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Graded crush of the rat optic nerve as a brain injury model: combining electrophysiological and behavioral outcome

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Abstract

In the present study, we have developed an animal model for central nervous system (CNS) damage using the graded crush injury of the adult rat optic nerve. This injury model has the following characteristics: (a) the injuries are reproducible; (b) the injuries can be created with controlled severity; and (c) variations in the severity of the injuries correlate with definable electrophysiological and behavioral outcomes. Self-closing forceps, modified by attaching a screw to the handle, were used as a lesion-causing device. Rat optic nerves were crushed in vitro. The severity of the injury, calibrated by a measured applied force, was determined, electrophysiologically, in vitro. Compound action potential was assessed and recorded by suction electrodes connected to both sides of the nerve. In vivo studies correlated the calibrated crush injuries with behavioral outcome. After severe crush (applied force: 205 g or higher), the rats were unable to orient towards visual stimuli presented in the visual field of the damaged nerve and only minor transient recovery was observed. After moderate crush (applied force: 120 g), however, there was a significant recovery of orienting performance up to the 6th postoperative day, followed by a gradual, secondary loss of function. In the mildly injured group (applied force lower than 30 g), functional improvement continued for up to 10 days and remained stable thereafter, with only minor secondary loss. This CNS graded injury model may be valuable to study the molecular and anatomical mechanisms underlying secondary degeneration and the potency of various posttraumatic treatments in leading to recovery of function.

Introduction

Injury to axons in the CNS leads to a variety of neurological and behavioral disabilities. These various disabilities are probably due to the primary loss of specific brain structures and/or their neuronal connections. In addition, however, further degeneration develops within a certain delay after the injury and may contribute significantly to the long term functional outcome (for review see ref. 14). Attempts to delay [1] or prevent this 'secondary' degeneration have been made using pharmacological treatments, such as GM1 gangliosides [9,10,15] and nerve growth factor (NGF) [5,6]. In order to assess the beneficial effects of the various treatments, and to gain insight into the mechanisms of their actions, a well-defined animal model is needed, utilizing a well calibrated graded injury.

Ideally, an animal model of brain damage should fulfill a number of criteria, among which are: (a) reproducible lesions; (b) lesions with controlled severity; and (c) variations in lesion severity, clearly correlated with

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definable outcome in physiological, behavioral or other parameters.

In the present study, we describe well-controlled graded crush injuries of the adult rat optic nerves and the correlation with the resultant electrophysiological and behavioral manifestations.

Materials and Methods

Calibration of the crush forceps and force estimation

To fabricate forceps with which crush lesions of varying intensity could be accomplished, we modified Castroviejo's cross-action (self-closing) capsule forceps in the following way: first, we filed down the cupped grasping jaws, and then polished the new tips having 1 mm width and attached a screw to the handle (Fig. 1). In this way, we could ensure constant and graded force (adjustable by the screw) at the grasping tips when no external pressure was applied to the handles.

Measurements of the force created by the forceps at different positions of the screw

The measurement of the force created by the forceps was done by Instron Universal Tester. The forceps' jaws were pulled in cross-speed of 0.5 cm/min until full opening. The force was recorded as a function of the change in the distance between the forceps' tips.

Dissection of the optic nerve. Rats were deeply anesthetized with pentobarbitone (70 mg/kg, intraperitoneally). The skin was removed from the rat's skull and the proximal ends of the optic nerves were transected near the eyeball. After decapitation, the skull was quickly opened with rongeurs and the brain was removed together with the optic nerves. The nerves were then dissected out at a maximal length of 5–8 mm and transferred to vials containing fresh cold Krebs solution (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 2.4 mM $CaCl_2$ and 11 mM D-glucose) aerated with 95% O_2 and 5% CO_2. In order to permit optimal stabilization of the action potential readings, the nerves were kept at room temperature under constant flow of the gas mixture for 1–2 h prior to the electrophysiological measurements. Measurements in 5 control nerves indicated that under these experimental conditions, the nerves maintain normal electrical activity for a period of at least 4 h.

In vitro electrophysiological studies. In vitro electrophysiological experiments were carried out with optic nerves excised from 4-month-old Sprague–Dawley rats weighing 300–400 g.

Electrophysiological measurement. In order to measure the action potential of the excised nerve, the nerve was immersed in a Petri dish containing 37 °C warm Krebs solution. The nerve ends were aspirated into two suction electrodes made of fine polished glass tubes with Ag-electrodes located inside each tube and the negative terminal of each electrode outside the tube. The bathing solution, in which the nerve and the electrodes were immersed, as well as the stimulator and amplifier, had a common ground. The electrode on the proximal end gave a stimulating pulse, while the distal electrode was used for recording. The stimulus was given by a Grass SD9 stimulator at an intensity of 2 V with a duration of 50 μs. The action potential signal was first transmitted to a Medelex PA63 preamplifier and then to a Medelex MS7 electromyograph with its AA7T amplifier. Following analog to digital conversion, the signal was averaged over 8 pulses at a frequency of 0.5 Hz. The amplitude of the nerves' compound action

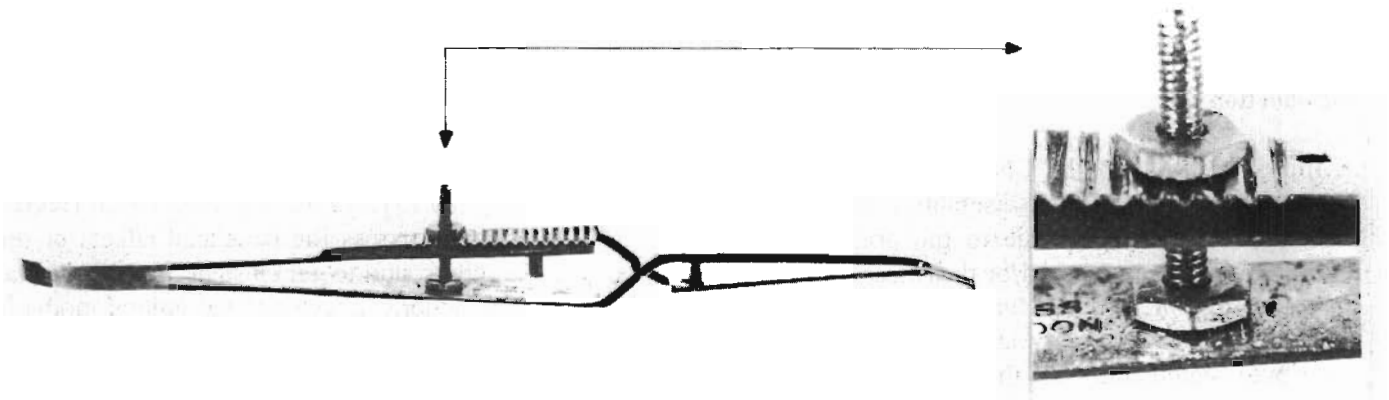

Fig. 1. Photograph of the cross-action forceps, showing the adjustable screw. By tightening the handles of the screw with the outer nut, the tips, with which the crush is applied, will be relieved of pressure.

potential (positive phase only) was measured and correlated with extent of lesion severity.

In vitro crush of the optic nerve. After measuring the action potential of the optic nerves, a crush injury was performed, with the cross-action forceps applied to the middle of the nerve for the duration of 10 s, while both ends of the nerve remained connected to the suction electrodes. Nerves were crushed with various severities. Each group consisted of 5 nerves. Action potential was recorded immediately following the crush and at 2, 15, and 30 min thereafter. For control purposes, 5 nerves were connected to the suction electrodes for 30 min but did not receive any crush.

Crush injury in vivo

Surgery. Forty-seven SPD rats (250–350 g) were randomly assigned to the different groups with the various crush severities. Under general Rompun (10 mg/kg, intraperitoneally) with Vetalar (50 mg/kg, intraperitoneally) anesthesia, a lateral canthotomy was performed under a binocular operating microscope and the conjunctiva was incised laterally to the cornea. After separation of the retractor bulbi muscles, the optic nerve was identified and exposed near the eyeball by blunt dissection for 2.5–3 mm. The dura was left intact and care was taken not to injure the nerve except with the subsequent crush. The crush was made by the previously calibrated cross-action forceps 1 mm distal to the eye, for a period of 30 s. After completion of the crush, the canthotomy was sutured. The animals were allowed to recover from the surgery for one day with free access to water before behavioral testing commenced.

Assessment of vision following crush injury

The experimenter was unaware of the group identity of individual rats while behavioral performance was assessed. Before surgery, all rats had undergone pretraining (acquisition of the behavioral task) for 6 days. The behavioral task involved presentation of a stimulus to the rat visual field and visual function was assessed by the rats' ability to orient towards the stimulus. The test is similar to perimetry testing in human patients and has successfully been used to assess visual performance in brain-injured hamsters [11]. In our experiments, the stimulus, a small rubber ball from which a small water canula protruded, was presented to the visual field of water deprived rats in their home cage. Water-deprived rats quickly learned to orient towards the stimulus and drink a drop of water as a reward. When the visual field is defective, the rats do not orient towards the stimulus presented in some or all sectors of the visual field. Control animals exhibited consistent orienting performance over the testing intervals. All operated animals were tested with both eyes. Testing of the intact eye was used as a positive control to ensure the animal performance capability.

Data analysis. For purposes of data collection, the visual field was divided into three regions on each side: nasal (0–45°), nasotemporal (45–90°) and temporal area (> 90°; see Fig. 2). We recorded whether the rats showed clear orienting response to stimulus presentation in each of the field sections (intact = 1, deficient = 0). In those cases where the responses were unclear, they were recorded as 0.5. With this procedure, we were able to map the intact and deficient sectors of the visual field for each individual rat. A total 'vision score' was then calculated as the average performance per visual section (a maximum of 1 and a minimum of 0 in each visual half field).

Statistical analysis

Electrophysiological activity and behavioral performance were analyzed by a two-way analysis of variance (ANOVA). Factor A was the crush severity and factor B the time after crush. Additional ANOVA was carried out for behavioral performance where crush severity was selected as factor A and visual field sectors as factor B (nasal, nasotemporal, or temporal). A repeated measure analysis was also used for behavioral performance.

Results

Calibration of the force created by the forceps

Fig. 3 shows the measurements of the force created by the forceps, expressed in grams. The force needed to

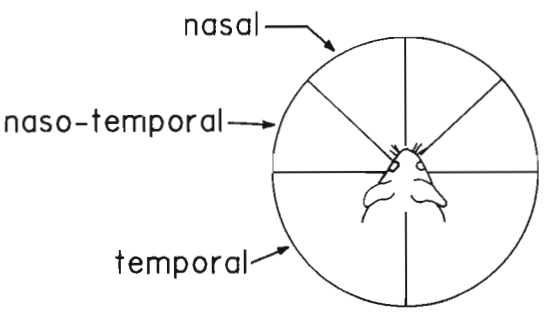

Fig. 2. Behavioral assessment: visual orienting ability was tested by presenting a water spout in various sectors of the visual field (nasal, nasotemporal, or temporal). Orienting responses which were rated to be intact were given the value 1, uncertain responses were given the value 0.5, and deficient areas were given the value 0.

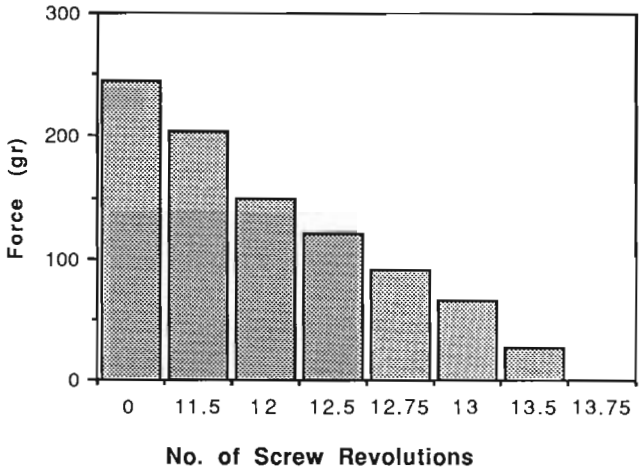

Fig. 3. The force of the forceps, as a function of the position of the screw nut (number of screw revolutions), measured by the Instron Universal Tester.

open the forceps' tips at each position of the screw nut are shown on the y-axis, whereas the x-axis shows the corresponding number of the screw revolutions.

Electrophysiological activity

Initial correlation, between the force created by the forceps at different opening stages and the resultant lesions, was achieved by in vitro electrophysiological measurement. Rat optic nerves were excised and were connected to two suction electrodes. The activity of the nerve prior to and after injury was recorded. The crush injuries were made while the two ends were connected. The average compound action potential amplitude of the normal non-injured rat optic nerve was found to be $4760 \pm 1459\ \mu V$ (mean $\pm$ SD), as recorded from the 20 nerves prior to injury (including the control group). Compound action potential amplitude did not change during the 30 min period in which the nerves of the control group were connected to the suction electrode (Table I; see also Fig. 4a). Various crush injuries were made by changing the screw nut position. Immediately after the crush, a marked initial impairment or loss of function occurred in all injury groups, the extent of which was dependent upon the crush severity. Based on the results, the following three groups of crush forces were selected: (a) a lesion (205 g) which resulted in only little, if any, recovery of the compound action potential amplitude after the 30 min period (Fig. 4d); (b) a lesion (120 g) which resulted in a gradual increase in action potential from 0 to $37 \pm 17.2\ \mu V$ (Fig. 4c; see also Table I); and (c) a lesion (<27.5 g) which resulted in a fast recovery (within 2 min) and reached $3400 \pm 1356\ \mu V$ within 30 min (Fig. 4b). These results of the in vitro electrophysiological calibration are summarized in Table I. These in vitro findings are documented by a statistical comparison of group means involving a two-way ANOVA with the factors 'crush severity' (factor A) and 'time after crush' (factor B). A significant effect was found for factor A ($F = 52.2$, df = 3, 80, $P < 0.001$) and factor B ($F = 36.1$, df = 4, 80, $P < 0.001$), indicating that the amplitude was significantly altered by the lesion severity and that it changed over time after the crush. Moreover, both factors showed a significant interaction $A*B$ ($F = 7.07$, df = 12, 80, $P < 0.001$).

Visual orienting behavior

Five groups of animals were tested, each group consisting of 7–9 animals. Crush injuries of ranging severity (from a force <27.5 g to a force >245 g) were performed. The visual ability was reported as described in Materials and Methods. The results were analyzed by a two-way ANOVA and by repeated measurement analyses.

A two-way ANOVA revealed that factor A ('crush severity') and factor B ('days after surgery') were significant (factor A – $F = 19.43$, df = 4, 420, $P < 0.0001$; factor B – $F = 6.12$, df = 11, 420, $P < 0.0001$), and the interaction $A*B$ was not ($F = 0.75$, P = n.s.). According to Duncan's multiple range test, the tested animals

TABLE I

Amplitude of compound action potential (μV mean $\pm$ S.D.)

Nerves were from 20 non-injured control animals and from animals with crushed nerves (5 animals in each group), and they were analyzed electrophysiologically by measuring compound action potentials. Results are presented by mean $\pm$ S.D.

Crush severity (force in g)	*Prior to injury*	*Minutes after injury*			
		0	*2*	*15*	*30*
Control	4760 ± 1459	4760 ± 1459	4760 ± 1459	4760 ± 1459	4760 ± 1459
<27.5	6600 ± 1854	18.3 ± 2.3	52 ± 39.6	2500 ± 1129	3400 ± 1356
120	4500 ± 632	0	14 ± 3.7	19 ± 11.1	37 ± 17.2
205	6500 ± 1224	0	0	0	0

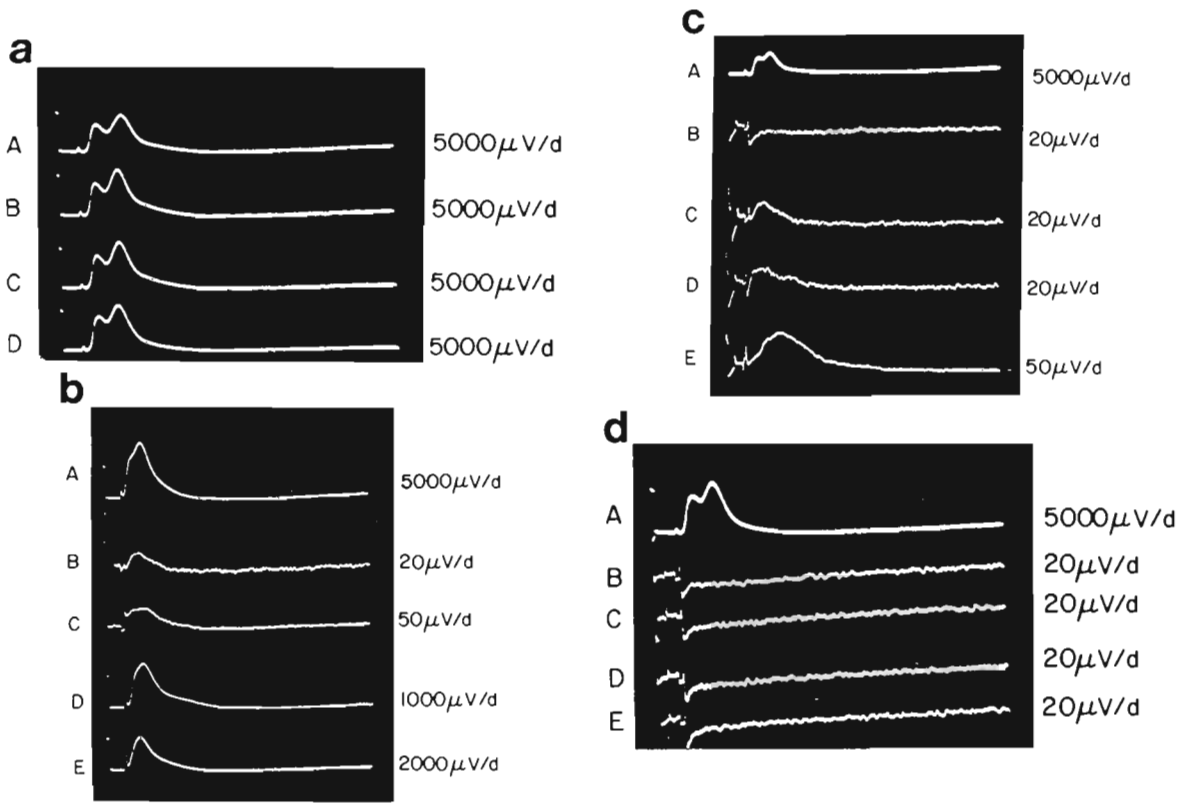

Fig. 4. Representative electrophysiological recordings of the compound action potential of selected optic nerves in vitro. a: compound action potential of a non-injured control nerve throughout a 30 min period. b–d: action potentials in rats of different crush severity of <27.5 g (b), 120 g (c), and 205 g (d) recorded before the crush (A) or at 0, 2, 15, or 30 min after the in vitro crush (B) through (E), respectively. The values to the right of the graph represent the maximum amplitude at each time point.

could be divided into 3 groups which corresponded to 3 types of crush severity. Table II shows the orienting performance following presentation of visual stimuli to rats with optic nerve crush of varying severity (for definition of the vision score, see Materials and Methods).

A similar pattern of behavior was observed in the first two groups, exposed to a force <27.5 g and to a force of 90 g. At 2 days postinjury, rats belonging to these groups did not orient towards stimuli (Fig. 5). Their visual ability improved up to about 60% by 7–9 days. The visual ability then decreased and led to a

TABLE II

Vision score in various regions of visual field and the crush severity

Animals which were exposed to crush injuries of various severities were examined for visual performance in the periods between day 2 and day 13 in the different regions of the visual field. Results are expressed by the mean ± S.E. of all measurements performed in each group. Numbers in parentheses are number of measurements.

Crush severity (force in g)	*Nasal*	*Region of visual field*			
		Nasotemporal (mean ± S.E.)	*Temporal*	*Vision score*	*Number of animals*
Control	1 ± 0	1 ± 0	1 ± 0	1 ± 0 (105)	5
<27.5	0.56 ± 0.06	0.24 ± 0.06	0.17 ± 0.05	0.32 ± 0.03 (153)	9
90	0.64 ± 0.07	0.30 ± 0.05	0.02 ± 0.01	0.32 ± 0.04 (147)	8
120	0.44 ± 0.05	0.10 ± 0.03	0.008 ± 0.008	0.18 ± 0.025 (101)	9
205	0.09 ± 0.04	0.09 ± 0.05	0.06 ± 0.04	0.08 ± 0.025 (102)	7
>245	0.06 ± 0.03	0	0	0.02 ± 0.01 (96)	9

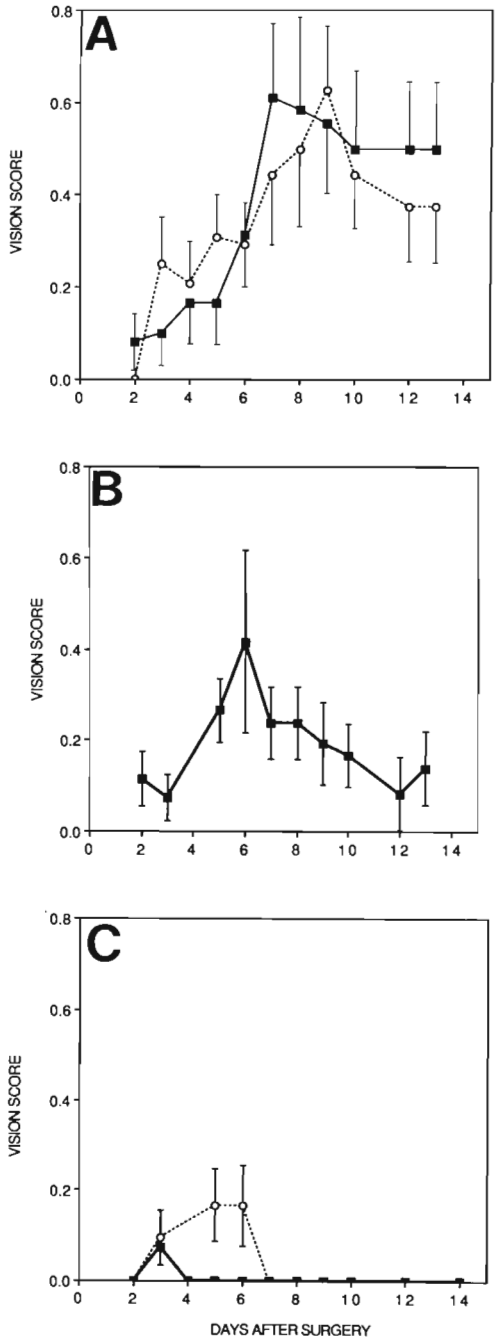

Fig. 5. Time course of orienting behavior (mean and SE) in rats with optic nerves crushed, with various forces. The placing of the five different crush groups in these categories was based on results of the statistical analysis. Note the delayed functional loss, especially in the severe and moderate cases. In (A), ---○--- represents crush severity of <27.5 g, and ——■—— represents crush severity of 90 g. In (B), the crush severity is 120 g, and in (C), ---○--- and ——■—— represent crush severities of 205 g and >245 g, respectively.

stable orienting ability, which was roughly maintained until the behavioral tests were terminated (2 weeks). A clear but not dramatic secondary functional loss was noticed in these two groups starting at 7–10 days.

Animals which were exposed to a force of 120 g displayed some residual function on the second day postinjury, with a marked recovery of up to about 40% performance on the 6th day. A secondary functional deterioration was seen in this group. By the 13th day postinjury, the average orienting performance had dropped back down to levels seen at 2 days after surgery.

Rats in groups which were injured with a force of either 205 g or >245 g showed the same initial deficit as the other groups. They did not orient towards visual stimuli 2 days after the injury and showed only little functional recovery (Fig. 5). A secondary loss of function was seen subsequent to the initial recovery phase and this secondary loss occurred 6 days (205 g) or 3 days (>245 g) after lesion. This behavioral deficit lasted throughout the remaining test period.

Fig. 6 shows that the duration of the apparent behavioral recovery process is a direct function of the crush severity and it is linearly related to the crush severity: the milder the crush, the longer the behavioral improvement continues. It should be noted that we did not assess the rats' behavior beyond the 2-week period and therefore, do not know if behavioral deterioration occurs at later periods of time, even in the mild injury groups.

In all lesion groups, the improvement of visually elicited orienting performance followed a clear nasal to temporal progression. A two-way ANOVA revealed a significant correlation between behavioral performance and both crush severity (factor A–F = 23.4, df = 4, 112, $P < 0.0001$) and the visual field (factor $B - F = 76.1$, df = 2, 112, $P < 0.0001$), as well as in the interaction between both factors ($A * B$, $F = 7.1$, df = 8, 112, $P < 0.0001$). Analyses by repeated measures revealed the same results. From these analyses and inspection of

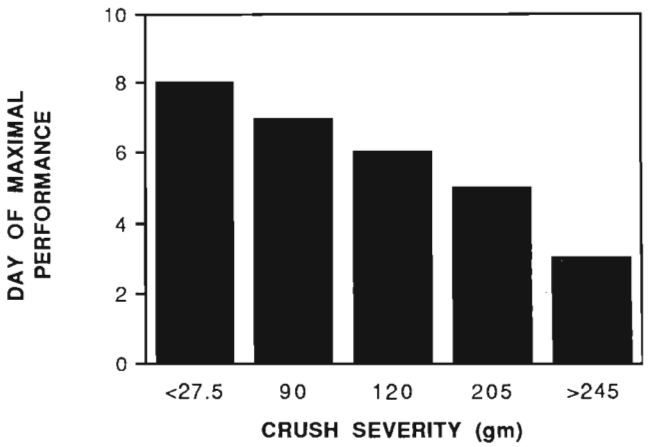

Fig. 6. The relationship between lesion severity and the first day of peak (maximum) orienting performance is shown here.

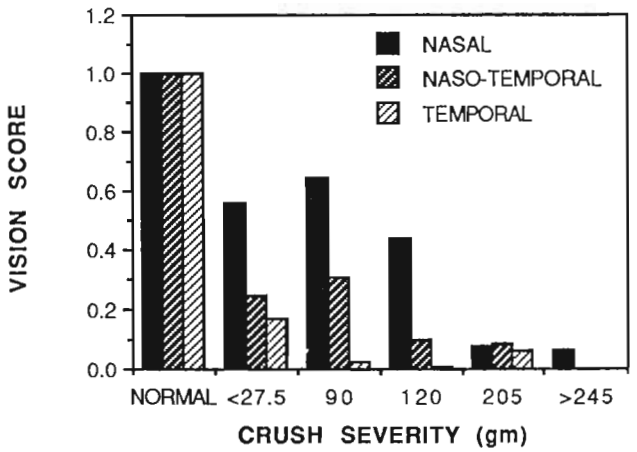

Fig. 7. The relationship between lesion severity and orienting performance (vision score, defined in Materials and Methods). Note that significant behavioral recovery occurs mostly in the nasal section of the visual field, while the nasotemporal and temporal areas recover the least. These data are pooled over the entire time course of the experiment (for standard errors, see Table II).

the individual data, it is clear that the improved visual ability of the nasal field was higher than the visual ability of the nasotemporal visual fields (Fig. 7; see also Table II).

Discussion

In light of recent observations that secondary degenerative events following brain lesion can be delayed or reduced by various treatment modalities such as gangliosides [9,10,15], NGF [5,6], or laser irradiation [1,8,12], a well controlled animal model of CNS injury was needed.

In the present study, we showed that the use of calibrated cross-action forceps allows us to apply crush lesions of defined force leading to well controlled lesions. The consequences of the lesions with the different severity were manifested both electrophysiologically and behaviorally. On the electrophysiological level, there was a lesion-dependent loss of conduction of the compound action potential across the crush site; the more severe the crush, the more severe the initial deficit and the smaller the recovery of the compound action potential. On the behavioral level, the loss of visual function (as defined by the rats' ability to orient toward a visual stimulus) and the subsequent recovery also depended on the lesion severity. The behavioral data indicate a secondary functional deterioration subsequent to the initial recovery, the duration of which correlated with the lesion severity.

Little is known about the anatomical, molecular, and behavioral aspects of secondary degeneration and this problem needs to be addressed now in greater detail. The graded optic nerve injury model may prove a valuable tool in this regard. To understand the advantages of the graded optic nerve crush as a brain injury model, it is important to consider the current knowledge pertaining to the study of brain injury using a variety of approaches. There are basically two types of approaches, the 'injury simulation approach' (see examples in refs. 3 and 4) and the 'neuroplasticity approach', both with their respective advantages and shortcomings.

The crush of the nerve maintains the morphological continuity of the nerve with the target and, therefore, has a major advantage over total nerve transection models, for example the one used by Carmignoto [2], which are of limited value for the study of recovery of function. Thus, avoiding the transection of the axons, graded crush injuries permit the study of mechanisms involved in the processes of secondary degeneration, recovery of function, and the action of drugs which interfere or delay degeneration [1,12] and affect CNS repair [7,10,13].

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