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Changes in the Cerebral Cortex after Dosed Craniocerebral Trauma in Rats of Different Ages

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Dosed lateral fluid percussion was used to model craniocerebral trauma (CCT) of moderate to severe intensity in one- and two-year-old rats. Brain sections were stained with cresyl violet by the Nissl method and with an immunochemical reaction for glial fibrillary acidic protein (GFAP) – a marker for astrocytes. The results provide evidence that zones of direct and remote injury formed in the side ipsilateral to the blow. The direct injury zone corresponded to the area of direct contact of the column of liquid with the dura mater, while the remote injury zone was positioned lateral and caudal to the direct injury zone. Morphological detection of trauma depended on the strength of the blow and was seen in both age groups as astrocytic gliosis, with thinning of layer I of the cortex due to death of neurons. Signs of ischemic changes to neurons were probably associated with local impairment to blood supply. Brain damage in one-year-old rats was local in nature but was more diffuse in two-year-olds, while gliosis was characterized by inhomogeneity. The reproducibility and appropriateness of the model allow it to be used for investigation of the molecular genetic mechanisms of the sequelae of CCT in humans and for identifying common mechanisms in the sequelae of CCT and the pathogenesis of major diseases comorbid with CCT, particularly depression and epilepsy.

Keywords: cerebral cortex, neurons, neuroglia, craniocerebral trauma, fluid percussion.

Craniocerebral trauma (CCT) is a widespread medical and social problem in the present world [13]. Depending on the biomechanics of CCT, injury can occur both in brain areas immediately adjacent to the site at which force is applied or in remote areas – contrecoup zones, generally located within the frontal and temporal lobes or at the base of the brain [7]. Direct brain injury in CCT is studied using a variety of animal models [12]. The most widely used is a lateral fluid percussion model (liquid percussive brain trauma). This model is characterized by highly reproducible results and the potential for using blows of widely varying forces to induce brain injury of different severities [10].

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The main primary sequelae of trauma induced by lateral fluid percussion are hemorrhages, acute neuron death, and impairment to the blood-brain barrier. Secondary (delayed) changes constitute a complex of biomechanical, structural, and molecular changes occurring as a result of the primary injury. These include inflammation, excitotoxicity, and neurodegeneration. Repair processes include neuro-, glio-, and angiogenesis [9, 15].

Trauma in older age is characterized by a long recovery period and a worse prognoses than with trauma at earlier ages [14]. This may be because of greater neurological inflammation and increased vascular permeability [11].

The aims of the present work were to study the characteristics of neurons and glia and to compare histological changes in the brains of rats of different ages after dosed CCT.

Materials and Methods

Experiments were performed on male Wistar rats (from the Stolbovaya supplier). Experimental manipulations were

TABLE 1. Assessment of the Extent of Damage to the Cerebral Cortex in Rats by Nissl Staining

Points	Measures evaluated
1	Visually – cortical layers well differentiated. Weak gliosis in damage focus: more than 10 glial cells
2	Visually – unclear separation of cortex into six layers at damage focus. Thinning of cortical layer I to 50%. Gliosis: more than 20 glial cells. Neurons with structural changes of the ischemic type
3	Visually – cortical layers not discriminated because of gliosis. Thinning of cortical layer I to 50%. Clearly apparent gliosis: more than 50 glial cells. Neurons poorly differentiated because of gliosis
4	Clear area of necrosis with unstained neuron outlines and glial cell nuclei

Note. The number of cells was determined in squares of size 150 × 150 μm.

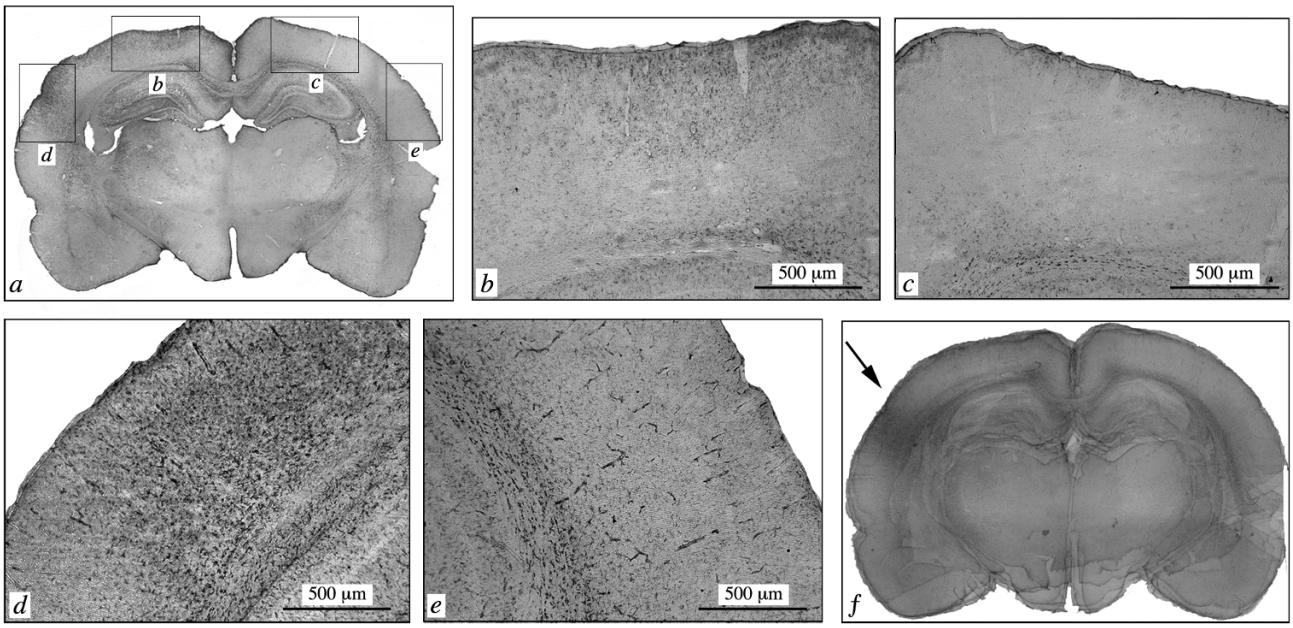


Fig. 1. Zones of direct and remote cerebral cortex damage after craniocerebral trauma in rats of group 1. *a*) Section at the level of 3.5 mm caudal to the bregma; *d–e*) areas identified by squares in (*a*); *b*) zone of direct damage: gliosis of cortical layers I–III, triangular shape with the base at the meninges; *c*) no gliosis in the symmetrical area of the contralateral hemisphere; *d*) remote damage zone, visible gliosis in all cortical layers; *e*) no gliosis in the symmetrical zone of the contralateral hemisphere; *f*) superimposition of brain sections from eight rats of group 1 the a level of 3.5 mm caudal to the bregma; the arrow shows the remote damage zone as darkening in the cortex of the ipsilateral hemisphere. Reaction for glial fibrillary acidic protein. *a*, *f*) objective ×10, ocular ×20.

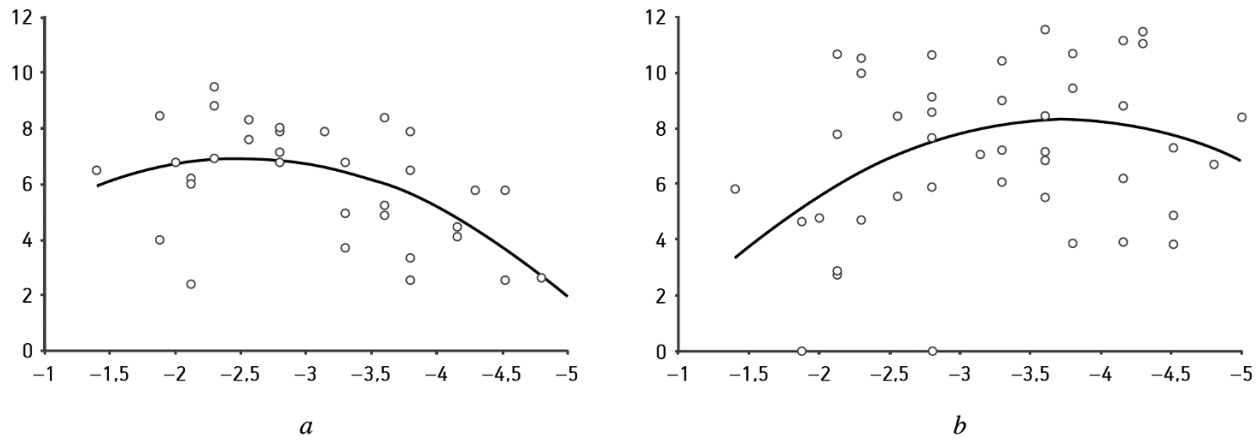


Fig. 2. Spatial characteristics of the zones of direct (*a*) and remote (*b*) damage after craniocerebral trauma in rats of group 1. The abscissa shows coordinates relative to the bregma (mm); the ordinates show damage area (units).

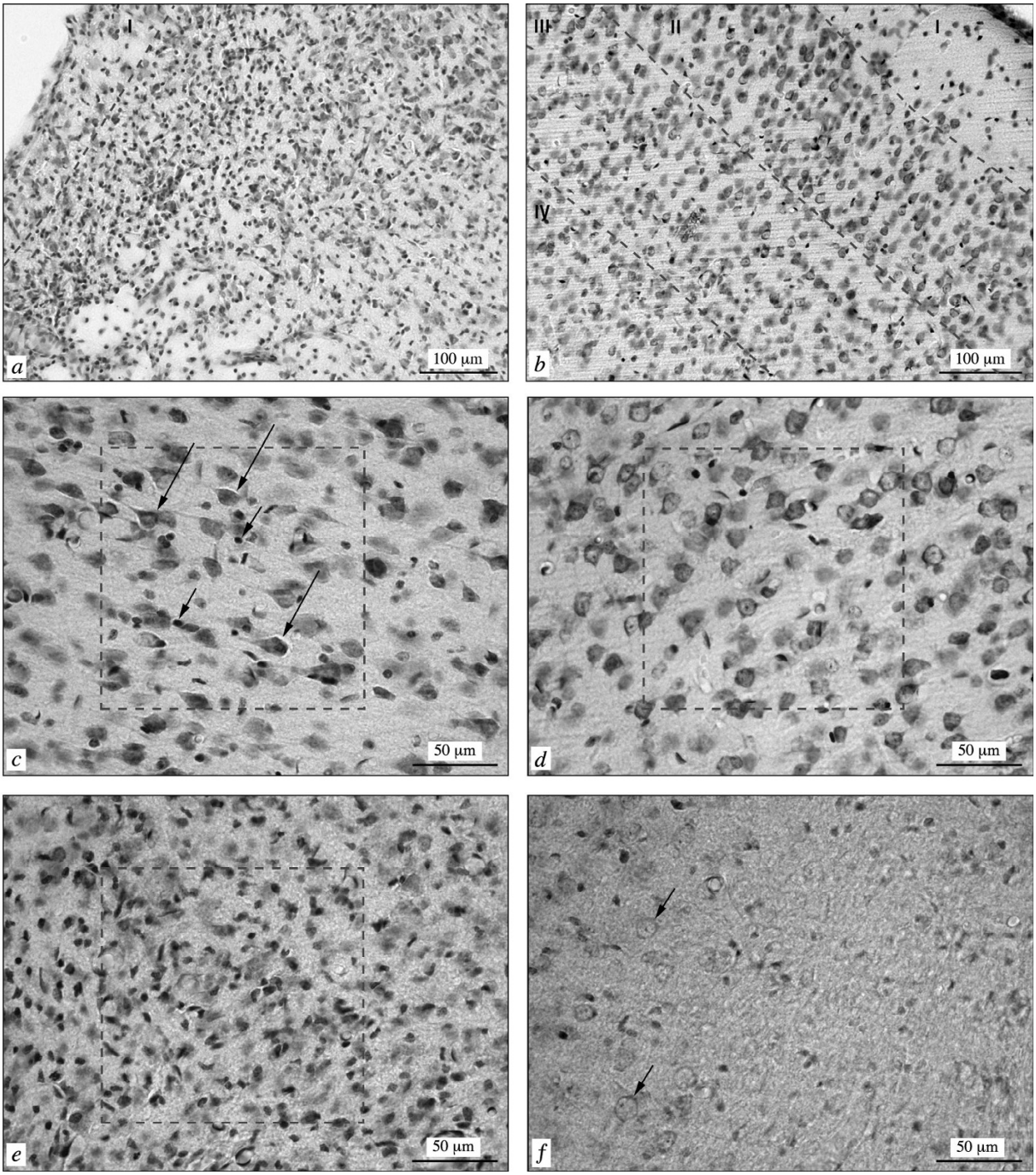


Fig. 3. Remote damage zone in the cerebral cortex after craniocerebral trauma in rats of group 1. *a*) Damage 3 points: cortical layers (I–IV) not differentiated visually, layer I thinned compared with the symmetrical area of the contralateral hemisphere (*b*); dotted lines show boundaries between cortical layers; *c*) damage (2 points) in layer II of the cortex: ischemically altered neurons (long arrows) and increased numbers of glial cells (short arrows); these changes were absent from the contralateral hemisphere (*d*); *e*) damage (3 points) in the layer II of the cortex: clear gliosis, such that neurons are poorly discriminated; *f*) damage (4 points) in layer VI of the cortex: neurons and glial cells with poorly stained outlines and nuclei (ghost cells – arrows). *c*–*e*) Dotted squares show areas evaluated. Stained with cresyl violet by Nissl method.

approved by the Ethics Committee of the Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences (protocol No. 10 of December 10, 2012) and were performed in compliance with the requirements of "Regulations for Studies Using Experimental Animals" (USSR Ministry of Health Decree No. 755 of August 12, 1977). The rats were divided into two groups: group 1 consisted of 13 animals aged 12 months and group 2 consisted

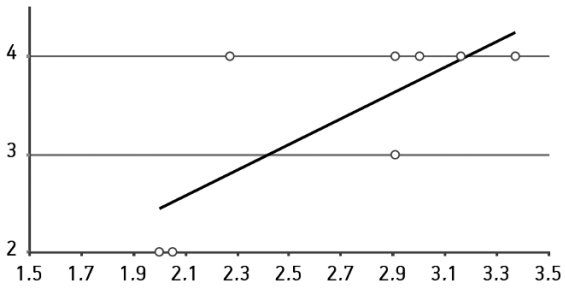


Fig. 4. Relationship between extent of damage in the remote zone in the cerebral cortex and blow force in rats of group 1 with model of craniocerebral trauma. The abscissa shows blow force, atm.; the ordinate shows the extent of damage (points): $F(df_1 = 1, df_2 = 6) = 7.78$, $p = 0.031$.

of nine animals aged 24 months. Body weight in group 1 was 355–467 g; that in group 2 was 430–627 g. CCT was modeled using the widely employed fluid percussion method [4].

CCT was modeled in eight rats of group 1 using different blow forces: three rats (2–2.27 atm., moderate) and five rats (3.3.37 atm., severe). Fluid percussion were delivered 2–3 h after trepanning and recovery from anesthesia. Controls consisted of three intact and two sham-operated rats: these latter were treated identically apart from not receiving fluid percussion.

Severe CCT was modeled in rats of group 2 using a blow force of 3–3.37 atm. (five rats). Trauma was applied one day after trepanning craniotomy. Controls consisted of two sham-operated animals and two intact animals.

Skull trepanning was performed under chloral hydrate anesthesia (395 mg/kg, i.p. [8]). The diameter of the aperture in the right parietal bone was 3 mm. The aperture was positioned 3 mm caudal to the bregma and 3 mm lateral to the midline. Seven days after the fluid percussion, brains were fixed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for histological and immunohistochemical investigations. This period was selected as the minimum period of time required for formation of a marked astrocytogliotic scar (1–4 weeks [4, 5]). Frontal vibrotome sections of thickness 50 μm were collected from the area delimited by the frontal planes made through a point with coordinates 1 and 5 mm caudal to the bregma. Analyses were performed using pairs of sequential sections separated by 600 μm (12 sections from each rat, six used for each type of staining). Half of the sections were stained with cresyl violet by the Nissl method. Levels of cortical damage were assessed using a Leica DM6000 B microscope (Leica Microsystems, Germany) (Table 1).

Intermittent filament glial fibrillary acidic protein (GFAP) was used as a marker for astrogliocytes, using antibodies diluted 1:500 (Dako, Denmark). Staining was detected using 3,3'-diaminobenzidine. Injury areas were evaluated using photomicrographs of sections using ImageJ. Areas of gliosis were identified manually, with calculation of mean values from the results of three measurements in each section of each focus in the cerebral cortex of the ipsilateral hemisphere.

Relationships between measures were evaluated by regression analysis.

Results

Group 1. Analysis of the sequelae of CCT on cerebral cortex sections ipsilateral to the trauma site identified zones of direct and remote damage (Fig. 1).

The zone of direct damage corresponded to the area of immediate contact of the column of liquid with the dura mater, while the remote damage zone was located distant from the first and was separated from it by unaltered nervous tissue.

Astrocyte gliosis in the zone of direct damage was seen on brain sections in six of the eight animals using the reaction for GFAP (see Fig. 1, *b*) and was absence from sham-operated and control animals. Gliosis was most marked in layers I–IV of the cortex on sections taken from the area 3 mm caudal to the bregma (primary somatosensory cortex, primary motor cortex). This area showed no changes to neurons on staining by the Nissl method.

The zone of direct damage corresponded to the shape of the trepanned aperture with its center at a point 3 mm caudal to the bregma. There was a tendency for the area to decrease both rostrally and caudally to this point: $F(df_1 = 2, df_2 = 30) = 6.277$, $p = 0.005$ (Fig. 2, *a*).

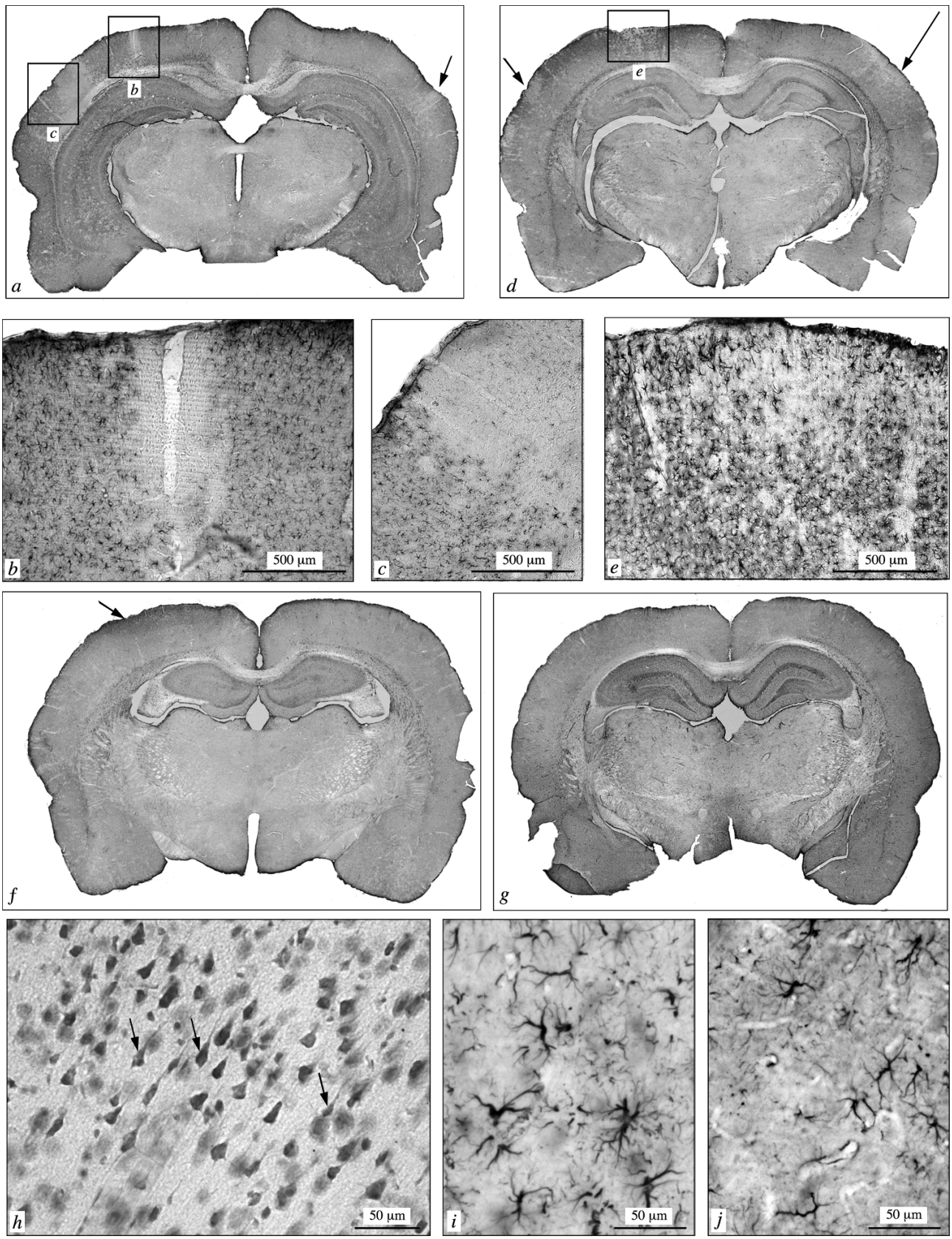
In the remote damage zone, astrocyte gliosis was seen on brain slices from eight out of eight animals using the GFAP reaction (see Fig. 1, *d*). The zone was located in layers I–VI of the lateral segments of the cortex (primary somatosensory zone, auditory cortex, visual cortex, some associative zones), and had a complex shape with a focus of direct damage ($F(df_1 = 2, df_2 = 42) = 3.189$, $p = 0.051$), though with a tendency to a more caudal location of the center of the damage zone (see Fig. 2, *b*). Brain sections from three of the six animals showed a layer of tissue with unaltered astrocyte glia between the zones; gliosis zones were visually discriminable in three of the six animals but were connected by a band of gliosis in cortical layers I–III. Thus, the remote damage zone was located ventrally, laterally, and caudally to the direct damage zone.

The position of the remote damage zone coincided in all animals with CCT (see Fig. 1, *f*).

Brain sections from the eight animals showed no clear relationship between the areas of gliosis in each of the zones or the total area in both zones with blow force.

Morphological changes identified in the cerebral cortex after CCT by staining using the Nissl method are shown in Fig. 3. The relationship between morphological changes in the remote damage zone and blow force on modeling of CCT was assessed on a single section from each rat taken from a level of 3.5 mm caudal to the bregma (Fig. 4).

Group 2. All rats of this group, including controls, were characterized by minor changes, with areas showing ischemically altered neurons (extended triangular shapes with dark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cytoplasm and nuclei, and twisted apical dendrites), probably located along the courses of vessels, along with a general increase in the immunoreactivity of the cortex for GFAP, a diffuse distribution of poorly staining foci ("cleared" zones), and deposits of lipofuscin in the extracellular space.

The sequelae of severe CCT in rats of group 2 were significantly different in terms of their histological changes from those in group 1. Astrocyte gliosis in the direct and remote damage zones in rats of group 2 had a different structure: cells were sparse, with "cleared" zones, and there were no astroglia along large vessels. In addition, significant diffuse damage in the cortex of both hemispheres was seen (Fig. 5, *d*, *h–j*).

The direct brain damage zones in all animals showed clear superficial gliosis with astroglia-free foci of "clearing" (see Fig. 5, *a*, *c*, *e*). Minor astroglial activation in this zone was seen in sham-operated rats but was absent from controls. There was an increase in the number of ischemically altered neurons. The direct damage zone in rats of group 2 had generally the same spatial characteristics as the direct damage zone in rats of group 1.

Damage in the remote zone was seen in four out of five animals with CCT and was absent from control rats. This zone included ischemic foci, along with a minor increase in the number of glial cells (damage rated at 1–2 points). Nonuniform gliosis was seen, with zones of "clearing" (see Fig. 5, *a*, *b*, *e*). The zones of necrosis typical of severe CCT in rats of group 1 were absent.

Diffuse brain damage (multiple ischemically altered neurons, absence of astroglia along vessels) was severe in both hemispheres of all rats with CCT. These profound changes were absent from control animals (see Fig. 5, *f*, *g*).

Discussion

Studies of histological changes in the brain after dosed CCT showed that the sequelae of fluid percussion consisted of the appearance of two zones of damage in the ipsilateral hemisphere. It is likely that the formation of zones in different locations and with different morphological features is associated with different biomechanisms for the trauma.

The origin of the first zone directly beneath the trepanned aperture appeared to be linked with a particular type of deformation – compression of brain tissue by the fluid percussion. This focus reproduced the shape of the trepanned aperture, the area of maximal damage being located beneath the center of the aperture, probably corresponding to the zone of maximal compression.

A different type of deformation probably occurred in the second zone – displacement of the layers of brain tissue relative to each other. This explains the locations of the foci – the area of maximal damage was located caudal and lateral to the zone of direct damage, i.e., at the boundary of the unaltered and "compressed" nervous tissue. Clear hemorrhage was seen in the remote damage area [4]. These changes are accompanied by edema and, later, formation of foci of coagulative necrosis [3], as demonstrated previously using the fluid percussion model [15]. Hemorrhagic stroke is known to be one of the major causes of convulsive disorders in elderly people [1]. Both the primary and secondary foci can lead to the formation of sources of convulsive discharges during the 10 days after CCT, with subsequent transformation into epilepsy [6].

Our experimental studies used rats of two age groups. The brains of 24-month-old rats showed moderate gliosis with poorly staining foci and some ischemically altered neurons. The histological sequelae of CCT in rats of this group were more diffuse than in rats aged 12 months, which may be due to lower reactivity and "superimposition" on concomitant age-related brain pathology. Brain damage in control and concomitant pathology in experimental rats at age 24 months may depend on age-related changes to the microcirculation. These changes were not seen in the brains of 12-month-old control animals – the sequelae of dosed CCT were local in nature.

It is likely that the vascular bed of the brain in Wistar rats is extremely vulnerable and has local instabilities in relation to external influences. Krushinskii–Molodkina rats, which have a genetic form of audiogenic convulsions, are derived from Wistar rats. Rats of this strain respond to loud sounds by developing convulsive seizures, and morphological examination demonstrates multiple intracerebral hemorrhages. Latent audiogenicity has been demonstrated in 10–15% of Wistar rats [2]. This limits their use in long-term chronic experiments not specifically addressing studies of convulsive activity.

The reproducibility and appropriateness of the model allows it to be used for investigations of the molecular genetic mechanisms of the sequelae of CCT in humans and to identify the common mechanisms of the sequelae of CCT and the pathogenesis of important diseases comorbid with CCT, particularly depression and epilepsy.

Thus, regardless of the animals' ages, fluid percussion induced the formation of two foci of damage to the cerebral cortex of the ipsilateral hemisphere. Damage depended on

Fig. 5. Cerebral cortex in rats of group 2 – controls and after craniocerebral trauma (CCT). *a*) Section at the level of 4.8 mm caudal to the bregma; *b*) changes in the direct damage zone (shown by square in (*a*)): a large vessel is surrounded by a zone with a weak reaction for glial fibrillary acidic protein (GFAP) (an analogous zone with a vessel of smaller caliber in the contralateral hemisphere in (*a*) is shown by an arrow); *c*) remote damage zone with focal absence of astroglia apparent as a "clearing" (shown by square in (*a*)); *d*) section at the level of 4.5 mm caudal to the bregma; *e*) part of the cortex with diffuse damage in the form of a "clearing" in the direct damage zone (shown in (*d*) by square, an area with similar damage is shown by arrows (short in the ipsilateral and long on the contralateral hemispheres); *f*) section of brain from a sham-operated rat with superficial gliosis in the craniotomy zone (arrow); *g*) section of brain from a control rat showing no damage; *h*) cortex of contralateral hemisphere of the brain from a rat with groups of ischemically altered neurons (arrows) after CCT; *i*) astrocytes in remote damage zone of the ipsilateral hemisphere and *j*) the contralateral hemisphere. *a–g*, *i*, *j*) reaction for GFAP; *h*) Nissl staining. *a*, *d*, *f*, *g*) Objective ×10, ocular ×20.

blow force and was characterized by changes to neuron structure and the formation of a homogeneous astroglial scar.

In rats aged 24 months, there was a predominance of diffuse damage with ischemically altered neurons along vessels, with formation of an inhomogeneous astroglial scar with diffusely located, poorly staining foci and weak neuron reactions, which was not characteristic of 12-month-old animals.

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