MORPHOLOGIC CHANGES IN PREGANGLIONIC SYMPATHETIC NEURONS OF RAT FOLLOWING EXERCISE AND AGING

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ABSTRACT

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By

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Morphologic changes in neurons placed under a functional load have been studied at several sites in brain and spinal cord. Chromatolysis (Mattahews and Raisman, 1972) and dimensional changes (Gilliam, 1973) are consistent findings in neurons subjected to increased functional activity. It is the purpose of this study to record changes in sympathetic preganglionic cells of the intermediolateral horn in rat spinal cord after exercise and aging.

Adult male Sprague-Dawley albino rats were forced to exercise on running wheels at two levels of intensity for periods of eight and 16 weeks. The spinal cord segments T1 through T5 were excised, fixed in Carnoy's solution, embedded in paraffin, sectioned and stained with gallocyanin-chromalum. Light transmission through cytoplasm was measured photometrically. Cross-sectional surface area of the nucleus and diameter of nucleolus were measured microscopically with an ocular micrometer. Finally, Nissl substance morphology was subjectively evaluated.

Significant age related changes were found. Older rats presented darker cytoplasm, smaller nuclei, larger nucleoli, and clumped Nissl bodies.

No significant effect of exercise was detected.

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DEDICATION

To Midge, for her love and understanding

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INTRODUCTION

Axon section, electrical stimulation, and exercise are known to produce neuronal alterations including increases or decreases in stainability with basic dyes, alterations in appearance of Nissl bodies, and dimensional changes in soma, nucleus, and nucleolus. Morphology and biochemistry within the neuron have been correlated to metabolic events such as increased protein synthesis. Investigators have studied changes in neuron structure of many brain and spinal cord areas, but none have been found which deal with the spinal cord component of the sympathetic nervous system: cells of the intermediolateral horn.

This investigation incorporated young rats subjected to exercise periods for several weeks and was conducted to answer the following questions:

1) Do intermediolateral horn cells of rats subjected to chronic exercise programs exhibit characteristic signs of increased neuron activity?

2) Are age changes in the intermediolateral horn detectable between 12-, 20-, and 28-week-old rats?

3) Does sacrifice time after exercise influence neuronal morphology?

REVIEW OF LITERATURE

Morphologic Changes in Neurons

Acute Exhaustive Exercise by Running

Mann (1894) exhausted a dog by subjecting it to 10 hours of running. He found the pyramidal cells of the motor cortex were less intensely stained than those of a control dog who had not run. The nuclei of the motor cells of the lumbar spinal cord were found to be more pale than the cytoplasm in the control dog. However, the nuclei were darker than the cytoplasm and "shriveled" in the fatigued dog.

Dolley (1909a) showed that in dogs exhausted by running on a treadmill and killed 4 hours after exercise, the numbers of hyperchromatic, hypochromatic, and "exhausted" Purkinje cells were increased. In hyperchromatic cells, the cell and nuclear volumes were smaller than controls, while both were larger than controls in hypochromatic cells. In the same study, one dog was killed immediately after exercise. A shift away from hypochromatic and exhausted cells toward the hyperchromatic cell type was observed.

Hyden (1943) found an increase in nucleolar size but no change in cell volume of anterior horn cells from guinea pigs forced to run to exhaustion on a work machine. Hyden stated that increased nucleolar size is an indication of "an intensively functioning protein-forming system in the cell."

Hochberg (1955) exhausted 20 male rabbits and stained the anterior horn cells of the lumbar spinal cord according to Einarson's gallocyanin-chromalum technique (Einarson, 1951) and measured basophilia photometrically. The animals were sacrificed 0, 2, 3, 4, 12, 15, 18, 24, 36, and 48 hours after exhaustion. The cells of the animals killed 2, 4, and 12 hours after exercise were predominantly hyperchromatic, while those killed at 0, 6, 15, 18, and 24 hours were hypochromatic. The nuclei of the hyperchromatic cells were also hyperchromatic while the nuclei of the paler cells were also pale. Nuclei of the paler cells were also pale. Nuclei were eccentrically located in the cells of 12- and 15-hour animals. The cells of the animals killed 48 hours after exercise had recovered to a normal resting level and were indistinguishable from controls.

In contrast to other studies, Kocher (1916) reported no changes in spinal cord cells nor brain cells of rats after the animals were exercised for 12 hours in a running wheel.

Acute Subexhaustive Exercise by Running

Only a few reports of subexhaustive acute running have been reported. Edstrom (1957) suspended the back legs of guinea pigs and forced them to run 20 to 30 minutes. He found the cytoplasm of the cervical ventral horn cells more pale than controls, but observed no changes in nuclear staining. Both cytoplasmic and nuclear volumes were increased 17% while nucleolar volume was not changed.

Chance, Lucas and Waterhouse (1956) looked at cells taken from the granular and molecular layers of the cerebellum in mice. They reported no change in the size of the nuclei of these cells after the

mice had run 100 feet in 5 minutes or 200 feet in 10 minutes.

In the report by Dolley (1909a) mentioned previously, three additional dogs were exercised short of exhaustion for 15, 30, and 60 minutes. The main difference between the control and the dog run for 15 minutes was the noticeably fewer numbers of "resting cells." After 30 minutes, there was an increase in numbers of chromophilic cells with smaller nuclear and soma volumes and chromophobic cells with increased soma and nuclear volumes. After 1 hour the numbers of chromophilic and chromophobic cells were increased more than at 30 minutes with a substantial number of cells in the late stages of chromatolysis or exhaustion.

Chronic Exercise by Running

Chronic exercise is that regimen which involves an extended period of training before the animal is sacrificed. Thus, an individual may be subjected to the same intensity of exertion every day for a month as opposed to an acute exercise which consists of only one exercise bout with no previous experience. Unfortunately, there are very few studies of this type.

Edstrom (1957) exercised guinea pigs between 10 and 40 minutes per day for 29 days and sacrificed after 18 hours of rest. Volume determinations revealed no change in nucleus or soma volumes. However, a 141% size increase in the nucleolus was reported. The nucleus and nucleolus were also noted to be paler than those of controls.

Gilliam (1973) demonstrated that changes occurred in the soma, nucleus, and nucleolar volumes and cytoplasmic staining in ventral horn cells of rats. He stated that the type of change depended on

the duration of the exercise period and the intensity of the exercise program. Animals exercised at low intensity for eight weeks had a greater number of large motor neurons than did the high intensity group, but at 12 weeks a decrease in the number of large motor neurons in the low intensity group had occurred. Gilliam's study also demonstrated a direct relationship between cytoplasmic volume and staining intensity which is in contrast to most other studies which describe the relationship as inverse.

Exercise by Swimming

The size of cell nuclei in the granular and molecular layers of the cerebellum of mice was shown, by Chance *et al*. (1956), to increase after swimming for 5, 10, and 12 minutes. Matz, Larina, and Geinismann (1970), however, reported that as swimming time for rats increased through 5, 10, 20, and 40 minutes, the volume of both nucleus and cytoplasm of ventral horn cells decreased.

Dye (1927) swam rats for various durations between 15 minutes and 5 hours 15 minutes, allowing only a 5-minute rest per hour during the longer sessions. He found an increasing number of dark and decreasing number of light staining Purkinje cells as the swimming time increased. Spinal ganglia and ventral horn cells demonstrated increased stainability and decreased soma and nucleus size and increased nucleolus size from 15 minutes to 1 hour. This was followed by a gradual loss of stainability and an associated increase in soma size.

Geinismann, Larina and Matz (1971) attached tail weights to rats amounting to 1/11 or 1/33 of total body weight. The animals swam 5,

10, 15, and 40 minutes and were sacrificed immediately. The cytoplasmic volume of anterior horn cells in rats with lighter tail weights was constant until 40 minutes had elapsed, at which time the volume had decreased. The cytoplasmic volume of cells from rats with the heavier weights demonstrated decreased cytoplasmic volume by 5 minutes and were smaller after 40 minutes than the group with lighter tail weights. The nuclear volume increased after 5 minutes but returned to normal by 40 minutes in animals with lighter tail weights. Rats with heavier tail weights showed a gradual decrease in nuclear volume beginning at 10 minutes.

Tumanov and Kriitskaya (1967) reported the results of a chronic exercise program by swimming. Rats were made to swim 2 hours twice per week for 6 months. The last exercise bout consisted of 8 trained and 8 previously untrained rats. Both groups swam 4 hours. Cells of the sensory-motor cortex of the trained animals were indistinguishable from controls while the cells of the untrained rats had larger soma and nucleus size and were hypochromatic.

Kocher (1916) reported no changes in any parameters after swimming.

Miscellaneous Reports

Several other reports describe the state of neurons under different types of physiologic stimuli other than exercise. Rods of a covered eye in dog and rabbit are smaller and richer in chromatin than rods of an uncovered eye. Also, the cells of the lateral geniculate bodies, corpora quadrigemini, and occipital cortex are smaller and darker in the pathway of the covered eye (Mann, 1894).

Another type of inhibition, where rabbits were placed in confinement cages made to the approximate size and shape of their body, was reported to cause increased staining in spinal cord motor neurons associated with increased nucleolar size (Mikhailenki, 1971). The staining intensity was greater as the confinement time was made longer. The cells of the motor cortex, however, were chromatolytic.

Einarson (1933) caused spastic extension of one limb and flaccidity of the other by rapid administration of ether. Ventral horn cells demonstrated chromophobia in cells innervating the spactic limb and chromophilia in cells innervating the flaccid limb. Einarson interpreted this a reflex inhibition phenomenon with the neurons innervating the flaccid limb being darker. The nucleoli of the cells innervating the spastic limb were larger than those of the flaccid limb.

It has also been demonstrated that hypoxia initially produces increased chromatin material and decreased cytoplasmic volume. This is followed by decreased chromatin and increased cytoplasmic volume until cell death, at which time no chromatin material is observed (Dolley, 1909b).

Quantitative Changes in RNA Content

In addition to morphologic changes in neurons subjected to alterations of functional level, the total cell content of RNA may also change. It is equally difficult, however, to define specific trends about changes in RNA content because of the lack of consistency among the experimental methods encountered in the various reports.

Two major cytological methods for studying RNA content are employed. One depends on the character of purine and pyrimidine bases to absorb ultraviolet (UV) light at the specific wavelength of 260 nm. A negative exposure of a nerve cell body is photographically recorded. The degree of darkening reflects the concentration of nucleic acid in any portion of the cell. Other than a small and uniform amount of DNA, the nucleic acid being recorded is RNA. A second method used to determine RNA content is quantitative histochemistry. The accuracy of this method depends on the ability of a histochemical stain to bind in stoichiometric amounts to nucleic acids. Einarson's gallocyanin-chromalum and Branchet's methyl-green-pyronin are considered to have this property (Pearse, 1961). The stained sections are analyzed photometrically with the amount of light transmitted reflecting the concentration of RNA. This measurement is analogous to darkening values obtained from UV cytospectrophotometry. In each method, the RNA content is evaluated by comparing the concentration of nucleic acid and the volume of the nerve cell body.

Changes in RNA Content of Nerve Cell Bodies Produced by Stressing the Intact Animal

One of the earliest attempts to quantify nucleic acids in nerve cells was by Hyden (1943). Four guinea pigs were made to run to exhaustion (about 2 hours) on a running wheel. Quantitative estimations of RNA content by UV absorption of anterior horn cells of the cervical, lumbar, and sacral spinal cord were determined. Cytoplasm of cells from control animals demonstrated high nucleic acid concentration while the cells from exhausted animals revealed a marked

decrease in concentration. Since changes in soma volume were not observed, it was concluded that the content of nucleic acid in the soma of cells from exhausted animals was 3 to 5 times lower than controls.

More recently, Pevzner (1971) investigated anterior horn cells of rats that had been swimming for 2, 3, and 4 hours. Ultraviolet absorption revealed a 40% and a 50% increase in RNA content after 3 and 4 hours, respectively. The RNA content of cells of animals that had swum 3 hours gradually returned to control values after 6 hours of rest. In the same study, rats were forced to stand on an electrified grid for 5, 10, 20, and 60 minutes. The RNA content of anterior horn cells increased by 11% after 5 minutes, then decreased to 10% below control after 10 minutes, came back to control after 20 minutes, then dropped to 20% below control after 60 minutes. The cause of "pseudonormalization" at 20 minutes is unknown. The cells of animals swimming for 60 minutes returned to normal after 18 hours.

Geinismann, Larina, and Matz (1970) used UV cytospectrophotometry to determine RNA content in anterior horn cells of rat spinal cord. They reported decreases in RNA content whenever artificial or natural stimulation was of sufficient intensity and duration.

Edstrom and Eichner (1958) reported a change in RNA content in the cells of the supraoptic nucleus of rats after sodium chloride administration for 2 months. RNA content was determined microchemically and observed to be higher in animals that had received sodium chloride than in those that had not. The investigators defined the stimulus as an increased hormone production.

Lodin, Flatin, Kazakasuili, Hartman and Muller (1968) flushed into one ear water at 50°C for one hour in each of 50 rabbits. The animals were sacrificed immediately after stimulation. Portions of the cerebellum were stained with Einarson's gallocyanin-chromalum and stainability determined photometrically. The RNA content increased 30% in Purkinje cells and 50% in Golgi and basket cells of the stimulated side. The increases were greatest in the nucleus and nucleolus while the chtoplasmic increases were less marked.

Morphologic and Composition Changes Produced in Nerve Cells by Axon Injury

Although axon ligation or section is not the specific problem of this research, the most uniform and consistent experimental results are recorded by these techniques of nerve cell stress. The changes produced by axon injury are mentioned here as a model for comparing and understanding the changes seen during less uniform types of neuronal stress such as incresaed functional activity.

Nicholson (1924) unilaterally ligated the hypoglossal nerve of rats midway between its foramen of exit and its attachment to the digastric muscle. The medulla was then sectioned and stained. Animals lived from 1 day to 6 months after the nerve action or ligation.

After ligation, degenerative changes were seen for the first 15 days. Regenerative changes occurred from 16 to 44 days after which time the cells were normal. Swelling of the cytoplasm began on the first day and increased gradually through the ninth day. This increase in cell size is seen only in frozen or fresh sections while shrinkage is observed in fixed sections. Some investigators (Barr

and Hamilton, 1948) feel that shrunken nerve cells are the result of histologic technique and not experiment. The swelling observed in fresh or frozen sections persists until 44 days and longer in some cells.

The Nissl substance began disappearing around the nucleus 24 hours after ligation and by the second and third days the remaining Nissl substance began to clump. From four to eight days, peripheral Nissl masses began breaking up and became more diffuse. From the ninth through 15th days, the Nissl substance disappeared. The reformation of Nissl substance, beginning on the 16th day, was marked by accumulations around the nucleus. It then spread peripherally until a normal Nissl picture was seen by the 44th day.

Gersh and Bodian (1942) looked at the lumbar anterior horn cells of monkeys after severing dorsal and ventral roots unilaterally using the contralateral side as a control. RNA and protein concentrations were measured by UV absorption. The concentrations of protein and nucleic acid were constant for control cells. A decrease in both nucleic acid and protein concentration was observed one day after nerve section and continued through the sixth day. These changes were associated with progressive chromatolytic changes in stained sections and were most severe after six days. The cells remained chromatolytic for 30 days and were normal by 80 days. The UV absorption of nucleic acid and protein increased from six days to control values by 80 days.

In this study, the relative concentrations of nucleic acid and protein were plotted. The slope of the curve (the ratio of nucleic acid to protein) for control and experimental groups was nearly

identical for one and three days. At six days, however, the curve for the experimental group was less steep. This trend persisted through the eighteenth day when it was considerably flatter. By thirty days the slopes were the same as the control animals. These changes in slope may represent either an increased rate of protein synthesis or increased rate of nucleic acid breakdown.

Hyden (1943) showed changes similar to those already mentioned in spinal and nodosal ganglia of rabbits. Ultraviolet absorption of nucleic acids was intense in a normal cell's cytoplasm and, except for a few cells, no change was observed 19 hours after nerve section. By three days after nerve sections, however, the cytoplasm had lost much of its absorbing substance. Fifteen days after nerve section, absorption of nucleic acids was essentially absent. Hyden calculated that, although dilution of cell contents may occur, an increase of 193% in cell volume would be necessary to produce a concentration change of this magnitude. Volume increases were not observed, and it was concluded that a net decrease in nucleic acid had occurred.

Cells observed 28 and 33 days after nerve section showed progressively more absorption of nucleic acids. The great majority of cells 150 days after nerve section were indistinguishable from uninjured cells.

Bodian (1947) described decreased UV absorption associated with decreased Nissl concentration in anterior horn cells of rat spinal cord. He also mentioned that the degree of chromatolysis is directly related to the distance from the cell body to the site of the axon section. Since an axon may represent a volume several hundred times that of the cell body, it is understandable how the amount of

"cytoplasm" removed is related to the regenerative efforts of the neuron and reflected by the degree of chromatolysis.

Barr and Hamilton (1948) cut the left sciatic nerve of 22 cats and stained the lumbar spinal cord using a Cajal reduced silver technique for morphologic measurements and cresyl violet to determine chromatolysis. The cells of the dorsolateral ventral horn were studied three to 136 days after axon section. Chromatolysis was quantified by assigning "N" for normal cells and "1, 2, 3, or 4" for the degree of chromatolysis, "4" being the most advanced stage. A formula was used to indicate the rate of chromatolysis. The rate depended on the percentage of each cell type observed. The calculated rate was then plotted against observation times of 3, 5, 10, 28, 50, 87, and 136 days after nerve section. Maximum changes occurred between 20 and 60 days but had not returned to normal by 136 days. They also found cell volume increased to a maximum of 20% greater than controls after 7 days and had returned to the control level by 4 months. These authors also felt that cell shrinkage is due to fixation and not to experimental treatment.

Brattgard, Edstrom, and Hyden (1957) produced a most informative study employing microchemical analysis to measure RNA content and concentration, protein and mass concentration, and cell volume. The hypoglossal nerve was crushed with a forceps cooled to -70°C. Cell volume increased in two stages. The first, eight to 12 days, was marked by a 50% increase in total organic mass but decreased RNA and mass concentration. The second stage, 12 to two or three months, constituted the largest volume increase. During this stage, RNA content and organic mass both increased 100%.

During the first nine days, RNA content was steady but, since the cell volume increased, the RNA concentration decreased. After nine days, RNA content began increasing and reached 2.2 times control by 48 days, at which time RNA concentration was again normal. It should be mentioned that during the period of decreasing RNA concentration a change in the state of aggregation of the Nissl granules takes place. This may reflect the result of a change in the activity of existing RNA.

Watson (1965) noted higher rates of incorporation of tritiated RNA precursors between two and 20 days in cells of the hypoglossal nuclei of mice whose axons had been sectioned than in normal cells. The maximum ratio of grain density in cytoplasm to grain density in nucleus was higher in injured cells than in normal cells, and never preceded any changes in cell volume. The ratio peaked in injured cells on the third day as did the cell volume. It was shown that when DNA-primed RNA synthesis was stopped with Actinomycin D, the rate of decay of RNA is faster in injured than in control cells. This lends support to the postulate of Brattgard *et al.* that the functional state of RNA is changed during nerve cell stress.

Specific Changes in Nissl Substance Structure

It has been well established that any change in the metabolic level of a neuron is accompanied by a series of changes in the structure and distribution of Nissl material. A well ordered sequence of events proceeds from the beginning of the metabolic influence which ultimately ends in cell death if that influence is of sufficient intensity. The number of stages through which a neuron

passes and the duration of each stage depends on the nature of the influence, type of neuron concerned, and age and species of the animal involved (Geist, 1933).

A normal cell might be considered as one that is in equilibrium with itself; supply and demand are in balance. In the efferent neurons of the sympathetic ganglia, Nissl material of normal cells is evenly distributed and in fairly dense clumps (Matthews and Raisman, 1972). Vraa-Jensen (1956) described the resting levels of neurons in a variety of other nuclei. Patterns in different nuclei vary in density and size of the individual Nissl bodies. Cells were categorized according to function (motor or sensory) with increasing coarseness of Nissl bodies indicating more mature neurons. The most mature cells, in terms of differentiation, are motor neurons. These cells have developmentally passed through the same series of events as less mature cells (Hyden, 1943) but, because of their particular metabolic load, require a more well developed metabolic machinery. When placed under stress, however, all nerve cells follow the same sequence of morphologic changes.

The first change produced by metabolic stress is chromophilia. Dolley (1909a) found an increase in amount and size of Nissl bodies of Purkinje cells. Granules were massed particularly around the nucleus. The density continued increasing to such a degree as to obscure other cell structures.

As the stress was continued, discrete areas of clearing were observed which progressed until most of the cytoplasm appeared much like that of a normal cell. However, the dendrites and dendritic poles were void of Nissl bodies.

The succeeding changes are chromophobic (Matthews and Raisman, 1972) and marked by dissolution of most Nissl bodies to a dustlike appearance. The remaining clumps become distributed around the periphery of the cell. This is the typical picture of "central chromatolysis."

Small aggregations of Nissl substance then appear around the nucleus indicating the beginning of a recovery period. Central aggregations of Nissl substance distribute themselves throughout the cytoplasm. These increase in size and density until a normal cell structure is observed.

These are typical events seen during the axon reaction. In an intact animal subjected to a stress such as exercise, however, neurons may pass only through a few stages before the sequence is reversed and recovery is accomplished.

MATERIALS AND METHODS

This study was designed to demonstrate the changes produced in the cells of the intermediolateral nucleus of the thoracic spinal cord as a result of exercise and duration (age). The experimental design, surgical technique, histologic treatment, methods of data collection, and statistical procedures are discussed in this section.

Research Design

The design for this experiment was a 3 x 3 x 2 factorial (Table 1). The first factor, exercise, consisted of 3 groups: 1) a control

Table 1. Experimental design and number of animals per cell (n)

	Duration					
	0 week		8 week		16 week	
	Sacrifice time		Sacrifice time		Sacrifice time	
Exercise	15 min	72 hr	15 min	72 hr	15 min	72 hr
Control	4	4	4	4	4	4
Short	4	4	4	4	4	4
Long	4	4	4	4	4	4

group, 2) a group running at high speed in short 10-second bursts, and 3) a group running at slower speeds for periods of 12 minutes 30 seconds. The second factor was treatment duration. The three

durations were 1) no training, 2) 8 weeks of training prior to sacrifice, and 3) 16 weeks of training prior to sacrifice. The third factor was sacrifice time in relation to the last training period. One group was sacrificed immediately (15 minutes) after the last training period, and the second group was sacrificed 72 hours after the last training period.

Eighty-four normal male Sprague-Dawley albino rats were used for the study. The animals were received in two shipments of 72 and 12 animals, respectively. The animals of the first shipment were randomly assigned to the various exercise and duration groups. A 10-day adjustment period was allowed prior to beginning the treatments. Animals of the second shipment were also allowed a 10-day adjustment period and then sacrificed to provide the untrained or zero week animals. All animals either began treatment (8- and 16-week animals) or were sacrificed at 84 days of age.

Since a cell size of n=4 animals was considered sufficient, those rats with the highest training performance were selected for sacrifice. Percent of expected meters run served as the criterion for the final selection of animals for each cell.

Exercise Groups

The three exercise groups were as follows:

Short (SHT). This treatment required short short duration bursts of high speed running. The program was gradually increased in intensity for 37 days. Thereafter, each animal was expected to complete eight exercise bouts with 2.5 minutes of rest between bouts. Each bout consisted of 6 repetitions with 10 seconds work and 40

seconds rest. This group was required to run a relatively fast speed of 99 meters/minute.

Long (LON). This group ran at slow speeds for a long period of time. The program was progressive so that the rats were running at the expected speed on the 37th day. These animals were expected to complete four bouts of exercise with a 2.5 minute rest period between bouts. Each bout consisted of one repetition of 12.5 minutes of continuous work. The animals of this group were required to run a relatively slow speed of 36 meters/minute during the work intervals.

Control (CON). These animals did not participate in any type of experimental exercise program and were maintained in individual sedentary cages until sacrifice.

Duration Groups

Three durations of exercise were studied. Eight animals from each exercise group were sacrificed after 0, 8, and 16 days of training. These animals were 84, 140, and 196 days of age, respectively, on the final treatment day.

Sacrifice Schedule

Four animals from each duration group were sacrificed 15 minutes after the last training bout. Four more animals were sacrificed 72 hours after the last training bout. These two groups represented the immediate (15 min) and 72 hour (72 hr) sacrifice times, respectively.

The exercise treatments were performed on a Control Running Wheel (CRW) developed at the Human Energy Research Laboratory, Michigan State University. The CRW has been described as "...a unique

animal-powered wheel which is capable of inducing small laboratory animals to participate in highly specific programs of reproducible exercise" (Wells and Heusner, 1971). The animals learned to run by avoidance-response operant conditioning. A low-intensity controlled shock current provided motivation for the animals to run. If an animal running on the wheel failed to reach or maintain the required speed, a light turned on inside the wheel. The rat then had two seconds to reach or exceed the expected speed. If he failed to do so, the light turned off and current was applied to the grid on which the rat was running and maintained until the required speed was attained.

The exercise regimens were electronically controlled. Several parameters were selected prior to the beginning of an exercise period. Running speed, length of exercise and rest periods, number of bouts and repetitions per bout were all pre-selected depending on the treatment (Table 2).

A repetition was defined as a running period followed by a rest period, and a bout consisted of several repetitions. Longer rest periods were allowed between bouts if several bouts were required for a complete training period. During the work periods, the running wheel was allowed to turn freely. A brake was automatically applied during the rest periods.

Body weight of each animal was recorded before and after every training period. All of the animals were housed in individual cages (24 cm x 18 cm x 18 cm) during the investigation. Since rats are normally more active at night, the room lighting was regulated to a more convenient schedule for the investigators. The lights were

Table 2. Control Running Wheel exercise programs for SHT and LON groups at the thirty-seventh day of training

Program	Acceleration time (sec)	Time per Rep (sec)	Rest (sec)	Repetitions	No. of bouts	Time between bouts (min)	Shock (ma)	Speed (m/min)	Total program time (min:sec)	Total expected meters run (m)	Total work time (sec)
SHT	2.0	00:12	40	6	8	2.5	1.0	99	52:10	792	480
LON	2.0	12:30	0	1	4	2.5	1.0	36	57:30	1800	3000

turned off between 1:00 p.m. and 1:00 a.m. Exercise periods were conducted between 12:00 p.m. and 9:00 p.m.

A relatively constant environment was maintained by daily handling of the rats as well as temperature and humidity controls. All animals had access to food (Wayne Laboratory Blox) and water *ad libitum*.

Surgical Technique

Each rat was anesthetized by intraperitoneal injection of sodium pentobarbital (Jensal), 4 mg/100 g body weight. The ribs and musculature surrounding the thoracic spinal cord were removed and the second thoracic vertebra was identified by its prominent dorsal spinal process. The intervertebral spaces C8-T1 and T5-T6 were then determined. The vertebrae and spinal cord were sectioned and removed caudal to T5. A circular incision was made between C8-T1 thus freeing vertebrae T1 through T5 from the more cephalic vertebral segments. The freed section of vertebrae was gently slipped from the spinal cord segments it enclosed, thus leaving T1-T5 bare. The cord was cut at C8 and placed in 70 cc of Carnoy's fixative solution. The spinal cord was in the fixative within 30 minutes of the animal's death.

Histologic Preparation

The spinal cord segments were left in Carnoy's solution overnight. The segments were dehydrated in graded alcohols, cleared in methyl benzoate, infiltrated with paraffin at 54°C, and placed in paraffin blocks (Table 3). The cords were oriented in the blocks such that segment T1 would be cut first.

Table 3. Histologic preparation schedule

Carnoy's solution	24 hr	
Wash in running water	all day	
30% alcohol	overnight	
50% alcohol	2 hr	
70% alcohol	2 hr	
95% alcohol	2 hr	
95% alcohol	2 hr	
100% alcohol	1 hr	
100% alcohol	1 hr	
Methyl benzoate-100% Alcohol (1:1)	overnight overnight	
Methyl benzoate	1 hr	
Methyl benzoate	1 hr	
Methyl benzoate-paraffin (1:1)	1 hr	
Paraffin	1 hr	Vacuum 400 mmHg
Paraffin	1 hr	
Embed		

The blocks were sectioned on a rotary microtome at 15 μm and mounted on 35mm leader film (Lab-Line Instruments, Inc.) as described by Pickett, Green, and Sommer (1964). The reels of film were allowed to dry overnight.

The sections were stained by the gallocyanin-chromalum technique of Einarson (1951) and modified by deBoer and Sarnaker (1956). There is substantial support for gallocyanin-chromalum as a specific stain which allows quantitative determinations of cellular RNA concentrations (Goncalves and Haddad, 1969; Pakkenberg, 1962; Pearse, 1961).

The leader film was coated with liquid acrylic (Lab-Line Instruments, Inc.) to serve as a cover slip.

Tissue Analysis

All cells from which data were obtained required a demonstrable nucleus and nucleolus under the light microscope. The first 50 cells for morphometric analysis that met these requirements and were within the boundaries of the intermediolateral nucleus were used (Figures 1 and 2). Every third section was observed to avoid the possibility of repeating measurements from the same cell.

Morphometric Analysis

Long and short axes of cell nuclei were measured by an ocular eyepiece in a Nikon binocular microscope. By knowing the major and minor axes of an ellipse, the cross-sectional surface area may be computed by the following formula: $A = \pi a_1 a_2/4$, where a_1 is the semi-major axis and a_2 is the semi-minor axis. All data for nuclei are therefore expressed as square microns (μ^2).

Figure 2. Cells of intermediolateral horn in rat spinal cord. Gallocyanin-chromalum. 260X.

Figure 1. Intermediolateral horn (arrow) in upper thoracic region of rat spinal cord. Gallocyanin-chromalum. 25X.

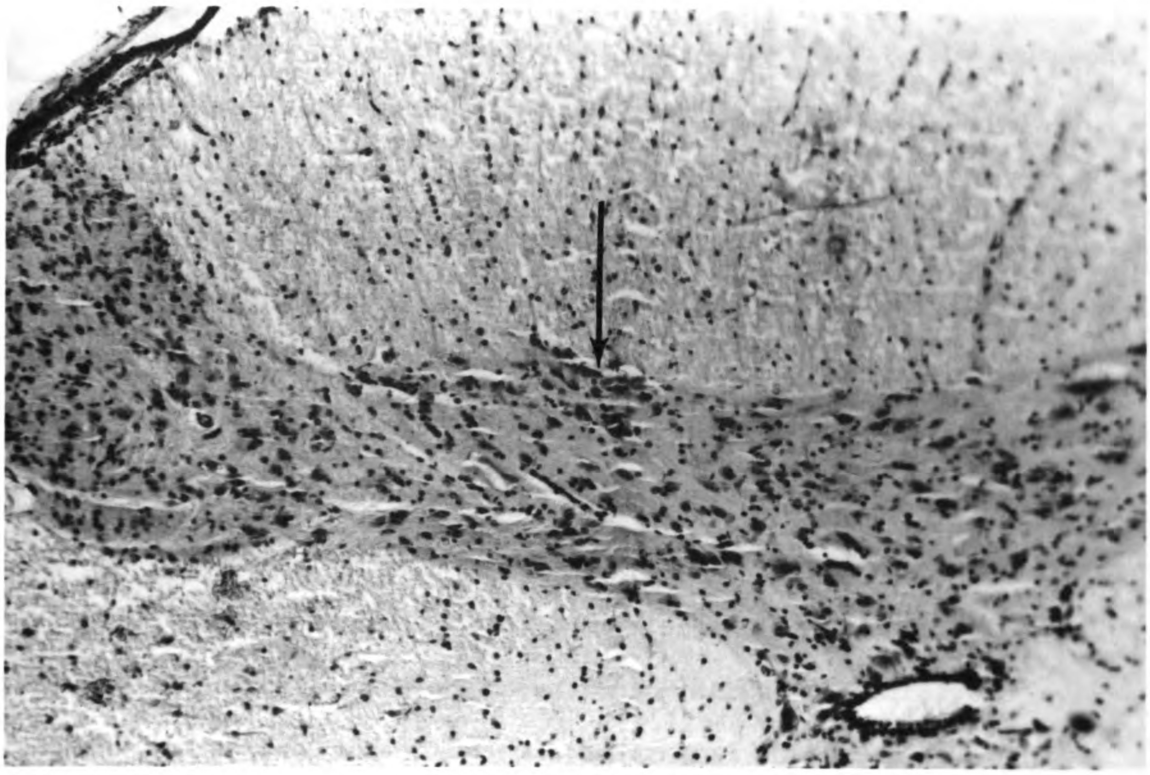

Figure 1

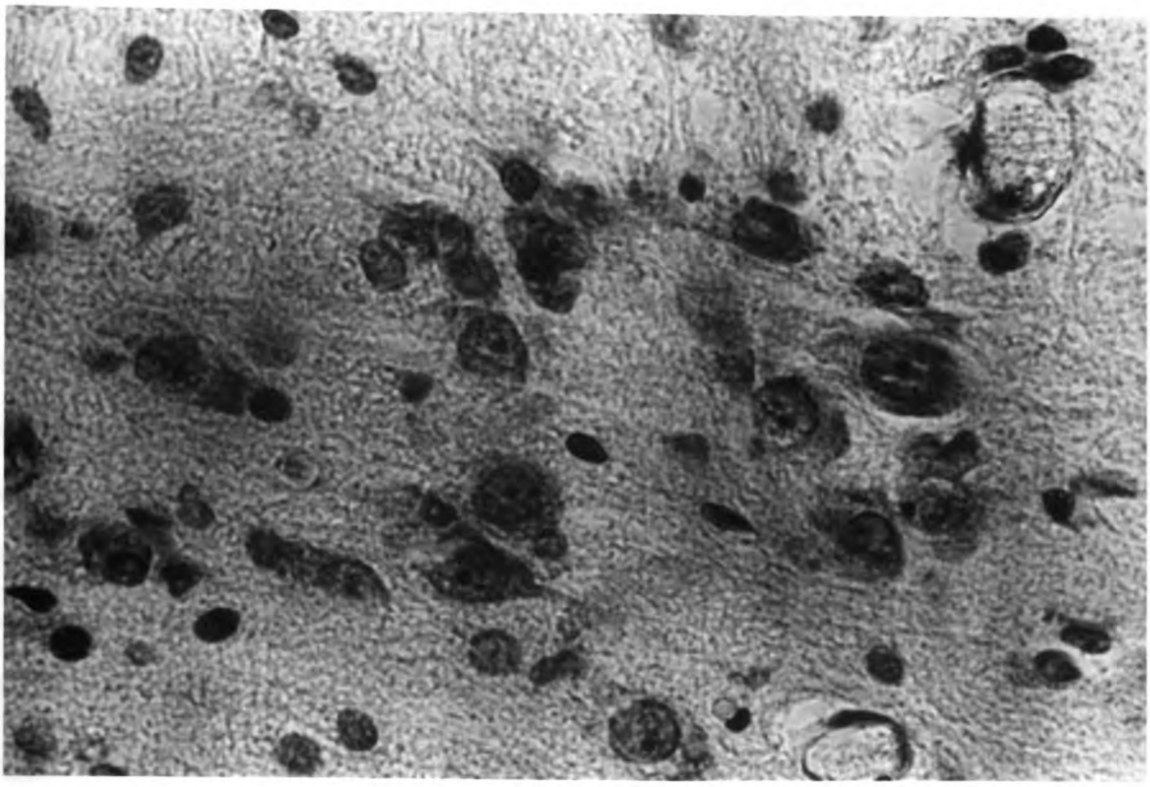

Figure 2

The nucleolus, defined as a sphere, required only one measurement of its diameter. All data for nucleoli are therefore expressed in microns (μm).

A subjective evaluation of Nissl structure was also recorded. All cells which had a granular appearance, but with visible cytoplasm were designated Type 1. Cells which demonstrated large, coarse, dark-staining granules were designated as Type 0 and corresponded to early nerve cell stress. Those cells in which Nissl structure appeared dust-like, diffuse, or homogeneously stained were designated as Type 2. These cells represented early chromatolysis. The designation of Type 3 was assigned to cells demonstrating marked chromatolytic changes. This group usually displayed central chromatolysis and represented advanced nerve cell stress.

Photometric Analysis

The staining intensity of individual cells was accomplished by measuring the relative amount of light transmitted through the cytoplasm with a histochemical photometer (Wells, 1972). The histochemical photometer "...consists of a projecting microscope, a photocell, and a digital display." A transmittance value of a particular cell was accomplished by positioning the area of cytoplasm to be measured over a hole beneath which a photocell is situated. The diameter of the hole was 1/16 inch. By measuring the total magnification with a calibrated glass slide, it was determined that approximately 5 μm of cytoplasm was measured. Total magnification was 330X.

The photocell was manually calibrated prior to scanning each section. Due to the high magnification required during measurement,

a calibration value of 100% transmittance was defined by a digital readout of 25%. The real percent transmittance through any cell was obtained by multiplying the readout by 4. Zero percent light transmittance was set by completely covering the hole through which light reached the photocell. One hundred percent transmittance (a readout of 25%) was defined as the amount of light passing through the acrylic cover slip and leader film. The photocell sensitivity was also calibrated electronically 15 times per second to avoid errors due to bulb aging, power-line voltage fluctuation, or tube fatigue.

Cytoplasm of the intermediolateral horn cells was systematically positioned over the hole. All cells in a section that met the requirements mentioned earlier were measured. Generally, not more than 5 cells were found per section, and both of the bilateral intermediolateral horns were scanned for admissible cells.

Statistics

All quantitative data (transmission and nuclear surface area) were analyzed by analysis of variance (Sokal and Rohlf, 1969). If significance was found, the Student-Newman-Keuls a posteriori stepwise test for comparisons among means was computed. Qualitative data (Nissl index and nucleolar diameter) were analyzed with a Chi-squared test for k independent samples (Seigel, 1956).

RESULTS

These results will be discussed in three parts, each part covering one independent variable. The first part will deal with age, the second with exercise, and the third with the effect of sacrifice time.

Effects of Age

The designations of 0 wk, 8 wk, and 16 wk were employed to define exercise durations. However, the actual ages of rats at the time of sacrifice were 12, 20, and 28 weeks, respectively.

The overall mean light transmittance was 39% at 12 weeks, 36% at 20 weeks, and 33% at 28 weeks (Table 4). Analysis of variance showed that the differences were significant at $\alpha = .05$. An *a posteriori* test (Student-Newman-Keuls) revealed that the comparisons of 12 to 20 weeks and 20 to 28 weeks were not significant. However, a significant decrease was present between 12- and 28-week-old rats. Staining intensity increased between 12 and 28 weeks.

Nuclear surface area for the three groups averaged 96 μm^2, 85 μm^2, and 83 μm^2, respectively (Table 5). After significance was found between these groups, the post hoc test indicated that the 20- and 28-week-old animals were not different but that both were different from the 12-week group. It was concluded that a decrease in

Table 4. Mean light transmission in each age group

Age	% Light Transmittance
12 weeks	39 ± 3
20 weeks	36 ± 6
28 weeks	33 ± 6

the nuclear surface area had occurred between 12 and 20 weeks and 12 and 28 weeks and no significant change from 20 to 28 weeks.

Table 5. Mean nuclear surface area in each age group

Age	Nuclear Surface Area ($m\mu^2$)
12 weeks	96 ± 7
20 weeks	85 ± 9
28 weeks	83 ± 9

Significant changes were also found in the distribution of nucleolar diameters between age groups. Table 6 compares the frequency of nucleolar diameters for each age. The χ^2 value for the contingency table was significant indicating that the distribution of diameters is affected by age. There were fewer small and more large nucleoli in the 28-week age group than in the younger rats. A trend toward larger nucleoli as these animals age must be concluded.

Table 6. Frequency of nucleolar diameters in each age group

Age	Nucleolar Diameters				
	≤1.5	2.0	2.5	3.0	≥3.5
12 weeks	16	336	522	308	18
20 weeks	21	326	532	301	20
28 weeks	2	300	518	356	24

Table 7 shows the frequency of each morphologic type within all age groups. The significant χ^2 value showed that the distribution of morphologic types is dependent on age. A tendency for the frequency of cells with well defined granules (Types 0 and 1) to increase with age is present. This finding may be related to lower light transmission properties of cells from 28-week animals: as particle size increased, light transmission decreased.

Table 7. Frequency of morphologic types in each age group

Age	Morphologic Type			
	0	1	2	3
12 weeks	332	372	376	122
20 weeks	385	362	356	97
28 weeks	424	412	327	37

Effects of Exercise

The mean percent light transmittance was 34% for the control group, 37% for the short, and 37% for the long group (Table 8). None of these values was significantly different from another. It may be concluded that exercise did not alter staining intensity.

Table 8. Mean light transmission in each exercise group

Exercise Group	% Light Transmission
CON	34 ± 6
SHT	37 ± 7
LON	37 ± 9

Table 9 compares the mean nuclear surface area in each exercise group. Nuclear surface area averaged 86 μm^2, 90 μm^2, and 89 μm^2 in the control, short and long groups, respectively. There was no significant effect of exercise on nuclear surface area.

Table 9. Mean nuclear surface area in each exercise group

Exercise Group	Nuclear Surface Area ($m\mu^2$)
CON	86 ± 13
SHT	90 ± 6
LON	89 ± 9

The distribution of nucleolar diameters remained statistically unchanged over all three groups (Table 10).

Table 10. Frequency of nucleolar diameters in each exercise group

Exercise Group	Nucleolar Diameters				
	≤1.5	2.0	2.5	3.0	≥3.5
CON	8	323	553	296	20
SHT	12	318	512	333	25
LON	19	321	507	336	17

There was also no significant effect of exercise on the distribution of morphologic type (Table 11).

Table 11. Frequency of morphologic types in each exercise group

Age	Morphologic Type			
	0	1	2	3
CON	390	387	343	80
SHT	353	378	376	93
LON	396	381	340	83

Effect of Sacrifice Time

There was no change in any parameter resulting from sacrifice time. Percent light transmittance averaged 36 and 35%, respectively, for animals sacrificed 15 minutes and 72 hours after exercise (Table 12).

Table 12. Mean light transmission in each sacrifice group

Sacrifice Time	% Light Transmission
15 minutes	36 ± 6
72 hours	35 ± 8

Nucleus surface area averaged 88 μm^2 in the 15-minute group and 88 μm^2 in the 72-hour animals (Table 13).

Table 13. Mean nuclear surface area in each sacrifice group

Sacrifice Time	Nuclear Surface Area ($m\mu^2$)
15 minutes	88 ± 10
72 hours	88 ± 10

The distribution of nucleolar diameters was likewise unaffected by sacrifice time. Table 14 shows practically no differences in frequency within each diameter.

Table 14. Frequency of nucleolar diameters in each sacrifice time

Sacrifice Time	Nucleolar Diameters				
	≤1.5	2.0	2.5	3.0	≥3.5
15 minutes	21	481	797	466	35
72 hours	18	481	775	499	27

Effects of sacrifice time on distribution of morphologic type were also not significant (Table 15).

Table 15. Frequency of morphologic types in each sacrifice time

Sacrifice Time	Morphologic Type			
	0	1	2	3
15 minutes	548	580	527	145
72 hours	591	566	532	111

DISCUSSION

Although some of the parameters studied here are quantitative values, care must be taken in their interpretation. For instance, although changes in light transmission properties may reflect actual *in vivo* changes in RNA concentration, other factors may alter the amount of light able to pass through the cell. Dehydration during tissue preparation may result in cell shrinkage. Gersh and Bodian (1942) found large ice crystal artifacts in chromatolytic cells of frozen sections and suggested that chromatolysis created higher osmotic pressure inside the cell thus increasing intracellular water. Dehydration would result in increased density of intracellular contents.

Attempts have been made to estimate relative cellular RNA concentrations by comparing the soma volume and relative light transmission. However, since the amount of cellular water changes under conditions of age and activity, the rate of shrinkage may vary. Even relative comparisons under these circumstances would be invalid.

Although specific statements about cellular RNA content and concentration may not be formulated with the resulted presented in this study, it was possible to determine that the internal environment of the neuron has been altered. Thus, changes in light transmission, nucleolus diameter, or nuclear surface area reflect some undetermined set of alterations within the neuron.

The observations on morphologic appearance may be considered more specific since the structure of Nissl substance is consistent with a particular fixation technique. Chromatolysis and breakup of large Nissl granules are consistent findings in neurons subjected to stimuli that cause variations from the resting functional level (Matthews and Raisman, 1972). Recent studies have shown that the activity of neuronal ribosomes in adult rats are more sensitive to alterations of the intracellular environment than those of younger rats or liver ribosomes. This special character of cerebral ribosomes may be related to changes in the protein synthesizing capabilities required for the neuron to change from one metabolic state to another (Zomzeley, Roberts, Gruber and Brown, 1968), and may be the functional correlate of chromatolysis (Roberts, Zomzeley and Bondy, 1970).

In an attempt to relate this study to the literature, one must appreciate that intermediolateral horn cells have not been studied by these methods prior to this time. Generalizations spanning from one neuronal type to another may be qualitatively justified, but quantitatively very dissimilar (Vraa-Jenson, 1956).

The results of this study have demonstrated no change in preganglionic sympathetic neurons as a result of exercise in and of the parameters observed. The basic conclusion of this finding is that the exercise treatment was not of sufficient intensity to cause a significant alteration from the resting level. The axons of these cells are fairly short compared with those of the ventral horn cells. Thus, the nerve cell body would have a relatively low metabolic requirement for maintaining axonal function. It might be expected

that exercise should cause a detectable increase in sympathetic nervous system activity which would be reflected by changes in the characteristics of the neurons of that system. However, since the primary efferent neurons of the sympathetic nervous system are in the paravertebral ganglion, more nervous activity might be expected there than at the next higher order (pre-ganglionic) cells.

Since there was no effect of exercise on these cells, it is not unexpected that there was also no change related to sacrifice time.

Biochemical changes in the CNS during postnatal development of the rat have been studied (Ford, 1973). Mean cellular RNA content increases rapidly during the first 3 to 4 postnatal weeks in the anterior horn cells of rats (Haltia, 1970), as does total brain RNA (Adams, 1966) and protein synthesis (Oja, 1966). These and other changes have been correlated with the manifestation of spontaneous behavior and CNS activity (Bolles and Woods, 1964). After this period, RNA content per anterior horn cell remains the same at least up to 180 days (Haltia, 1970), but decreases from 8 weeks to 36 months in hippocampal cells of rats (Ringborg, 1966). Protein synthetic activity has been shown to be highest in young animals (Jakoubek, 1968; Oja, 1966 [abst.]). The results in this study have demonstrated decreased frequency of cells with diffuse Nissl substance from 12 to 28 weeks. This finding is consistent with the picture of decreasing protein synthesis with age. Light transmission results also seem to be consistent with the conclusion of higher synthetic activity in younger rats. However, considering the effect of fixation techniques on intracellular post-fixation appearance, this conclusion must be drawn with some reservation.

Reports of morphologic alterations during maturation are few. Donaldson and Nagasaka (1918) found increased diameters of dorsal root ganglion cells and slight increases in anterior horn cell diameters in rats 17 to 360 days old. Nuclei of the same cells showed only slightly increased diameters with age. Ford and Cohen (1968) showed no volume changes in ventral horn cells from 25 days up to 180 days.

This study has demonstrated decreased nuclear surface area and increased nucleolus diameter in older animals. In view of the conflicting reports relating changes in nuclear and nucleolar dimensions with functional activity, a conclusive statement as to the meaning of these changes is impossible without further investigation.

SUMMARY AND CONCLUSIONS

Seventy-two young male Sprague-Dawley strain rats were forced to exercise daily on controlled running wheels for long periods at low intensity or short bursts at high intensity. Since the study spanned 16 weeks, maturation from 12 to 20 and 28 weeks was also recorded. Cells of the spinal cord component of the sympathetic nervous system (intermediolateral horn) served as the focus for the observations. To determine if changes had occurred in these neurons, variables which might be expected to change were measured. Those variables were cross-sectional area of nucleus, diameter of nucleolus, morphologic presentation of Nissl substance, and percent of light transmitted through the cell cytoplasm after staining by an RNA specific reaction.

It was found that no changes had occurred in any of the dependent variables after either high intensity or low intensity running. Since these cells are second order motor neurons, the stimulus could have been too low an intensity to cause an observable change. Also, the axons of these cells are relatively short and consequently would require less energy for maintenance than longer axons such as those arising from the cells of the paravertebral ganglion. Future studies in this area might be more appropriately directed toward the ganglion cells.

Maturational changes were found from 12 to 28 weeks of age. These changes consisted of decreased nuclear surface area, larger nucleoli, more granular Nissl substance, and darker-staining cytoplasm. Previous reports dealing with dimensional changes in nucleoli and nuclei have varied considerably. It is therefore impossible to draw any conclusive statement as to intracellular changes based on these data. However, condensation and clumping of Nissl substance has been consistently observed during recovery of neurons from many types of stimuli. It is logical to conclude that the neurons in this study are adjusting toward an equilibrium condition characterized by granular Nissl substance and increased staining intensity.

Although changes in the rate of protein synthesis are accompanied by alterations in Nissl structure and staining intensity, the exact nature of chromatolysis remains to be defined.

The major criticism of this work is the same question that afflicts others working in this area: Are the observed changes the result of treatment or of histologic technique? Certainly changes have been detected in this study which can be directly related to age, but whether the direction and magnitude of those changes reflect *in vivo* conditions is a matter for further investigation.

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