep anesthesia using gas containing a mix of isoflurane (5%; Novaplus, Irving, TX, USA or Abbott Lab, North Chicago, IL, USA)/N_2O (700 ml/min)/O_2 (300 ml/min). Isoflurane level was reduced to 2.5% after approximately 5 min and then under aseptic conditions, a midline incision was made in the scalp and the fascia was retracted to expose the cranium, after which a craniotomy was made with a high-speed drill (5 mm circular). Contusions were made immediately anterior to bregma (measured from the skull) with a circular 5-mm stainless impactor attached to a piston activated with compressed air (Sutton et al., 1993). The pneumatic piston was mounted to the carrier of a stereotaxic apparatus.

To create the injury, the tip of the piston was first gently placed on bregma to fix the extent of piston incursion into the cortex. The height of the tip was recorded so that upon contact, the piston would compress the cortex to a depth of 2 mm. Impact was made with a velocity of 2.25 m/s, and duration of the contact with the brain was 500 ms. After impact, cortical surface hemorrhaging was stopped and the fascia and scalp sutured. Sham-operated rats (SV) were not submitted to craniotomy and lesion in this experiment because previous work showed no detrimental effect of this manipulation on Morris water maze (MWM) behavior. Using a SurgiVet (model V3304; Waukesha, WI, USA) pulse oximeter, blood SpO_2 was monitored and maintained at levels ≥90%. Body temperature was maintained at 37 °C with a homeothermic heating blanket system (Harvard Apparatus, South Natick, MA, USA).

Neurosteroid administration

Rats were randomly assigned to the different hormone treatments. In the short-term survival condition ($N=3$/group, total of 18 rats), the animals received injections at 1 h and 6 h after the injury and in the long-term survival condition ($N=5$/group, total of 30 rats), the rats received the same two injections, plus additional injections every day after the injury for 5 consecutive days. The injections were made as follows: the sham (SV) group received only vehicle (β-cyclodextrin) and injured rats received vehicle (LV), PROG (16 mg/kg), or the three doses of ALLOP (4, 8 or 16 mg/kg, respectively LA4, LA8 and LA16). The dose used for the PROG injections was based on previous results comparing different doses of PROG and showing that 16 mg/kg of PROG in 2-hydroxypropyl-β-cyclodextrin was more efficient than 8 or 32 mg/kg to reduce brain edema and to improve cognitive outcome (Goss et al., 2003; Wright et al., 2001).

PROG (Sigma; P-0130; St. Louis, MO, USA) and ALLOP (Calbiochem; 127100; San Diego, CA, USA) were dissolved in 22.5% β-cyclodextrin solution (Sigma; C4767). The first injections were given i.p. to ensure rapid absorption, and the subsequent injections were given subcutaneously for more gradual absorption. Animals were killed at two different times after injury for tissue assays (24 h or 21 days).

Histology and tissue assays

The rats were deeply anesthetized with an overdose (75 mg/kg) of Nembutal sodium solution (NDC-0074-3778-04; Abbott Laboratories). For Thionin staining, they were perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH=7.4) followed by 4% paraformaldehyde in PBS (PF) and then decapitated. Their brains

were removed and placed at 4 °C overnight in a solution of 4% PF. Fifty-micron-thick brain slices were then cut with a vibratome. For caspase-3 activity and DNA fragmentation assays, animals were decapitated after deep anesthesia, the brains rapidly removed from the cranium and put on a cold plate (approximately 0 °C). The lesion site (5 mm of thickness directly anterior to bregma) and a region distant from the lesion site (5 mm thickness and 2 mm posterior to bregma) were cut, rapidly submerged in cold 2-methyl-butane, then stored at −20 °C until the day of quantification of caspase-3 activity or DNA fragmentation.

Caspase-3 quantification

A caspase-3 colorimetric assay (R&D Systems, Minneapolis, MN, USA; BF3100) was used to detect the activity of the caspase-3 enzyme. Briefly, the samples obtained as described in the histology and sample assay section were slowly thawed, kept at 4 °C for about 15 min, and then centrifuged for 1 min at 10,000×*g*. The pellet was discarded and the cytoplasmic solution (supernatant) transferred to an Eppendorff tube. Fifty microliters of this solution (about 200 μg of total protein) were transferred to an enzyme-linked immunosorbent assay (ELISA) plate well with the same volume of reaction buffer/Dithiotreitol. Five microliters of caspase-3 substrate (DEVD-pNA) were then added, and the whole solution was gently shaken at 300 r.p.m. The ELISA plate was then heated for 1–2 h at 37 °C for the enzymatic reaction. A negative control was obtained by replacing, in the previous steps, the tissue sample with the same volume of lysis buffer. A positive control was obtained by replacing the sample with a recombinant caspase-3 (R&D; catalog no. 707-C3). The samples were placed in duplicate in the wells of the ELISA plate, and the colorimetric reaction was read at 405 nm on a Spectra reader (SLT Spectra reader 00252785; SLT Labinstrument, Salzburg, Austria) at the end of the reaction. To compare results between samples, we determined protein concentration for each sample (Protein BCA assay kit 23227; Pierce, Rockford, IL, USA), and applied a correction factor to obtain values corresponding to the same protein amount in each sample.

DNA fragmentation quantification

A cell death detection ELISA Plus photometric kit (Roche Molecular Biochemicals, Nutley, NJ, USA; 1774425) was used as described above. Briefly, the samples were slowly thawed and centrifuged for 10 min at 200×*g*. The supernatants were carefully placed in Eppendorff tubes, and 20 μl of this solution was then put in a well of a streptavidin-coated ELISA plate. Positive and negative controls were obtained using respectively the same volume of a positive control (given in the kit) and the same volume of the lysis buffer instead of the sample. Then to each well we added 80 μl of an immunoreactive solution containing a biotinylated anti-histone and a peroxidase-coupled anti-DNA antibody. The plate was then covered and remained for 2 h at room temperature under gentle shaking at 350 r.p.m. The excess of solution was then carefully removed from the wells by gentle adsorption, rinsed three times with 250 μl of incubation buffer, and then 100 μl of ABTS peroxidase substrate was added. After 10–20 min color reaction under gentle shaking, the plate was read in a Spectra reader at 405 nm under ($\lambda_{reference}$=492 nm). To compare the results between samples, we determined protein concentration in each sample (Protein BCA assay kit 23227; Pierce), and we corrected each value by a protein amount factor, to obtain values corresponding to the same protein amount between each sample.

Cell counts

Standard Thionin staining was performed on fixed sections mounted on glass slides. Neurons from the mediodorsal nucleus (MDN) of the thalamus were located under light microscope at ×10 magnification on sections cut at −2.8 mm posterior from bregma. MDN neurons were then counted under the ×100 immersion oil objective. Each slide was coded to be blind to the treatment the animals received. Eight consecutive areas of a total area of 767 μm width and 285 μm height were chosen in the MDN region at ×1000 magnification. Spared neurons were distinguished as previously described (Djebaili et al., 2001). Briefly, spared cells were distinguished as rounded, with a clear cytoplasm with no condensed chromatin or organelles. The counts from the eight areas were summed for each section and averaged between each distinct group at the end of the counting.

Lesion reconstruction

Nineteen days after injury, the rats were perfused as previously described, and Thionin-stained, coded sections were analyzed under light microscope at ×1 magnification. Five sections for each rat brain (4.7, 3.7, 2.7, 1.7 and 0.7 mm anterior to bregma) were chosen according to the atlas of Paxinos and Watson (1986). These sections were viewed under a micro-projector (Fig. 1), and the perimeter of the lesion as well as the remaining tissue were traced on a computer-interfaced, digitizing pad, and quantified (Image Pro system, Media Cybernetics, Silver Spring, MD, USA) for statistical analysis. The five measures obtained were expressed as a ratio to the remaining section volume, and averaged together in order to obtain an estimation of the cavity size per remaining tissue. To maintain consistency for behavioral analyses, any rat with a lesion that averaged less or more than 45% of total section size was eliminated. One rat was eliminated on this basis.

Memory and learning performance

The MWM was used to evaluate the effects of PROG and ALLOP on functional recovery (Roof et al., 1994; Shear et al., 2002). Performance was evaluated in a 135 cm diameter white plastic pool located in a room with numerous extra-maze visual cues such as doors, tables, and shelves. The maze was filled with water at approximately 21 °C and made opaque by adding white, non-toxic water-soluble paint. The "safe" platform consisted of a 12×12 cm white Plexiglass sheet submerged 1.5 cm below the surface of the water. An overhead camera and computer-assisted tracking system (San Diego Instruments Inc., San Diego, CA, USA) recorded the rat's position in the maze, swim distance, strategy (distances swum in the inner and in the outer part of the maze) and time taken to find the platform (latency). Seven days following injury, all rats were tested for acquisition in the MWM. Each rat received two trials/day in two 5-day blocks, for a total of 20 trials. A trial consisted of placing the rats in the pool at one of four equally spaced starting positions (N, S, E, W) in a randomly selected order and with the rats facing the wall at the beginning of the trial. The rats were allowed to swim in the pool until they reached the platform located in the southwest quadrant of the tank, or until 90 s had elapsed. The experimenter guided the rats to the platform if the rats were unable to find it in 90 s. Rats stayed for 20 s on the platform, and were then removed from the pool for 30 s. The second trial began from a new pool position under the same time constraints. Latency, swimming strategy (ratio between distances swum in the inner part and in the perimeter of the maze), speed, and distance to reach the platform were recorded.

Statistical analysis

All results are expressed as mean±S.E.M. The data were tested for normality and homoscedasticity before being analyzed by either non-parametric (Mann-Whitney U) tests when the *N* was 3/group (caspase-3 and DNA fragmentation results), parametric two-tailed, one-way, analysis of variance (ANOVA), or parametric two-tailed, repeated measures, ANOVA. Outliers (statistically defined as cases

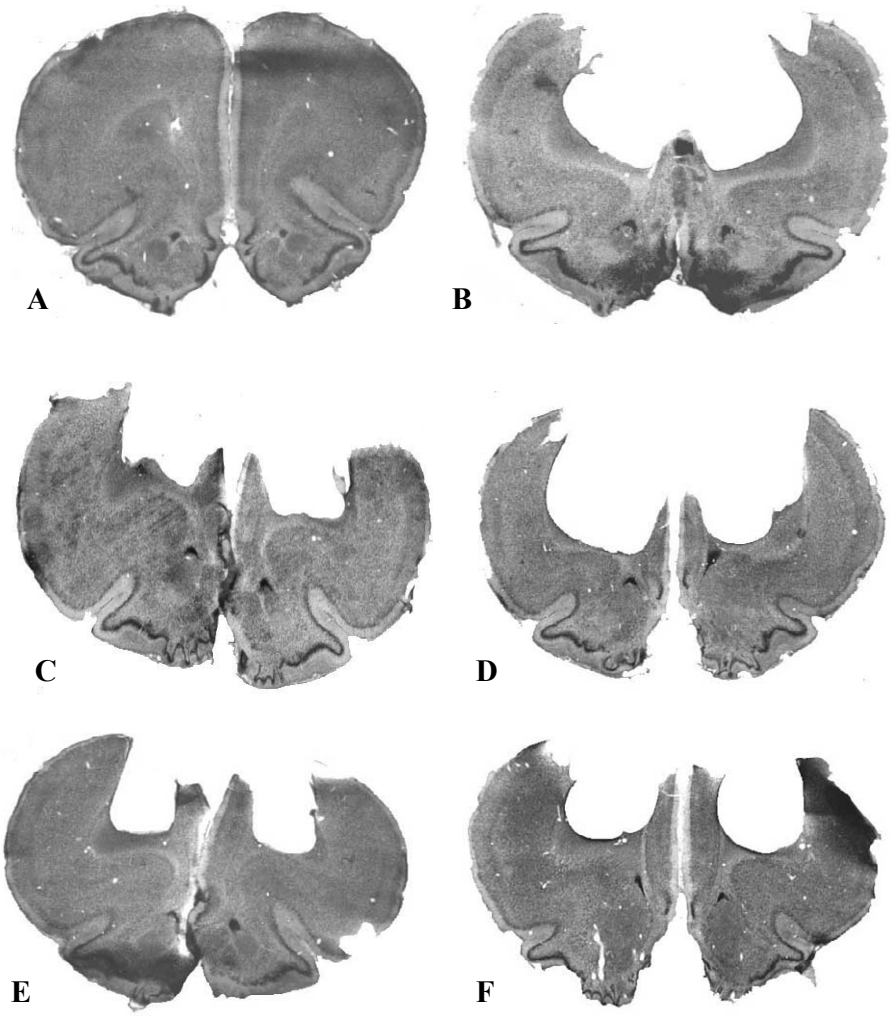

Fig. 1. Typical necrotic cavity 19 days after injury (B–F) compared with a sham brain (A). Brain slices from a sham rat (A) and from injured animals injected with vehicle (B), PROG (C), ALLOP (4 mg/kg) (D), ALLOP (8 mg/kg) (E) and ALLOP (16 mg/kg) (F) on sections taken 2.7 mm anterior to bregma.

in each group with values between 1.5 and 3 interquartile range (box) lengths from the upper or lower edge of the box) and extremes (statistically defined as cases in each group with values more than three box lengths from the upper or lower edge of the box) were removed from the data before individual comparisons were made. Differences among the groups were evaluated using a Fisher post-hoc test when homogeneity of the variances between groups was observed (Levene test). When the homogeneity of variance was not observed, a Games-Howell post-hoc test was performed or a logarithm transformation of the data was made in order to obtain homogeneity of variance. The criterion chosen for rejecting the null hypothesis was set at $P<0.05$. Correlations between each measurement were determined using a bivariate, two-tailed test and Pearson's correlation factor (r). The calculations were obtained using SPSS 11.0 software.

RESULTS

Short-term effects of neurosteroids after two injections at 1 h and 6 h post-injury

Neurosteroid effect on caspase-3 activity at the lesion site. Twenty-four hours after injury, logarithm transformation of caspase-3 activity data showed a significant difference among groups ($F_{(5,35)}=3.583$, $P<0.05$) and injured rats given vehicle or ALLOP (4 mg/kg) were the only ones to show significantly higher caspase-3 activity compared with shams ($P<0.05$; Table 1).

Table 1. Caspase-3 activity in the lesion site 24 h after injury[a]

Caspase-3 activity	Mean	S.E.M.
SV	100	13.58
LV	182.72*	35.8
LP	148.89	15.17
LA4	195.06*	26.012
LA8	126.91	14.2
LA16	127.9	4.94

[a] Logarithm transformation of caspase-3 activity data show that only injured rats given vehicle or ALLOP 4 mg/kg (LA4) had significantly higher caspase-3 activity compared to shams. Results are represented as percent change compared to shams (SV).
* $P<0.05$ compared to shams.

Table 2. DNA fragmentation 24 hours after injury[a]

DNA fragmentation	Mean	S.E.M.
SV	100	17.06
LV	137.54*	13.65
LP	158.36	23.89
LA4	81.91	8.53
LA8	94.88	20.82
LA16	68.26#	11.26

[a] There is no lesion effect on the DNA fragmentation between injured rats given vehicle (LV) and shams (SV) but injured rats given ALLOP (16 mg/kg; LA16) showed significantly higher DNA fragmentation compared to LV. Results are represented as percent change compared to shams (SV).
* Significantly different from LV.
# $P<0.05$ compared to LV.

Neurosteroid effect on DNA fragmentation. One day after the injury, post hoc tests failed to show a significant difference between groups ($F_{(5,17)}=2.452$, $P=0.094$). No significant difference between shams and lesion rats given vehicle (LV) was observed ($P=0.275$), probably because variability in the shams was high compared with the LV group (17% compared with 10% of deviation from the mean). However, on the same day, injured rats given ALLOP (4 or 16 mg/kg; respectively LA4 and LA16) showed significantly less DNA fragmentation than injured rats given vehicle alone (LV) ($P<0.05$), although variability was also relatively higher in the LA16 compared with LV (16.5% compared with 10% of deviation from the mean; Table 2).

Long-term effects of neurosteroids after seven injections at 1 h, 6 h, 24 h, 2 days, 3 days, 4 days and 5 days post-injury

Weight. Repeated measures ANOVA on percentage of weight loss for the animals on the 5 first days after injury revealed a group ($F_{(4,21)}=16.559$, $P<0.001$) and group by day ($F_{(5,21)}=3.369$) effect. Fisher post hoc analysis over all days showed that sham-operated animals weighed significantly more than all the injured groups (Table 3).

Nineteen days after the injury, a one-way ANOVA revealed a significant effect among groups in percent of weight gain ($F_{(5,22)}=3.8$, $P<0.05$). On this day, only injured rats given ALLOP (8 or 16 mg/kg) weighed significantly less than sham-operated rats (Table 4).

Table 3. Percentage of weight loss of rats compared to pre-surgical weight during the 5 first days after injury[a]

	Weight loss, %	S.E.M.
SV	3.566	0.633029
LV	−9.678***	1.097772
LP	−8.365***	2.275659
LA4	−11.0525***	1.742256
LA8	−12.5575***	1.778334
LA16	−12.732***	1.778447

[a] All injured animals weigh significantly less than their pre-surgery weight compared to shams (SV). There is no significant difference in weight loss between injured rats given PROG (LP) or ALLOP (4, 8 or 16 mg/kg; LA4, LA8 and LA16 respectively) and injured rats given vehicle alone.
*** $P<0.001$ compared to SV group.

Table 4. Percentage of weight gain of rats compared to their pre-surgical weight up to 19 days after injury[a]

Weight gain percent	Mean	S.E.M.
SV	20.28	0.76
LV	10.25*	2.02
LP	20.83#	6.05
LA4	16.78	2.72
LA8	4.6775**	3.7
LA16	4.528***	2.55

[a] Only injured rats given PROG (LP) and ALLOP (4 mg/kg; LA4) have weight similar to shams (SV), whereas injured rats given vehicle or ALLOP (8 or 16 mg/kg; LA8 and LA16) weigh significantly less than shams. Injured rats given PROG weigh more than injured rats given vehicle alone.
* $P<0.05$, ** $P<0.01$ and *** $P<0.005$ compared to shams (S); # $P<0.05$ compared to LV.

Neurosteroid effects on necrotic cavity. Since there was no necrotic cavity in the shams, they were not included in the analysis of mean difference among groups. A one-way ANOVA showed a significant effect among groups ($F_{(4,\ 20)}=3.042$, $P<0.05$) in the size of the necrotic cavity. Injured rats given ALLOP (4 mg/kg) had a significantly larger necrotic cavity than injured rats given PROG or ALLOP (8 or 16 mg/kg; Fig. 1 and Table 5), and the group given ALLOP 4 mg/kg was not significantly different in necrotic cavity size compared with injured rats given vehicle alone.

Neurosteroid effects on cell death in the MDN of the thalamus. The one-way ANOVA did not show a significant difference among groups ($F_{(5,\ 20)}=1.723$, $P=0.190$) in the number of MDN cells of the thalamus. Fisher post hoc analysis showed that only injured rats given vehicle or ALLOP (4 mg/kg) had significantly fewer spared cells in the MDN compared with shams. The other injured groups given PROG or ALLOP (8 or 16 mg/kg) were not different from shams ($P>0.05$; Table 6).

Table 5. Lesion volume reconstruction 19 days after injury[a]

% of lesion/remaining tissue	Mean	S.E.M.
SV	0	0
LV	39.3	2.92
LP	31.5¥	2.47
LA4	51.1	4.35
LA8	28¥¥	2.73
LA16	34.2¥	5.41

[a] There is no significant difference between groups given neurosteroids and the lesion-only group (LV) but there is a significantly larger necrotic cavity in the brains from injured rats given ALLOP (4 mg/kg; LA4) and injured rats given ALLOP (8 or 16 mg/kg; LA8 and LA16) or PROG (LP).
¥ $P<0.05$ and
¥¥ $P<0.01$ compared to LA4.

Table 6. Spared cells in the MDN 19 days after injury[a]

MDN spared cells	Mean	S.E.M.
SV	126.5	12.08
LV	67.25*	8.31
LP	98.67	23.44
LA4	68*	22.72
LA8	101.33	10.07
LA16	80	16.65

[a] There are significantly fewer spared cells in the MDN region of the injured rats treated with vehicle (LV) and ALLOP 4 mg/kg, compared to shams (S). Rats given PROG (LP), ALLOP 8 (LA8) or 16 (LA16) mg/kg were not different from shams.

* $P<0.05$ compared to S.

Neurosteroid effects on learning and memory. In our analysis of the data, we separated the first and the second trial performance because in our opinion, these two trials reflect long-term and short-term memory respectively. We found that the treatment effects on the first trial had less variability among the different groups than on the second trial. We looked at distance (Fig. 2A), latency, (Fig. 2B), strategy (Fig. 2C) and speed (Fig. 2D) that the rats need to find the platform. A repeated measures ANOVA on latency showed a significant group ($F_{(5,21)}=3.215$, $P<0.05$) and days ($F_{(9,189)}=15.571$, $P<0.001$) effect. A repeated measures ANOVA test on distance revealed both group ($F_{(5,21)}=2.864$, $P<0.05$) and days ($F_{(9,189)}=13.801$) effects. The same repeated ANOVA analysis on swim strategy showed a significant effect of groups ($F_{(5,21)}=4.54$, $P<0.01$), and days ($F_{(9,180)}=7.932$, $P<0.001$). Finally, a repeated measures ANOVA analysis on speed showed a significant difference between groups ($F_{(5,17)}=3.38$, $P<0.05$) only.

Post hoc analysis over days revealed that injured animals given vehicle alone (LV) had significantly higher latency ($P<0.05$), longer distance ($P<0.01$), higher speed ($P<0.05$) and more thigmotaxic strategy ($P<0.05$) compared with shams. Injured rats given PROG showed improvement in their strategy, speed and distance (no difference from shams and shorter distance than LV; $P<0.05$) and in their latency (no difference from shams). Injured rats given ALLOP (8 mg/kg) also showed improvement in their swim strategy (no difference from shams; $P>0.05$; and better strategy than LV; $P<0.005$), in their speed and distance (no difference from shams; $P>0.05$; and shorter distance than LV; $P<0.05$) and in their latency (no difference from shams; $P>0.05$). Injured rats given the highest dose of ALLOP (16 mg/kg) showed only intermediate effects on all of the measurements (no difference from shams), except for latency, which was higher than for shams ($P<0.05$). Injured rats given the lowest dose of ALLOP (4 mg/kg) had longer latency ($P<0.05$), distance and swim strategy ($P<0.05$), and higher speed ($P<0.05$) than shams and were not different from LV ($P>0.05$).

Correlation between measurements

There was a positive correlation between maze strategy ($r=0.467$, $P<0.05$) on trial 1 and the number of spared cells in the MDN (Fig. 3).

DISCUSSION

These results suggest, in general, that ALLOP and PROG produce long-term memory improvements after a bilateral injury to the pre-frontal cortex in rats. In addition, animals treated with either neurosteroid at 24 h post-injury showed a decrease of caspase-3 activity, and at 19 days after the injury, a decrease of cell death in the MDN of the thalamus, a region distal to the lesion site. Generally, rats given ALLOP (8 mg/kg) were similar in performance to their sham-operated counterparts. The effects of ALLOP (8 mg/kg) and PROG (16 mg/kg) were similar except that ALLOP improved swim strategy (less thigmotaxis, i.e. wall hugging) compared with injured rats given vehicle only, whereas PROG did not have this effect. We also noticed that injured rats that did not show any improvement in the water maze (LV and LA4) had a higher swim speed than shams. This behavior could be due to higher levels of stress caused by not being able to find the platform more rapidly. This speculation is reasonable because we did not see a difference in the speed among groups at the very beginning of the test when all rats had to learn to locate the platform in the maze.

Rats given PROG and ALLOP 8 or 16 mg/kg did not have smaller necrotic cavities than injured rats given vehicle; however, we did observe that the highest doses of ALLOP and PROG decreased the size of the lesion compared with injured rats given the low dose of ALLOP. In previous studies PROG was also shown to reduce cavity size (Shear et al., 2002). In the present experiment, we did not see this effect. This could be due to our use of another carrier, β-cyclodextrin, instead of peanut oil, a more effective solvent for PROG. We chose the β-cyclodextrin in this study because ALLOP is very difficult to dissolve in oil, and we needed to use the same vehicle for both neurosteroids to compare their effects. In the light of reduced MDN cell loss and less caspase-3 and/or DNA fragmentation at the lesion site, we were surprised that there was no effect of the neurosteroids on size of the cortical injury.

The cognitive deficits we observed in our model of injury probably resulted from fronto-cortical cell loss and damage to other inter-connected brain structures damaged after the injury, such as the MDN or the nucleus basalis magnocellularis (NbM, or basalis nucleus of Meynert). There was clear damage to the frontal cortex (cingular, frontal and orbital lateral cortex) 24 h after the injury and in the MDN 19 days after the lesion. These two brain regions, along with the NbM (Majchrzak et al., 1990), are known to have substantial neuronal loss after cortical injury (Elliott et al., 1989; Cuello et al., 1990; Roof et al., 1994; Hoffman and Stein, 1997). We hypothesize that the learning and memory deficits we observed are probably due to the loss of MDN cells, since we found a positive relationship between swim performance on first trial and the number of spared cells in the MDN. Moreover, only injured rats given either the vehicle or the ALLOP (4 mg/kg) had fewer spared cells in the MDN compared with shams. This was not the case of ALLOP- (8 or 16 mg/kg) or PROG-treated rats, which showed better cognitive recovery.

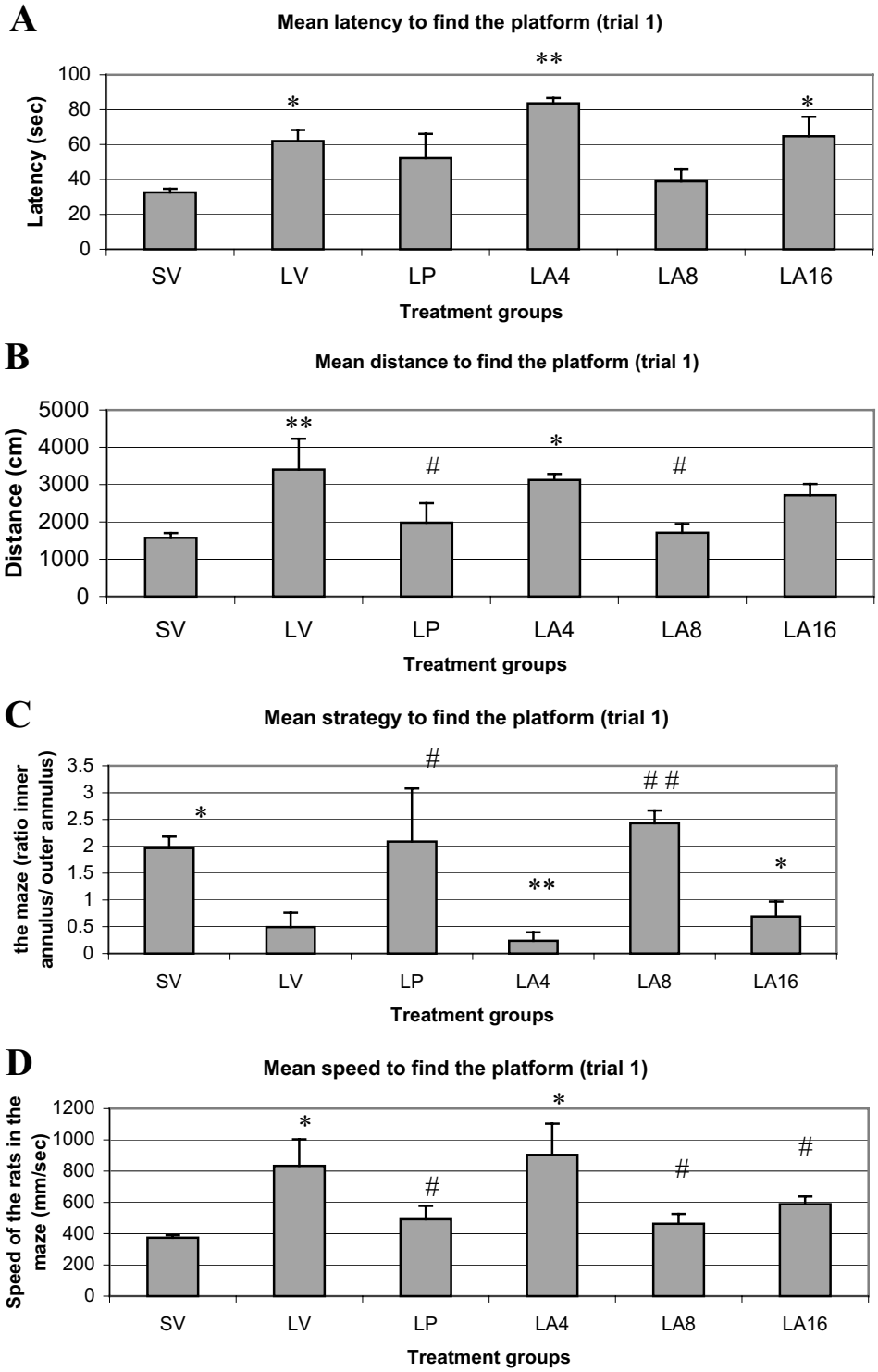

Fig. 2. A and B. Means of latency (A) and distance (B) swum by the rats to find the platform on the first trials through all 10 days of testing. The injured rats given vehicle (LV), ALLOP (4 or 16 mg/kg; LA4 and LA16) had significantly longer swim times (A) and distances (B) compared with shams (SV). Injured rats given ALLOP (8 mg/kg; LA8) or PROG (16 mg/kg; LP) were equivalent to shams (A and B), and significantly different from LV (B). * $P<0.05$, ** $P<0.01$ compared with SV, $^{\#}$ $P<0.05$ compared with LV. Data are means±S.E.M. C and D. Means of strategy (C) and speed (D) used by the rats to find the platform on the first trials over 10 days of testing. The injured rats injected with vehicle (LV) or ALLOP (4 or 16 mg/kg; LA4 and LA16) showed significantly more thigmotaxis (C) and higher swim speeds (D) than shams (SV). Injured rats given ALLOP (8 mg/kg; LA8) or PROG (16 mg/kg; LP) showed significantly less thigmotaxis (C) and lower swim speeds (D) than the injured-only group (LV). Rats given ALLOP (16 mg/kg) showed less thigmotaxis (C) compared with shams and lower swim speeds (D) compared with LV. * $P<0.05$, ** $P<0.01$ compared with SV, $^{\#}$ $P<0.05$ and $^{\#\#}$ $P<0.01$ compared with LV. Data are means±S.E.M.

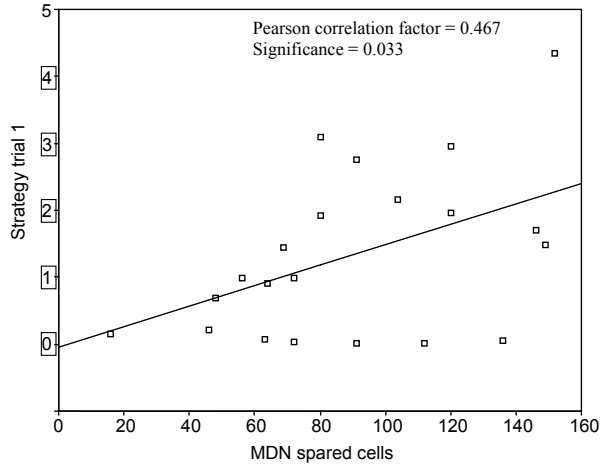

Fig. 3. Correlation between spared cells in the MDN of the thalamus and strategy on the first trial of the MWM test. There is a positive (Pearson correlation factor=0.467) and significant (P=0.033) correlation between the number of spared cells in the medio-dorsal nucleus (MDN) of the thalamus and the strategy used by the rats to find the platform in the MWM test.

As noted above, the MDN thalamic region is a structure known to have reciprocal connections with the medial frontal cortical area and is thought to play a role in learning and memory (Dubois and Pillon, 1997; Everitt and Robbins, 1997). We have previously shown that ALLOP (4 mg/kg) in 2-hydroxypropyl-β-cyclodextrin reduces the loss of MBN cholinergic neurons after an injury in the prefrontal cortex. PROG (4 mg/kg) in peanut oil was also demonstrated to reduce neuronal degeneration in the MDN region and to increase cognitive performance compared with injured rats treated with vehicle alone 21 days after a frontal TBI (Roof et al., 1994). Our results confirm these findings with a dose of 16 mg/kg for PROG and 8 mg/kg for ALLOP in β-cyclodextrin. The better recovery observed in our study with ALLOP and PROG is then probably due to both effects: the sparing of MDN neurons and the decrease of cortical cell death.

All the injured rats lost between 9 and 12% of their pre-injury weight during the first 5 days after injury, while shams gained 3% from their pre-surgery weight. Nineteen days after injury, lesion animals treated with PROG had about the same weight as shams and better weight recovery than the brain-injured rats treated with vehicle. The effect of PROG on weight is likely to be due to its effect on food intake and activity, since the hormone is said to be anxiolytic (for review, Zinder et al., 1999), acting through its metabolite ALLOP (Wieland and Dar, 1991; Bitran et al., 1993), and could result in less of the huddling or freezing behavior that is sometimes seen after a TBI. There is some evidence that a negative relationship between endogenous PROG and food intake exists (Rabiee et al., 2001), suggesting that PROG is implicated in metabolism. Injured animals treated with ALLOP 8 or 16 mg/kg weighed less on day 19 after the injury compared to rats given vehicle only, but they still had better cognitive performance than the lesion only group. Animals treated with the lower dose of ALLOP had about the same weight as shams 19 days after the injury, whereas injured rats treated with moderate and high doses of ALLOP had lower weights than shams.

The detrimental effect of the moderate and high doses of ALLOP on weight gain 19 days after the injury is more difficult to explain, especially given that the moderate dose of ALLOP had the best effects on cognitive outcome. Since it has been reported that ALLOP as well as PROG can induce hyperphagic effects (Chen et al., 1996; Reddy and Kulkarni, 1998), and since we also know that ALLOP, as a GABA-A agonist, is a potent sedative, we suggest that the low dose of ALLOP (4 mg/kg) is enough to induce hyperphagic effects. The 8 and 16 mg/kg doses of ALLOP also induced anesthetic effects that overwhelmed the hyperphagic effects observed with the lower dose. Moreover, the dose used in mice where hyperphagic effects were noticed was 2 mg/kg (Chen et al., 1996), which is close to the low dose of ALLOP used in this study. We did observe some anesthetic effects on animals given ALLOP (16 mg/kg) injections about 20 min after treatment.

ALLOP (8 or 16 mg/kg) and PROG (16 mg/kg) were also able to decrease caspase-3 activity compared with injured rats given the vehicle alone. However, the ability of ALLOP to decrease DNA fragmentation compared with injured vehicle-treated rats 1 day after the injury was found only at the highest dose (16 mg/kg). The inhibitory effect of ALLOP on apoptosis has been reported in other injury models showing that pre-treatment of NT2 neurons with ALLOP reduces the number of TαT-mediated x-dUTP nick end labeling (TUNEL)-positive cells after NMDA administration (Lockhart et al., 2002). At present no molecular mechanism of this anti-apoptotic effect of ALLOP is known. We report here that ALLOP (8 or 16 mg/kg) reduces caspase-3 activity in the lesion site of the injured frontal rat cortex. Caspase-3 was found to be critical in neuronal apoptotic processes occurring after stroke, spinal cord trauma, head injury and Alzheimer's disease (Robertson et al., 2000). Caspase-3 activity has also been described as the major effector of apoptotic processes after CNS injury (Yakovlev and Faden, 2001; Springer et al., 2001) and is specific to apoptotic cell death (Wang, 2000). In the light of these results, we suggest that, given soon after TBI, ALLOP can inhibit cell death by reducing apoptotic processes.

In the current study, we also found that PROG reduced caspase-3 activity but not DNA fragmentation 24 h after the injury, even though the hormone was able to reduce MDN thalamic cell loss 19 days after the injury. Perhaps its effect on apoptosis was not detected at the DNA level because a significant effect of the injury alone on DNA fragmentation was not significant 24 h after the injury in our model. This is more probable given that caspase-3 activity increase occurs before, or is associated with, apoptotic DNA fragmentation after brain injury (Cernak et al., 2002; Yakovlev et al., 1997), and that caspase-3 is implicated in activation of deoxyribonucleases enzymes after a brain injury (Luo et al., 2002b; Wang et al., 2001). Other investigations using later time points (48–72 h) after injury where apoptosis was reported to be at its maximum level

(Cernak et al., 2002; Beer et al., 2001; Raghupathi et al., 2000) would be useful to confirm this idea.

The literature on PROG effects on apoptosis is controversial. In one study, it was shown that a combination of different compounds, including PROG, could protect against oligodendrocyte apoptotic death after growth factor deprivation (Mayer and Noble, 1994). In another recent report, PROG treatment was demonstrated to increase the anti-apoptotic protein bcl-2 in hippocampal neuron culture after glutamate administration (Nilsen and Brinton, 2002). Still other research showed that PROG could increase the number of TUNEL-positive (apoptotic) neurons in a model of experimental allergic encephalitis (Hoffman et al., 2001) and inhibit the anti-apoptotic effect of estrogens in the hypothalamic arcuate nucleus of female rats (Garcia-Segura et al., 1998). These conflicting data on PROG effects on apoptosis show that the mechanism is complex and probably depends upon the endocrine (estrogen), the cellular (effects on neurons or glia), the brain location (region of the brain concerned), and the apoptotic stimuli context.

Our results showing that both neurosteroids reduce cell death after brain injury are supported by previous research demonstrating that both hormones are neuroprotective after excitotoxic damage (Goodman et al., 1996; Lockhart et al., 2002). It is now well established that apoptotic cell death takes place in the brains of mice and rats beginning the day after the injury and continues for up to 2 weeks. This implicates molecules like the bcl-2 proteins family and the caspase-8, -9 and -3 as perhaps the most important species in triggering cell death (Keane et al., 2001; Luo et al., 2002a). Caspase-3 activity has been found in the cerebrospinal fluid of humans 2–5 days after TBI. The anti-caspase-3 effects of ALLOP that we report here could then represent an important step in developing new treatment strategies for TBI in humans.

In summary, only the moderate dose (8 mg/kg) of ALLOP and PROG (16 mg/kg) had beneficial effects on cognitive performance, sparing of MDN cells and apoptosis. The “U”-shaped curve effect observed with the three doses of ALLOP is not surprising since the same dose-response curve was reported for PROG on learning and memory performance (Goss et al., 2003). The highest dose of ALLOP, which seemed to be the most efficient in reducing caspase-3 activity and DNA fragmentation, was not the most efficient dose in sparing MDN cells and in producing cognitive recovery; while the moderate dose of ALLOP, by inhibiting some apoptosis, protects the brain from extensive apoptotic cell loss and improves behavioral outcome. These results suggest that partial inhibition of apoptosis may be more beneficial than a more complete inhibition by allowing the elimination of the most dysfunctional cells, while preventing the loss of repairable cells, thus leading to improved behavioral outcome.

In summary, this study comparing PROG to three dosages of ALLOP after injury to the medial frontal cortex in rats, shows that both ALLOP and PROG can decrease cell loss in regions distal to the lesion site by a mechanism likely involving a decrease of apoptotic processes that are caspase-3-dependent. The prevention of apoptotic secondary cell death may then lead to better cognitive performance.

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