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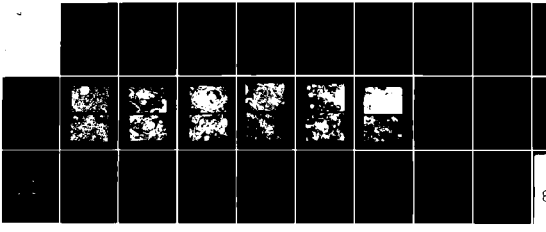

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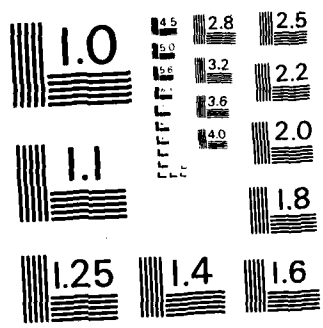

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RESPONSES OF CHOLINERGIC AND NONCHOLINERGIC RENSHAW CELL RECEPTORS AFTER ACUTE AND CHRONIC EXPOSURE TO ANTICHOLINESTERASES (U)

ANNUAL SUMMARY REPORT

WILLIAM G. VAN METER, Ph. D.

July 1983

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Investigation of cat lumbar 7 segment (L-7) Ventral horn cells (motoneurones and interneurones-Renshaw cells) in the presence of excess acetylcholine (ACh) are being studied as models of a central cholinergic transmitting synapse. Preliminary studies to investigate effects induced by chronic low dose exposure to diisopropylfluorophosphate (DFP) have begun with a two week exposure protocol with DFP doses of 0.1, 0.2, 0.5, 0.75, and 1.0 mg/kg/day s.c. Cats tolerate doses of 0.1 to 0.5 mg/kg/day s.c. with

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20. ABSTRACT

symptoms of increased defecation, moderate constriction of pupils, slight ataxia and weight loss. The degree of severity increases with dose. However, with doses of 0.75 and 1.0 mg/kg/day s.c., DFP induces profound weight loss, ataxia, and marked dehydration after three days of treatment. If the 1.0 mg/kg/day s.c. DFP is discontinued after three days and replacement fluids instituted as well as atropine methyl nitrate (0.3-1.0 mg/kg/day s.c.), cats die in two to five days post DFP. On the other hand, if DFP in doses of 0.75 mg/kg/day s.c. are discontinued after three days, the cats survive two weeks with or without replacement fluids and/or atropine methyl nitrate.

Renshaw cell unit potentials (RUP) and their field potentials (RFP) are being studied to determine effects from exposure to DFP in doses according to the protocol above. In control animals, RFP respond similar to RUP during and subsequent to repetitive stimulation at 20 Hz. to include post tetanic depression and post tetanic potentiation. On the other hand, animals treated with DFP in doses from 0.1 - 0.5 mg/kg/day s.c. for fourteen days show an enhanced RFP which is more readily obtained than in untreated controls. The RUP are less readily observed in these animals but response to repetitive stimulation is similar to untreated controls.

Effects of drug treatment on the ventral horn neurones of L-7 are being studied with light microscopy (LM) and electron microscopy (EM) histochemistry. Control sections show L-7 ventral horn to have predominatly dark motoneurones. The copper trapping method shows reaction product in rough endoplasmic reticulum and preliminary observations have indicated the feasibility of combining the phosphotungstic acid stain for synapses with the cholinesterase histochemical methods to permit assessment of cholinesterase containing synapses and changes therin as a result of the chronic treatments. Finally, preliminary data indicate motoneurone degeneration and some demyelination in cats treated with DFP 0.2 mg/kg/day s.c. for fourteen days.

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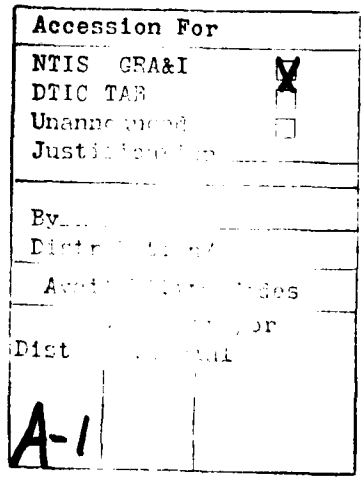

ABSTRACT:

Investigation of cat lumbar 7 segment (L-7) ventral horn cells (motoneurones and interneurones-Renshaw cells) in the presence of excess acetylcholine (ACh) are being studied as models of a central cholinergic transmitting synapse. Preliminary studies to investigate effects induced by chronic low dose exposure to diisopropylfluorophosphate (DFP) have begun with a two week exposure protocol with DFP doses of 0.1, 0.2, 0.5, 0.75, and 1.0 mg/kg/day s.c. Cats tolerate doses of 0.1 to 0.5 mg/kg/day s.c. with symptoms of increased defecation, moderate constriction of pupils, slight ataxia and weight loss. The degree of severity increases with dose. However, with doses of 0.75 and 1.0 mg/kg/day s.c., DFP induces profound weight loss, ataxia, and marked dehydration after three days of treatment. If the 1.0 mg/kg/day s.c. DFP is discontinued after three days and replacement fluids instituted as well as atropine methyl nitrate (0.3 - 1.0 mg/kg s.c.), cats die in two to five days post DFP. On the other hand, if DFP in doses of 0.75 mg/kg/day s.c. are discontinued after three days, the cats survive two weeks with or without replacement fluids and/or atropine methyl nitrate.

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Effects of drug treatment on the ventral horn neurones of L-7 are being studied with light microscopy (LM) and electron microscopy (EM) histochemistry. Control sections show L-7 ventral horn to have predominatly dark motoneurones. The copper trapping method shows reaction product in rough endoplasmic reticulum and preliminary observations have indicated the feasibility of combining the phosphotungstic acid stain for synapses with the cholinesterase histochemical methods to permit assessment of cholinesterase containing synapses and changes therein as a result of the chronic treatments. Finally, preliminary data indicate motoneurone degeneration and some demyelination in cats treated with DFP 0.2 mg/kg/day s.c. for fourteen days.

FOREWARD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION:

Renshaw cell activity in the cat lumbar spinal cord provides a model for the study of cholinergic neurotransmission in the CNS (Eccles et al 1954). Organophosphates have been reported to prolong the repetitive discharge of these cells to antidromic stimulation of the ventral spinal roots (Eccles et al 1956), supposedly by a persistence of the action of acetylcholine. Furthermore, chronic exposure to DFP (diisopropylfluorophosphate), has been shown to decrease the number and sensitivity of muscarinic cholinergic receptors in the rat caudate nucleus (Ehlert and Kokka 1978).

The present report contains preliminary morphological, and electrophysiological/pharmacological, data on the responses of these cells and on neurones in the anterior horn of the cat lumbar spinal cord to electrical stimulation and to chronic exposure to DFP. In addition, gross behavioral observations have been made on cats during the period of chronic exposure to DFP.

METHODS:

A. MORPHOLOGY:

GENERAL-- A total of 34 spinal cords and 8 brains of normal and DFP treated male and spayed female cats have been fixed and are being processed for electron microscopic analysis of motoneurones and synaptic complexes. To date, only the spinal cord lumbar segment 7 (L-7) has been studied (1,226 specimens have been embedded and 290 of these have been sectioned).

Glutaraldehyde and formaldehyde have been selected as immersion fixatives because of the preservation of fine structure. However, a compromise between detailed fine structure and suitable reaction product with histochemistry of cholinesterases had to be made, and the following concentrations of the aldehydes were used; 1) 5% glutaraldehyde, 2) 3% glutaraldehyde + 2% paraformaldehyde, 3) 3% formaldehyde + 2% glutaraldehyde, 4) 2.5% parafromaldehyde + 1% glutaraldehyde. The fixed samples were then treated as follows; 1) osmium tetroxide (OsO_4) + uranyl acetate (UA) for conventional electron microscopy, 2) (ChE's) a modified thiocholine procedure to visualize cholinesterases at the EM level, 3) ethanolic phosphotungstic acid (E-PTA) for the demonstration of synapses and 4) a combination of 2) and 3) to demonstrate synapses that stain for ChE's.

CONVENTIONAL EM (OsO_4 + UA)-- After fixation of 17h to 48h, tissue blocks were washed three times in phosphate buffer and 4% glucose (pH 7.3-7.4). Postfixation for 1 to 1.5h in veronal acetate buffered 1% OsO_4 was followed by 3 washes in veronal acetate buffer (pH 7.3-7.4). Uranyl acetate (2%) in veronal acetate was applied en block. Tissues were then dehydrated in a series of graded ethanol solutions, quickly passed through acetone and embedded in a Poly-Bed 812/Araldite mixure, Spurr, or Maraglas.

HISTOCHEMISTRY FOR CHOLINESTERASES--A modified copper trapping method was used to demonstrate the presence of inhibited (irreversible) cholinesterases at the LM and EM levels. After fixation, the tissues were either washed 3 times in Tyrode's solution (pH 7.3-7.4) or were incubated for 2-5min in a buffer solution with 10^{-3}, 10^{-7} M DFP followed by a 3 washes in Tyrode solution. Incubation Medium I contained sodium citrate, cupric sulfate, phosphate buffer and acetylthiocholine iodide (AcThCh-I).

Tissues were incubated 40 min to 1.5hours after which they were washed 3 times in Tyrode's solution and incubated for 20-60 minutes in Medium II (potassium ferricyanide). Specimens were postfixed in 1% OsO_4 (in phosphate buffer), dehydrated, and embedded (Poly-Bed 812/Araldite or Spurr).

ELECTRON MICROSCOPIC VISUALIZATION OF SYNAPSES--The ethanolic phosphotungstic acid (E-PTA) procedure was used for selective staining of paramembranous material in synapses to permit their exclusive visualization with the electron microscope. After fixation, specimens were washed 3 times in phosphate buffer with 4% sucrose followed by dehydration in graded ethanol. The tissue blocks were then stained in 1% E-PTA (in 100% ethanol + 3 drops of 96% ethanol per 10ml solution) 2 times for 1 hour, rapidly rinsed in propyleneoxide or acetone and embedded in Spurr or Maraglas.

AChE + E-PTA--The procedures for cholinesterases and for synapses were combined in an attempt to demonstrate cholinesterase containing synapses and to distinguish these from either non cholinesterase containing synapses or from synapses whose cholinesterase has been inhibited irreverisbly. Subsequent to fixation, the specimens were washed and incubated in Media I and II as above, followed by dehydration and E-PTA staining also as above, then embedded in Spurr or Maraglas.

B. ELECTROPHYSIOLOGICAL/NEUROPHARMACOLOGICAL:

Adult (2.0-3.0kg) male or spayed female mongrel cats were conditioned for three weeks prior to use in any of the experimental protocols. All animals were taken off feed 24 hours prior to surgical procedures with water available ad libitum. A laminectomy was performed after anaesthetization with DIAL (diallyl barbituric acid and urethane) in doses of 80.0mg/kg i.p. supplanted with minimal amounts of pentobarbital as needed. Respiration was unsupported unless muscle fasiculations, movements or respiratory embarassment necessitated the use of gallamine triethiodide (1.0mg/kg) followed by a bilateral pneumothorax and artificial ventilation. Expired CO_2 was monitored throughout by a Beckman LB2 monitor. Blood pressure was continuously monitored and maintained above 60 torr by i.v. infusions of 5% dextrose in saline if needed. Penicillin G was administered since the experiments routinely exceeded 12 hours. The lumbosacral spinal cord was exposed by laminectomy with the cord transected at L-1/T-13 after local injection of 2% procaine. Ipsilateral dorsal and ventral spinal roots of spinal segments S-1 through L-6 were isolated and transected prior to being placed on bipolar stimulating electrodes for ortho and antidromic stimulation. After surgical preparation and recovery (1-2hours), the cats ere placed in a spinal unit and the cord covered with a deep layer of medicinal paraffin held in a pool over the back by retained skin flaps. Body and paraffin temperature was kept above 35 degrees celsius by heating pads and infra-red lamps.

Extracellular Renshaw cell unit and field potentials were recorded by conventional means from 2.7M NaCl filled glass micropipettes (tip diameter, 1.0-1.5micron). Data was either recorded on film or was recorded on FM tape for subsequent analysis.

In one group of cats, chronic administration of DFP in sub LD50 doses (0.1-0.75) mg/kg or its vehicle polyethylene glycol (PEG 0.1ml/kg) was

given every 24h for a period of 3 to 21 days. These animals were housed separately and had food and water available ad libitum until the day preceding the laminectomy at which time access to the food was withdrawn. The cats were weighed daily and gross observations were made for symptoms of toxicity. In another group of cats, DFP was administered acutely in sub LD50 and above LD50 doses during the electrophysiological experiment by means of an indwelling catheter in the external jugular vein. All anaesthetized cats were killed at the termintion of the experiment either by overdosing with pentobarbital or by lethal injection of magnesium sulfate.

C. BEHAVIORAL:

Behavioral observations were made on a group of cats given subcutaneous injections of DFP (0.1mg/kg -1.0mg/kg) daily for 3-19 days, and on control cats given 0.1ml/kg polyethylene glycol (PEG, the vehicle for DFP) daily for 14 days.

RESULTS:

A. MORPHOLOGY:

I. CONVENTIONAL ELECTRON MICROSCOPY (OSMIUM TETROXIDE AND URANYL ACETATE)

a. MOTONEURONES--Cat motoneurones of the most ventral part of the anterior horn of spinal segment 7 were studied. About 50% of the motoneurone population show dark cytoplasm and occasionally pyknotic nuclei. These neurones are referred to as 'dark' motoneurones (Fig. 1), and they are present in normal as well as in DFP treated animals. No significant differences could be observed between the cytoplasm of normal and treated animals. However, the number of dark neurones seems to be higher in DFP treated cats. It remains unclear at this point whether these neurones are in a process of degeneration or if their dark appearance is due to fixation and/or post-mortem artifacts. According to Cammermeyer (1972), dark neurones and pyknotic nuclei occur unavoidably in tissues fixed by immersion. Furthermore, dark neurones could represent postmortem artifacts since a few minutes elapses between removal of the spinal cord and fixation of the tissue (Cammermeyer, 1972).

The other part of the motoneurone population is represented by neurones with less electron density. The cell organelles are dispersed throughout the cytoplasm of both neurone types in a similar manner, however the cisternae of rough endoplasmic reticulum (rER) are more dilated in dark then in light (pale) motoneurones (Figs. 1 & 2).

b. SYNAPSES--In preliminary observations, the presynaptic terminals of DFP treated animals show differences when compared to nontreated animals. One of the most striking changes is the increased number of coated vesicles in some terminals (Fig. 3). Furthermore, microtubules and neurofilaments are present in some of the boutons making synaptic contact with either a motoneurone-soma or dendrite (Fig 4).

c. NEUROPIL--The axons and axon terminals of normal animals contain a moderate number of clear synaptic vesicles and mitochondria. Degenerating nerve fibers are occasionally present in control animals whereas in treated animals many different stages of degenerating axons and axon terminals can be observed (Fig. 5,6). Also, an increased amount of smooth axonal endoplasmic reticulum (sAER) can be seen in some axon profiles (Fig. 7), such profiles are occasionally seen in the ventral part of the anterior horn of untreated animals.

II. ACETYLCHOLINESTERASE (AChE) HISTOCHEMISTRY--Vibratome sections are used in this method of visualizing AChE. Such sections have shown positive staining of cisternae of rER in spinal motoneurones (Fig. 8). After establishing the staining procedure in our laboratory we attempted to develop the AChE stain for en block tissue samples, which, up to this point, has been unsuccessful. In material, which is not counterstained with lead citrate like the material described above, vesicles, multivesicular bodies and lysosomes are stained (Fig. 9).

III. ETHANOLIC PHOSPHOTUNGSTIC ACID (E-PTA)--This staining procedure is now functional in our laboratory and is now successfully used for material of nontreated as well as of treated animals (Fig. 10).

IV. AChE + E-PTA--This procedure is still in the stages of development. However, the E-PTA part of this stain is now routine (Fig. 11). Also, parts of the myelin sheaths show positive reaction which could be caused the AChE stain. Vesicle-like structures appear to stain readily with thi method (Fig. 12).

FIGURE LEGENDS:

Fig. 1: 'Dark' motoneurone in a control cat. Note the dilated cisternae of rough endoplasmic reticulum (arrows). N-Nucleus. Bar: 1 micron.

Fig. 2: 'Light' motoneurone in a control animals. The cisternae of rough endoplasmic reticulum (arrow) are not as dilated as in the 'dark' motoneurones. G-Golgi complex. Bar: 1 micron.

Fig. 3: Axo-dendritic synapse of an animal treated with 0.1mg/kg DFP for 13 days. Note the large number of coated vesicles (arrows) in the presynaptic terminal. Bar: 0.5 micron.

Fig. 4: Presynaptic terminal after treatment with 0.2mg/kg DFP for 14 days. Note the large amount of neurofilaments (arrow) which are usually absent in presynaptic terminals. Bar: 0.5 micron.

Fig. 5: Myelinated axon after treatment with 0.2mg/kg DFP for 7 days. Degeneration is indicated ty the presence of lamellar and dense lamellar bodies (arrows). Bar: 1 micron.

Fig. 6: Degenerating axo-dendritic synapse after treatment with 0.3mg/kg DFP for 21 days. Note the small number of synaptic vesicles and the presence of dense lamellar bodies (arrow). Bar: 0.5 micron.

Fig. 7: Nerve fiber after treatment with 0.5mg/kg DFP for 16 days. The profile contains a large amount of sAER (arrows). Bar: 0.5 micron.

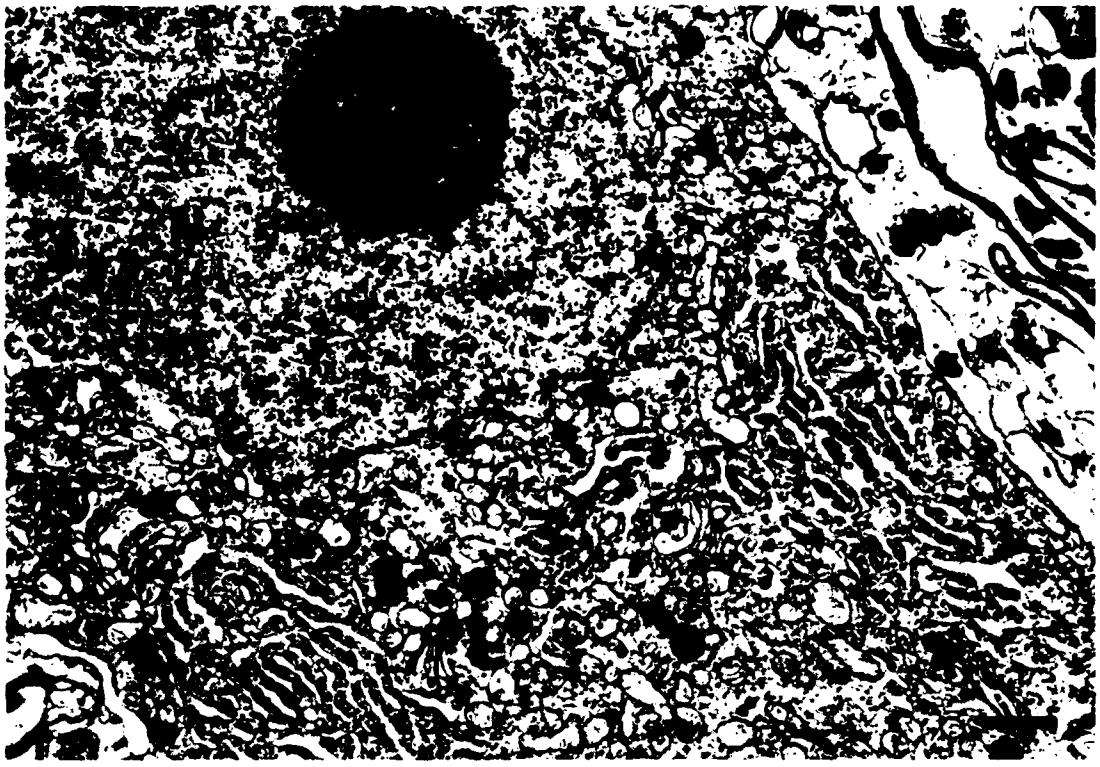
Fig. 8: Motoneurone of a control cat. Positive staining for AChE is present within the cisternae or rough ER. Bar: 1 micron.

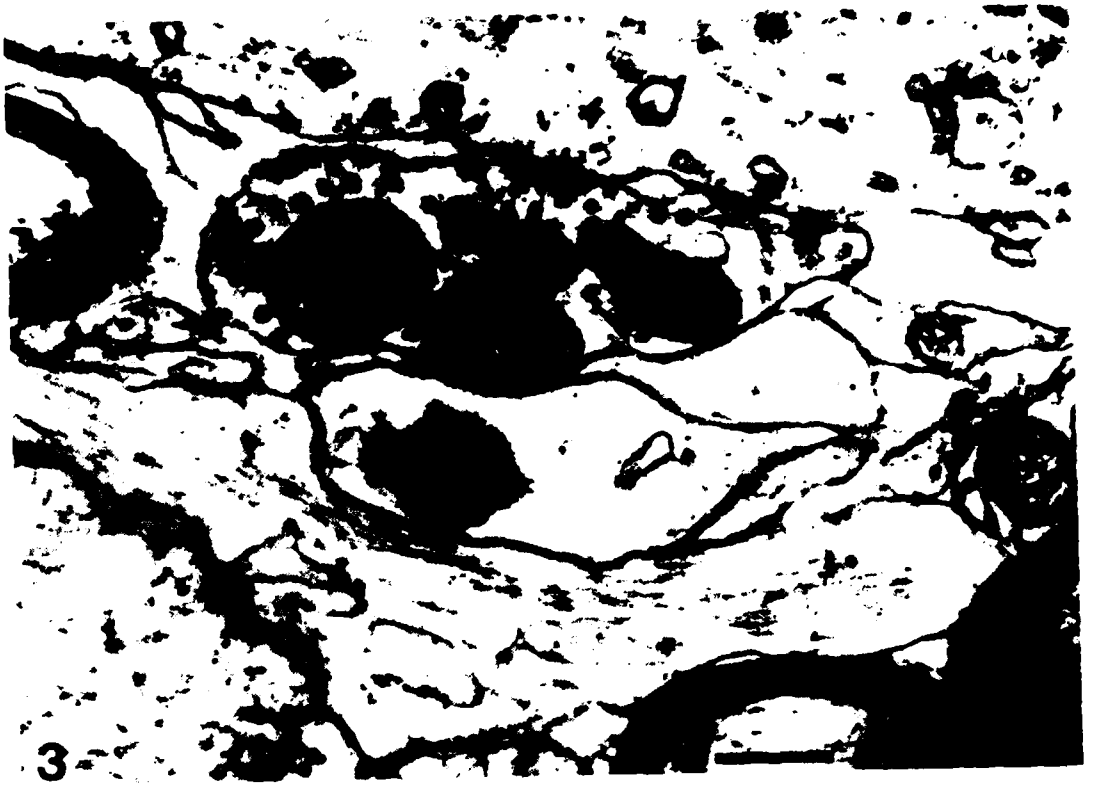
Fig. 9: Positive staining for AChE within lysosomes located in a motoneurone soma of a control animal. Bar: 0.5 micron.

Fig.10: Synapses (arrows) in a control animal stained with E-PTA. Bar: 0.5 micron.

Fig.11: Synapses (arrow) in a control animal stained for AChE + E-PTA. Bar: 0.25 micron.

Fig.12: Vesicle like structures in a control cat positively stained with the AChE + E-PTA. Bar: 0.5 micron.

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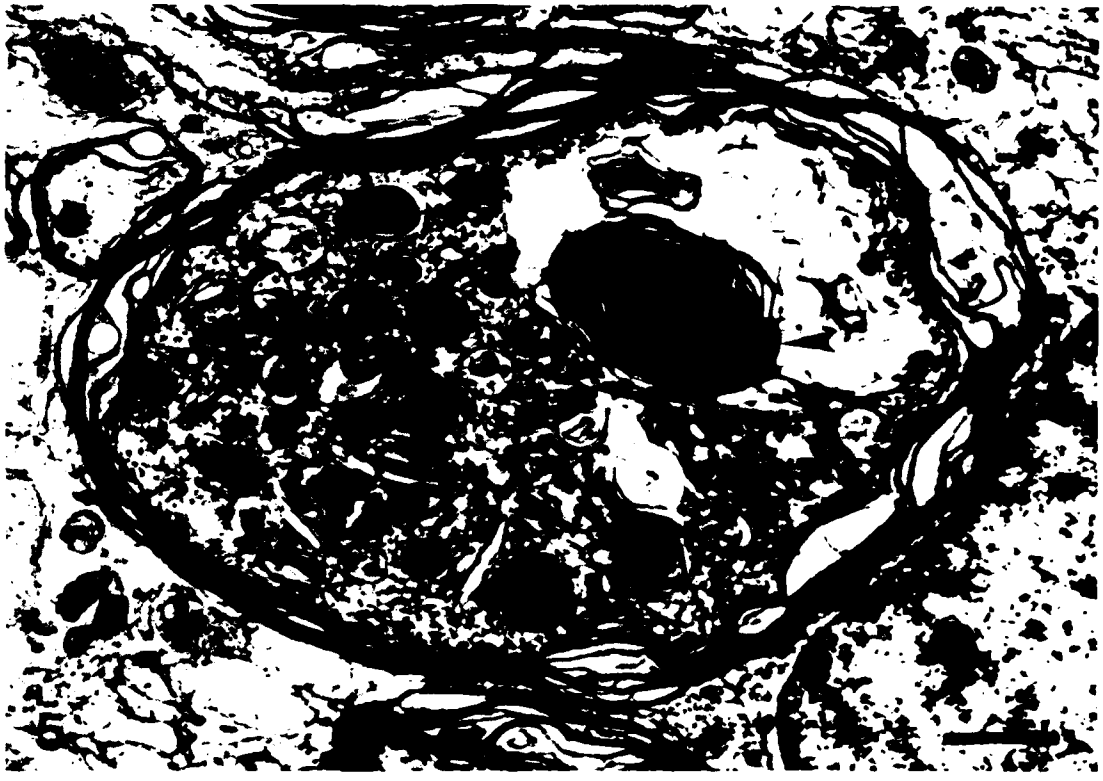

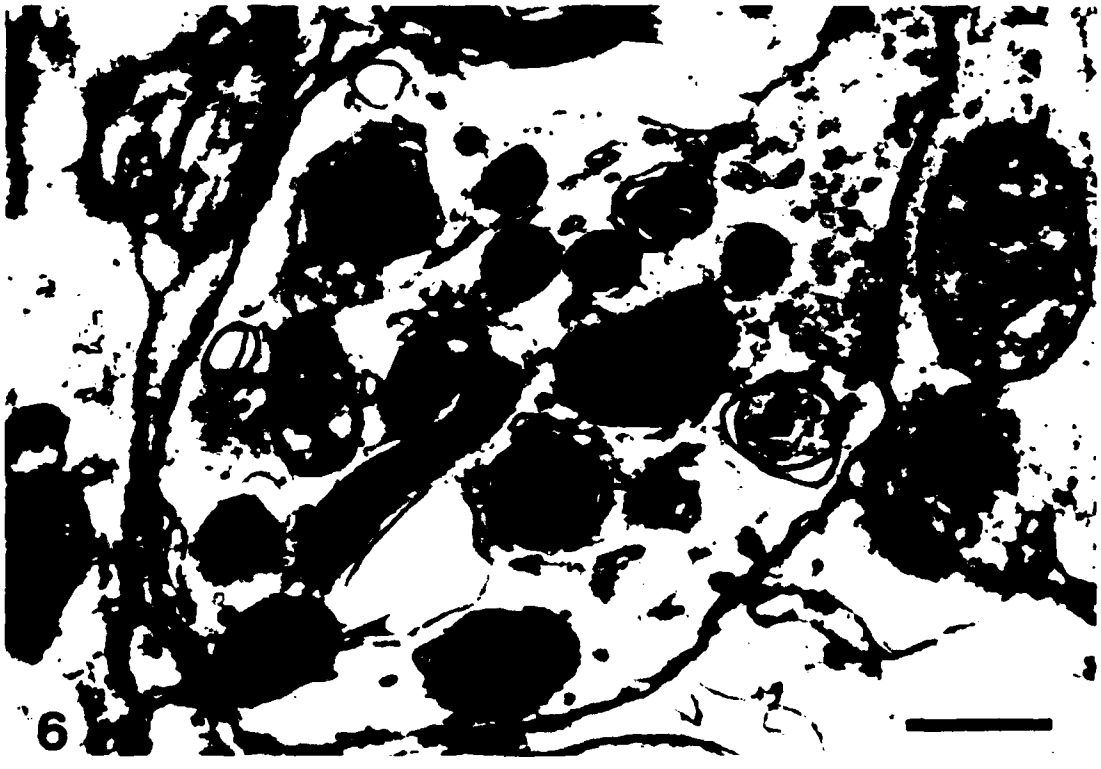

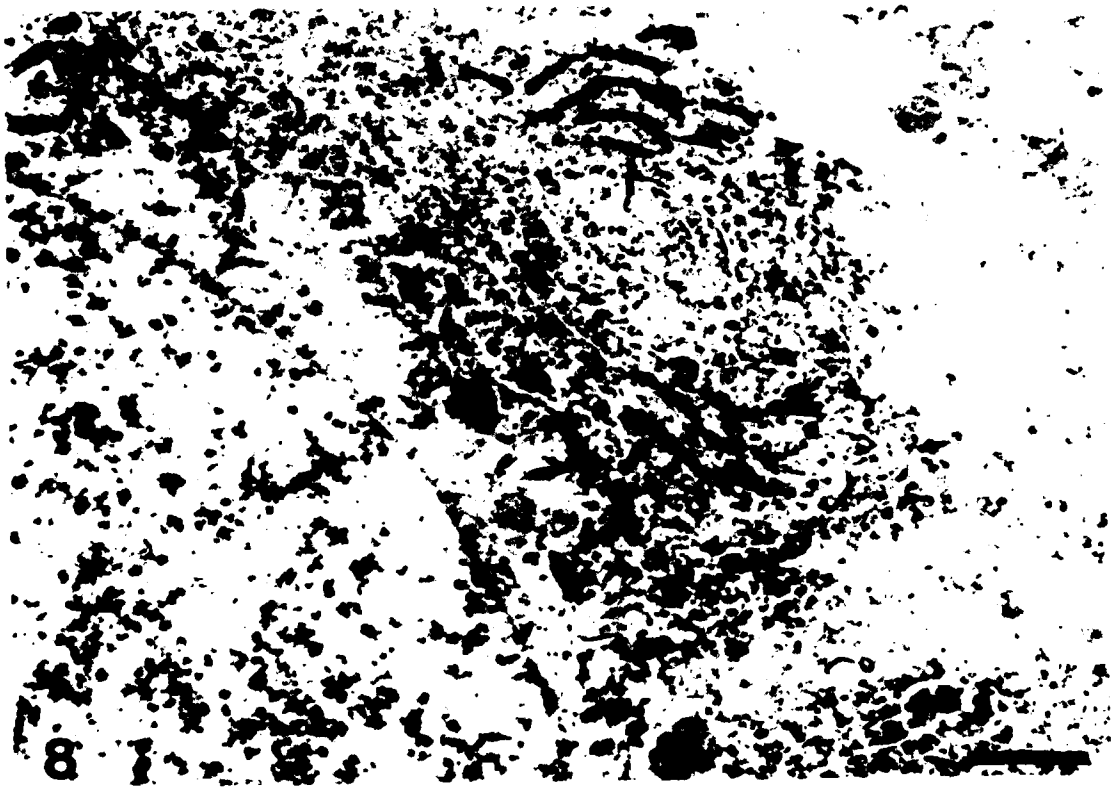

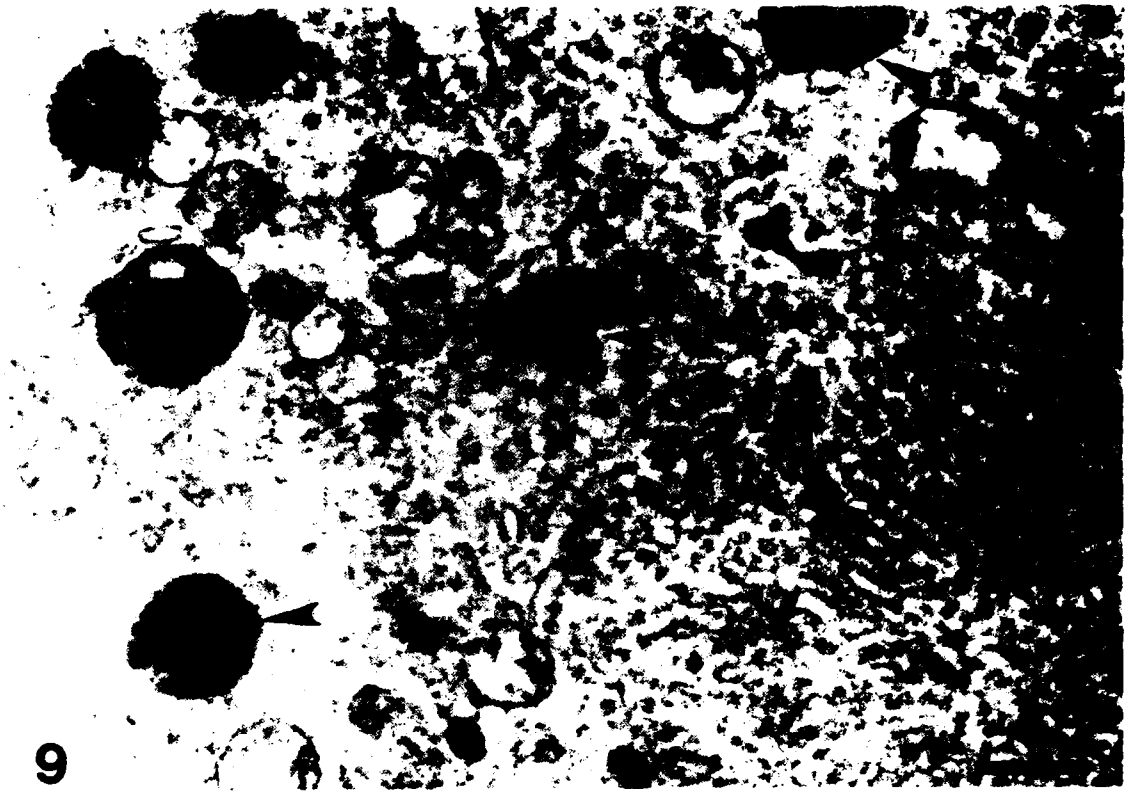
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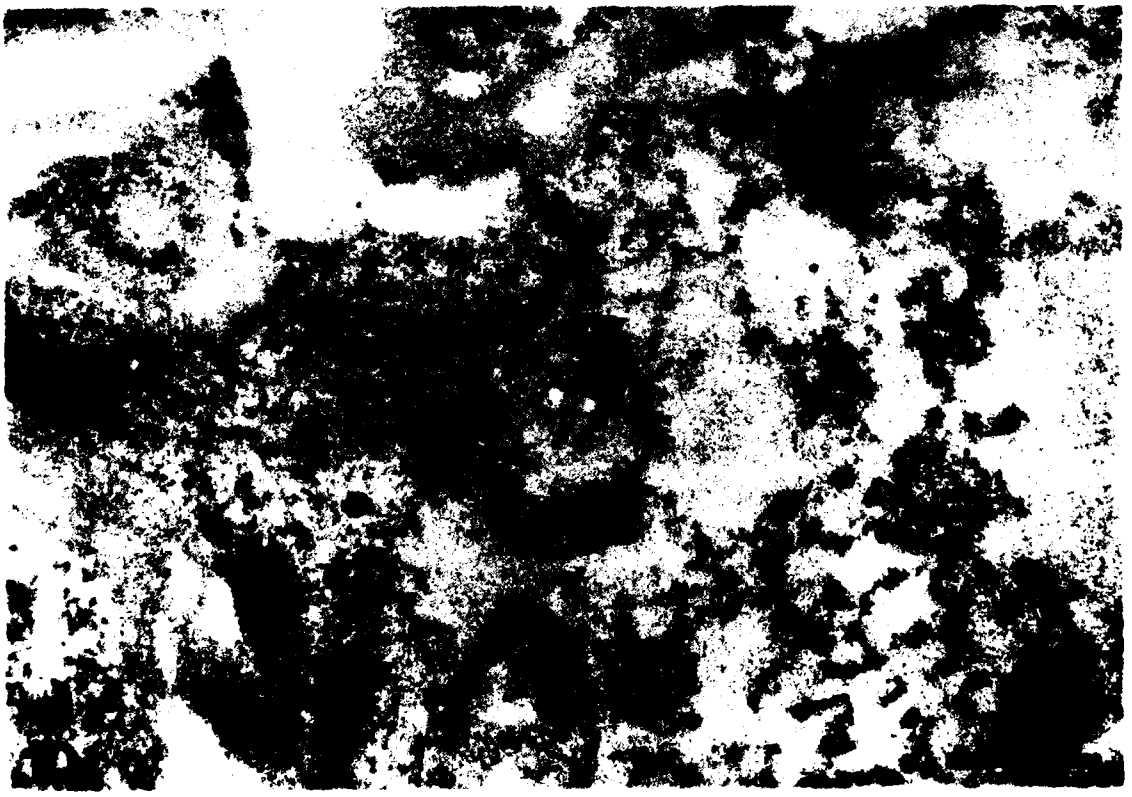

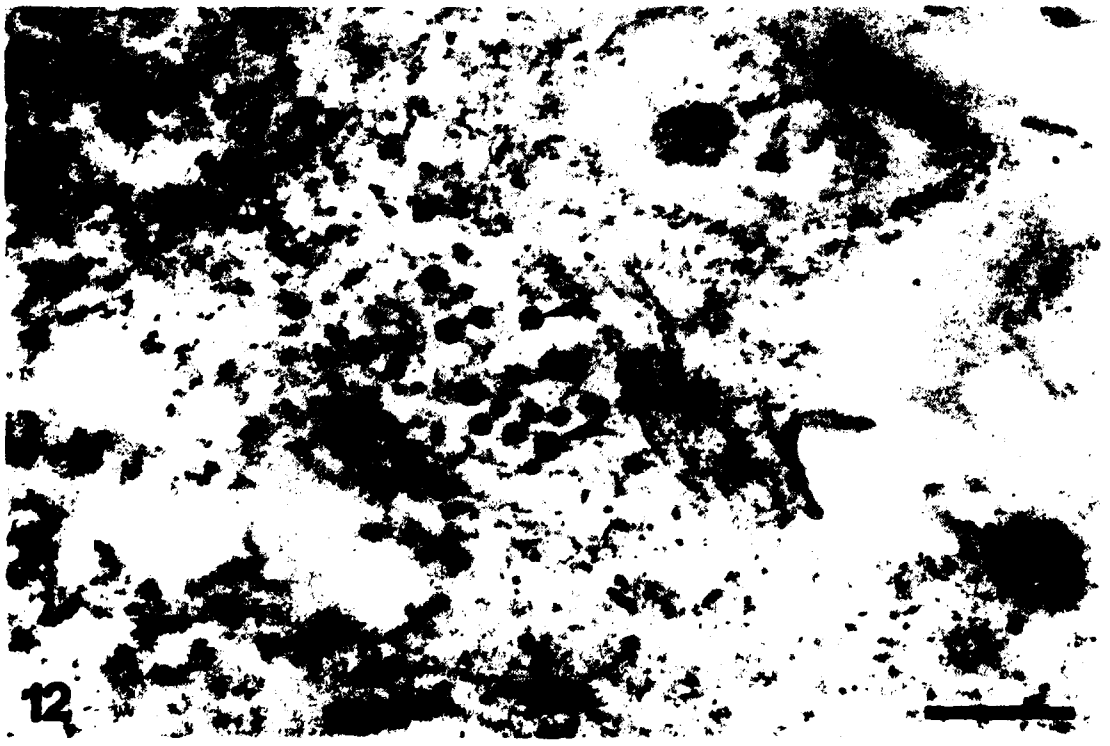
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B. ELECTROPHYSIOLOGY/PHARMACOLOGY:

Experiments have been carried out on 50 cats with a criteria of repetition of responses in 6 cats.

A comparison of RFP (Renshaw cell field potentials) (Fig. 13a-e) and RUP (Renshaw cell unit potentials) (Fig. 13g-i) evoked by subthreshold to suprathreshold intensity antidromic stimulation of lumbar segment L-7 ventral spinal roots, reveals that both have a sharply defined response at threshold (Fig. 13a subthreshold and 13b threshold for RFP cf, to Fig. 13g subthreshold and Fig. 13h threshold for RUP). However, with increasing stimulus intensity, the RFP characteristically show an increase in amplitude (Fig. 13c through 13f at 2X, 4X, 6X, and 8X threshold of 13b). On the other hand, RUP show an increase in duration with an increase in stimulus intensity as is shown at 2X (Fig. 13i) threshold (Fig. 13h).

Figure 14 shows the sequence of RUP responses during and subsequent to 60 secs of 20 Hz supra threshold antidromic stimulation of L-7 ventral roots. The control response to 2Hz suprathreshold antidromic stimulation immediately before repetitive stimulation is seen in Fig. 14a and 14b at different CRO sweep speeds. At 15, 30, 45, and 60 seconds of repetitive stimulation (Fig. 14c through 14f) there is a characteristic decrease in frequency, and duration of the burst response until it is entirely depressed as is seen in Fig. 14f. Immediately after the stimulation there is a characteristic lack of response to 2Hz stimulation. This post tetanic depression (PTD) may persist up to 5 minutes in exceptional instances (Fig. 14g-i) but usually recovers within 1-2 minutes. In addition, the response shows a marked delay as is seen in Fig. 14j through 14l (note the different time and amplitude calibrations as indicated in the figure legend).

On the other hand, some cells fail to show a depression of response after repetitive antidromic stimulation as is seen in figure 15. Control responses to 2 Hz antidromic ventral root stimulation are shown at increasing sweep speeds in Fig. 15a through Fig. 15c just prior to 20 Hz stimulation. Figure 15d through Fig. 15h are at 5 through 90 secs. of repetitive antidromic stimulation at 20 Hz. The response to 2 Hz antidromic stimulation recovers immediately (Fig. 15i) and shows post tetanic potentiation (PTP) as the response at 5 minutes after repetitive antidromic stimulation (Fig. 15j-l cf with controls Fig. 15a-c).

In three animals, even less antagonism was seen (Fig. 16). In Figure 16 repetitive antidromic stimulation at 20 Hz was continued for 2 minutes, during which time complete depression of RUP was not obtained (Fig. 16g and 16h). Moreover, PTP did not occur (Fig. 16k cf. 16b).

The response of Renshaw cell field potentials (RFP) before during and after repetitive antidromic stimulation is shown in Figure 17. Control responses are shown in Fig. 17a. Responses recorded at 5, 15, 30, 45 and 60 seconds (Fig. 17b-f) show an initial decrease in frequency and a subsequent depression of response amplitude with time. During recovery at 2Hz antidromic stimulation (Fig. 17g-l) PTD is seen initially followed by PTP (Fig. 17l) revealed as an increase in amplitude of the RFP (cf. Fig. 17a). Also, RFP did not show a delayed response during recovery as was often seen with the RUP (cf. Fig. 14j-l).

In cats treated with chronic sub LD50 doses of DFP, RUP did not show significant differences in response to 2 Hz antidromic stimulation from control animals. On the other hand, RFP demonstrated a marked

increase in amplitude of response as is seen in Figure 18. A response of control RFP from antidromic stimulation at 2 Hz is shown in Fig. 18a. In comparison, cats receiving 0.5mg/kg s.c. DFP per day for 14 days show a marked potentiation of the RFP (Fig. 17b). Figure 17c and 17d are RFP recorded from close proximity to a Renshaw cell in untreated control cats, for purposes of comparison.

COMPARISON OF RENSHAW CELL UNIT & FIELD POTENTIALS

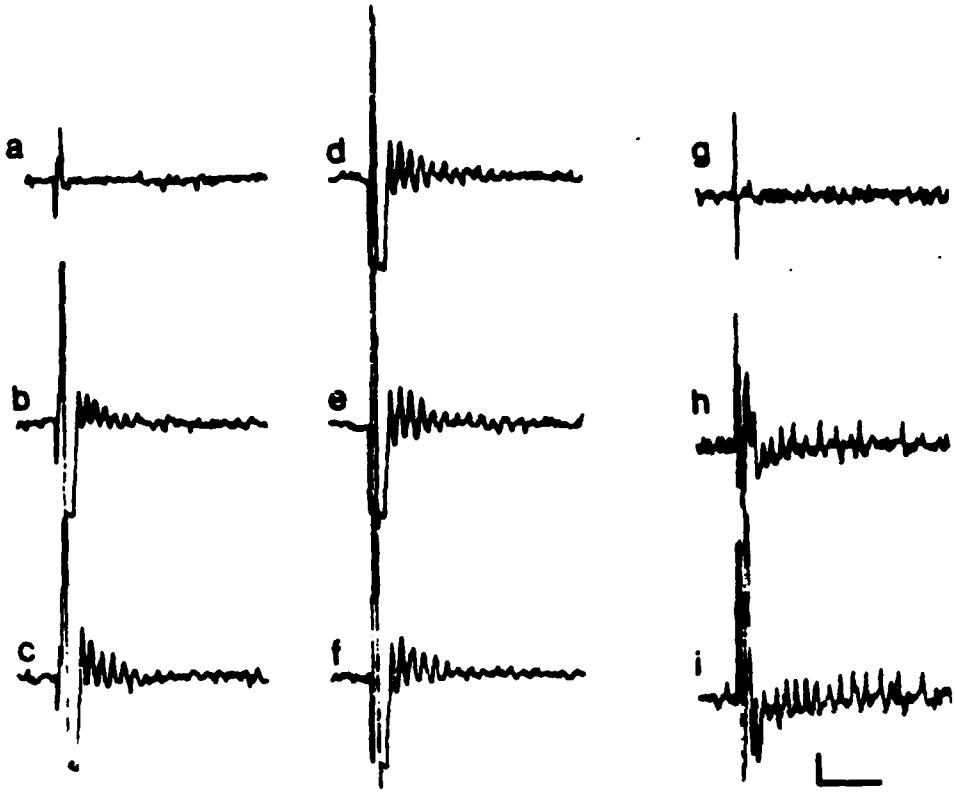

FIGURE 13. Renshaw cell field potential (RFP) -(a-f), In a, the potential is just below threshold and shows the stimulus artifact and the M (motoneurone) field potential. In b, the RFP is recorded at threshold . Note the presence of RFP oscillations (1000Hz). In c, the RFP are recorded at 2X threshold. Note the increase in amplitude of the response. In d, the RFP is evoked at 4X threshold. Note little to no further increase in amplitude but a slight increase in the duration of the burst. Renshaw cell unit potential(RUP)-(g-i), In g, the RUP is recorded at just below threshold(cf. a.)with only the stimulus artifact present. In h, the RUP is recorded at threshold. Note the presence of an M potential and the unit discharge duration. In i, the RUP is recorded at 2X threshold. Note the increase in the duration of the discharge with little to no change in amplitude nor frequency. Calibration signal = 100 μV/5 msec.

Inhibition of Renshaw Cell Unit Potentials by Antidromic Stimulation

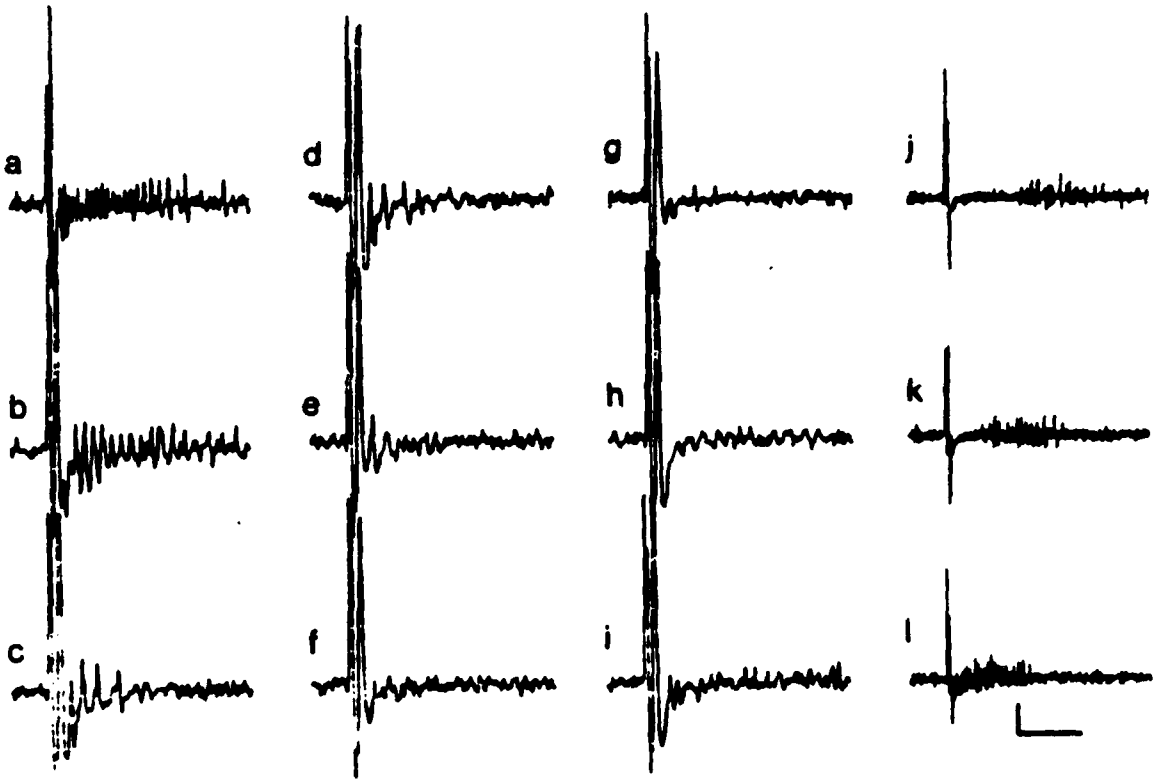

Figure 14. Response of a Renshaw cell to repetitive antidromic stimulation of L-7 ventral spinal roots(60 secs.@ 20Hz). Traces a and b are controls at different sweep speeds. Traces c-f show the depression of responses during 15,30,45 and 60 seconds of repetitive stimulation respectively. Note the progressive decrease in frequency and duration of activity until maximal inhibition at f,which persists during recovery as post tetanic depression (g-i). Trace g is 2 seconds after repetitive stimulation,h is one minute and i is two minutes post stimulation. Trace j is recorded two minutes after repetitive stimulation but at a slower speed,k is 4 minutes post repetitive stimulation and l is 5 minutes post repetitive stimulation. Calibration signal= 50 μV/10 msec for a, 50 μV/5 msec for b-i and 50 μV/20 msec for j-l.

Absence of Post Tetanic Depression of Renshaw Cell Unit Activity I.

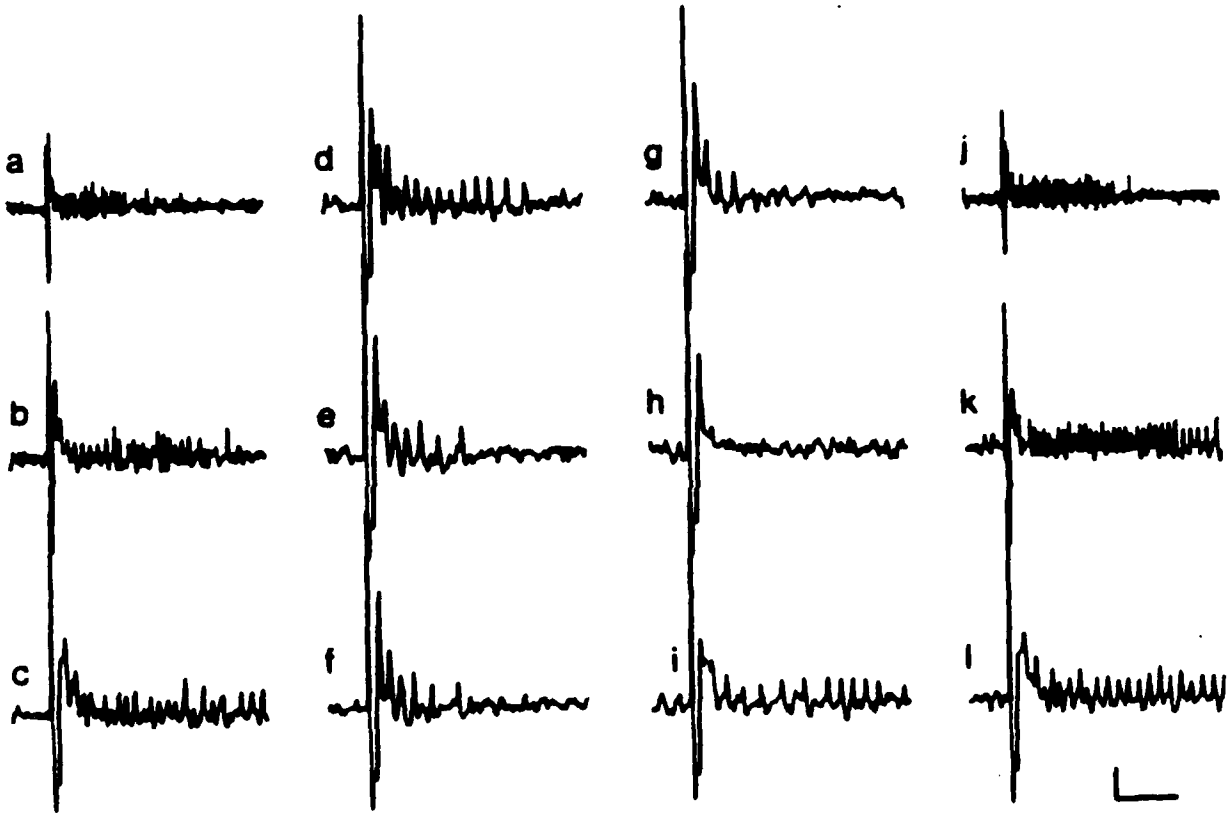

Figure 15. In a-c, RUP are recorded at increasing CRO sweep speeds during control. In d-h, RUP are recorded during 5,15,30,60 and 90 seconds of repetitive antidromic stimulation. Note the depression of unit potentials at h, and the immediate recovery as seen in i. In j-l traces are recorded at increasing CRO sweep speeds , 5 minutes after cessation of repetitive stimulation. Note the post tetanic potentiation (cf with control traces at same sweep speeds in a-c). Calibration signal= 50 μV/5 msec (c-i & l), 50 μV/10 msec (b & k), 50 μV/20 msec (a & j).

ABSENCE OF POST TETANIC DEPRESSION OF RENSHAW CELL UNIT ACTIVITY II.

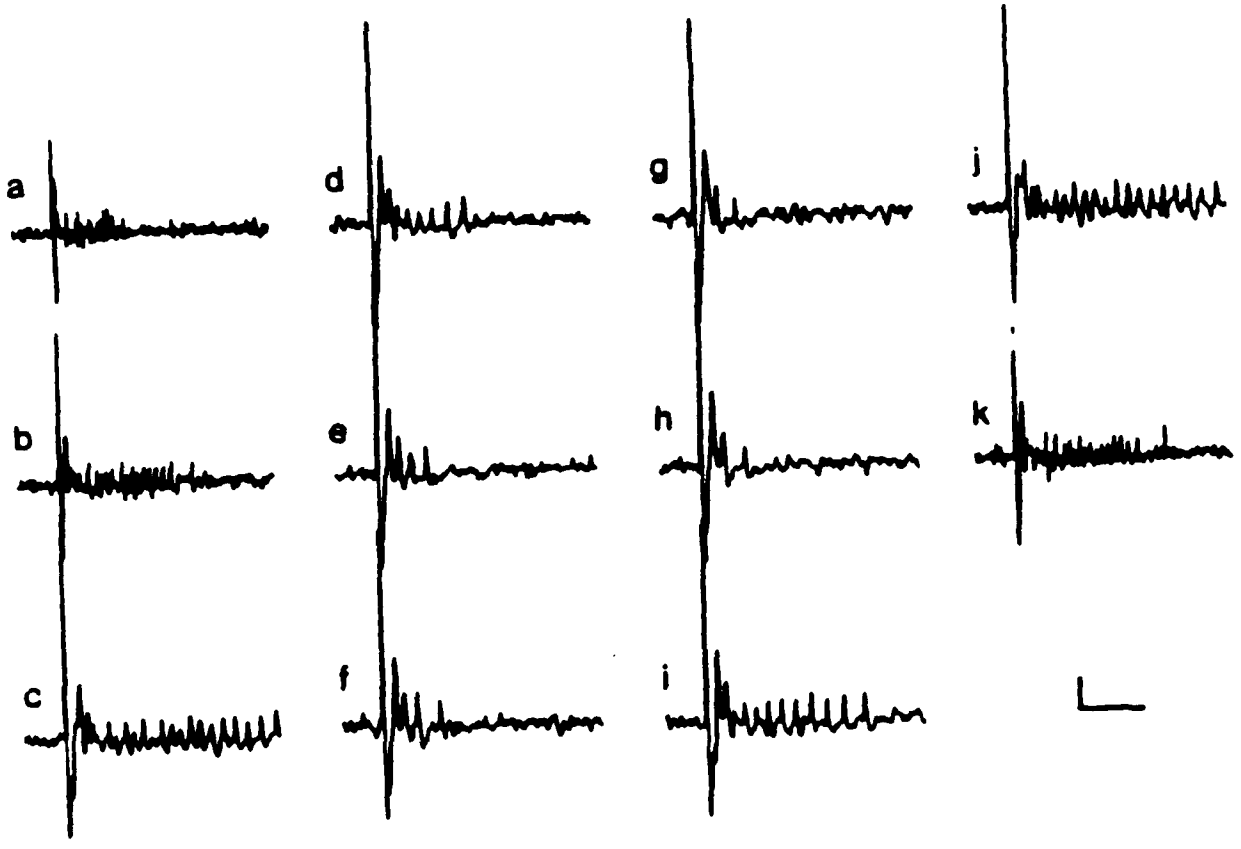

Figure 16. In a-c, RUP are recorded at increasing CRO sweep speeds during control. In d-h, RUP are recorded during 15,30,60,90 and 120 seconds of repetitive antidromic stimulation. Note the presence of unit potentials after two minutes of stimulation (h) and the immediate recovery of response (i). Also note the absence of post tetanic potentiation 5 minutes after cessation of repetitive stimulation. (j and k). Calibration signal= 50 μV/5 msec (c-j), 50 μV/10 msec (b and k), 50 μV/20 msec (a).

INHIBITION OF RENSHAW CELL FIELD POTENTIALS BY ANTIDROMIC STIMULA

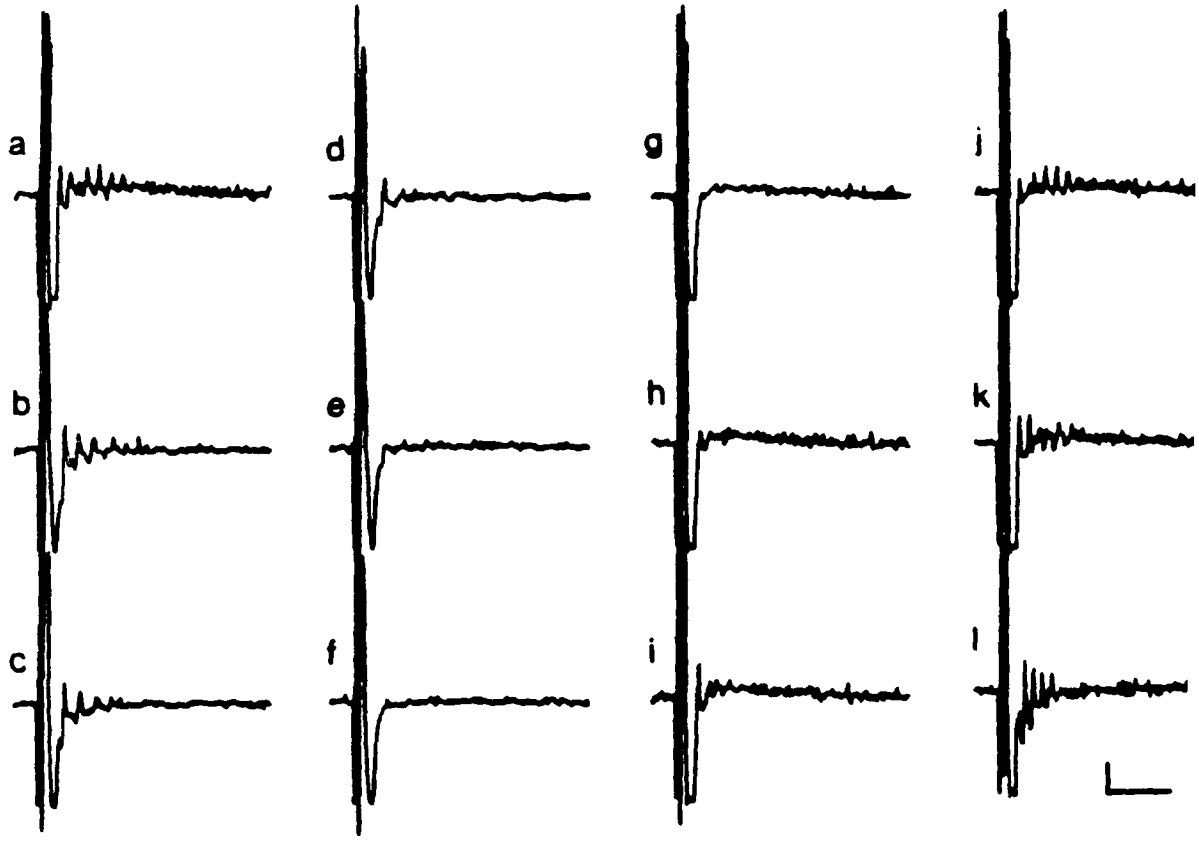

Figure 17. Responses of Renshaw cell field potentials (RFP) to repetitive antidromic stimulation of L-7 ventral roots. In a , a response to 2 Hz stimulation is recorded during control. In b-f are the responses during 5,15,30,45 and 60 seconds of repetitive antidromic stimulation of L-7 ventral roots. Note an initial decrease in frequency followed by depression of amplitude of RFP. Post tetanic depression of RFP is seen in g-h (15 and 30 seconds after cessation of stimulation), with recovery beginning at 60 seconds (i). The post tetanic depression is followed by post tetanic potentiation 120 seconds (k) and 180 seconds(l) after cessation of repetitive stimulation. Calibration signal= 50 μV/5 msec.

RENSHAW CELL FIELD POTENTIALS & CHRONIC SUB-LD50 DFP

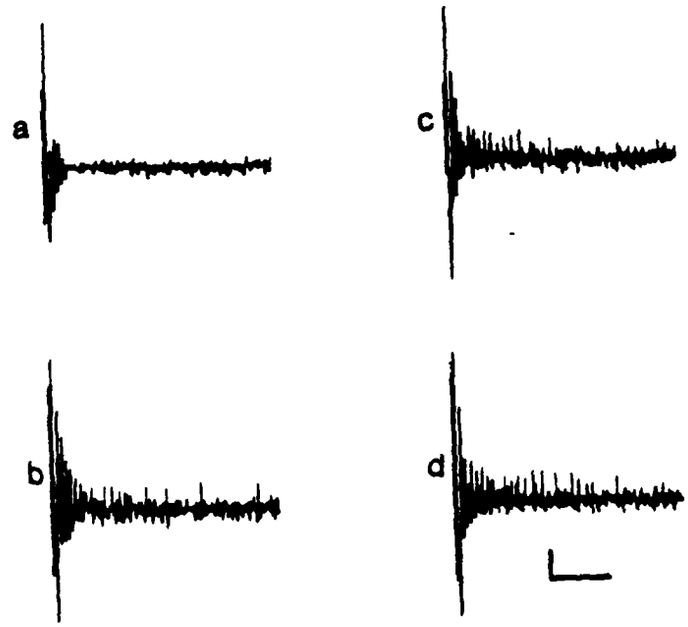

Figure 18. Renshaw cell field potentials (RFP) after treatment with DFP (0.5 mg/Kg/day s.c.) for 14 days. Renshaw cell field potential from an untreated control cat is shown in a. In b, the RFP from a cat after DFP is recorded in response to 2 Hz stimulation. In c and d RFP are recorded from untreated cats with the electrode in close proximity to a cell. Note the mixed unit potentials with the RFP. Calibration signal=50 μV/20 msec.

C. BEHAVIOR (TABLE 1):

In cats treated with low doses of DFP (0.1mg/kg and 0.2mg/kg) for 14 days, mild symptoms of DFP poisoning such as transient pupil constriction occured within a few hours of each injection and increased defecation with solid stools was observed. The cats remained active and alert throughout treatment. Mean weight loss for these cats was 2.2% (range 0-10%, n=10).

Cats treated with 0.3mg/kg DFP daily for 7 days showed the same symptoms as the low-dosed animals, but after 7 days an increase in severity of symptoms appeared: excessive urination; semi-solid or watery stools with blood occasionally present; reduced food intake and stiffness of the hind limbs. Though no significant weight loss occured in animals treated at this dose for 7 days, there was a 22% weight loss ocurred in one of the animals treated for 14 days.

Increased severity of symptoms was seen in animals treated with 0.5mg/kg DFP for 12, 14 and 19 days. Pupil constriction occurred within hours of the first injection and usually persisted between injections. Watery stools, with blood, were present 5-7 days after the first injection, as well as excessive salivation; excessive urination; severely reduced food intake; stiffness of hind limbs leading to reduced activity and difficulty in walking; trembling and vomition. Weight loss in these cats ranged from 7-23%. Even though activity in these animals was reduced, they remained alert and nonaggressive.

Animals given high doses of DFP (0.75mg/kg - 1.0mg/kg) were treated for 3,4 or 5 days only, due to the severity of symptoms. In cats administered 0.75mg/kg DFP, there was maintained constriction of pupils, excessive salivation, excessive urination, no food intake after 3 days, trembling, vomition, agitation, and rapid shallow breathing. One to three hours after the third injection, the cats assumed a defensive posture with piloerection and hissing when approached. This condition was maintained for 1-2 hours, after which time the cats, though not very active, were nonaggressive and could be handled. A 7% weight loss was seen in one cat treated for 5 days. Another cat was treated for 3 days and then allowed to recover for 11 days. Recovery started on day 5, the cat started to eat again on day 6 and became active again on day 7. There was a 17% weight loss in this cat over the 14 day period of treatment and recovery.

One cat only was treated with 1.0mg/kg DFP for 4 days, and this cat died 2 days after treatment was stopped. The cat became less active on day 1 and on day 4 the cat was recumbent, with rapid and shallow breathing, tremors, excessive urination, salivation, and dilated pupils. A 12% weight loss occured over 5 days. Intramuscular injections of atropine methyl nitrate (1.0mg/kg every 4 hours) given on days 4 and 5 did not reduce symptoms.

No symptoms were observed in control cats.

Morphological investigations as well as electrophysiological experiments have been performed on all of these animals and the results are being analysed.

Table 1

CHRONIC SUB-LD50 DIISOPROPYLFLUOROPHOSPHATE

Dose (sc) mg/Kg/day	Symptom Severity	Duration of Treatment	Total Dose mg/Kg	XLD50*
0.1	+	14 days	1.4	0.47
0.2	++	14 days	2.8	0.93
0.3	+++	12 days	3.6	1.2
		14 days	4.2	1.4
		19 days	5.7	1.9
0.5	++++	14 days	7.0	2.33
0.75	+++++	3 days	2.25	0.75
	+++++	4 days	3.0	1.0
	+++++	5 days	3.75	1.25
1.0	+++++	3 days	3.0	1.0

*LD50 (sc) based on an acute dose of 3.0 mg/Kg.

DISCUSSION:

MORPHOLOGY:

Histopathological changes in the central nervous system due to the neurotoxic effect of organophosphates are described to be most evident in the anterior columns of the thoracic and lumbar spinal cord (For review see Abou-Donia et al 1980). Changes mostly involve nerve fibers and terminals however, pathological alterations of the perikaryon are described by Bischoff (1970) four days after the onset of clinical signs of neurotoxicity. Our studies are in general agreement since acute exposure to DFP does not alter motoneurone cell bodies. However, our preliminary studies on chronic treated animals suggest an increase of lysosomal elements within the soma of motoneurones. Further studies are needed to determine the extent of these changes.

An increase in the number of coated vesicles in nerve terminals was observed after acute as well as chronic DFP administration. Lakowski et al (1977) find an accumulation of coated vesicles in the nerve terminals of motor endplates after a single dose of paraoxon and the authors propose excessive vesicle turnover to be responsible. Further studies are being undertaken to determine whether or not acute and chronic treated animals show a dose-dependent increase in coated vesicles.

The histochemical demonstration of inhibition of cholinesterases after acute exposure to organophosphates (Koelle and Gilman (1949), Poirier et al (1977), and Toth et al (1980)) has been used for numerous light (Koelle, G.B. (1954), Butcher et al (1977), and Van Meter et al (1978)) and electron microscopic studies (Kaiya et al (1980), Shimizu and Ishii (1966), and Somogyi and Chubb (1976)) on the organization of cholinergic systems in brain and spinal cord. In all of this studies thick sections of the tissue were used rather than tissue blocks. In order to follow consecutive areas more precisely for ultrastructural effects, small tissue blocks would be of more use than a series of sections. At this point however, en block histochemistry for cholinesterases has not been successful.

The staining of synapses with E-PTA to investigate changes in the membranous components of synapses after treatment with organophosphates as well as for quantification of synapses will be evaluated. If this method can be successfully coupled with cholinesterase histochemistry, the identification and quantification of putative cholinergic synapses might be possible.

ELECTROPHYSIOLOGY AND PHARMACOLOGY:

Two types of Renshaw cell responses can be recorded from the anterior horn of spinal segment L-7 of the cat evoked by antidromic stimulation of the severed ventral spinal roots. The first type of potential, the Renshaw cell field potential (RFP) is diffuse, has a well defined threshold and is rather easily recorded over a large range of the segment. (cf. Fig. 13 and Eccles et al 1954, Eccles, 1957, Willis and Willis 1966, Willes et al 1969). The second type of potential is discrete, is recorded with some difficulty, and from a restricted area in close proximity (a few microns) to the individual cell (Eccles et al 1954).

The RFP is generated by a somewhat homogeneous population of Renshaw cells at a given segmental level of the spinal cord. These cells tend to discharge synchronously during the initial part of their response which

results in a characteristic oscillatory field potential. (Eccles et al 1954, Frank and Fuortes 1956, Willis and Willis 1966, Willis et al 1969). Early studies attempting to localize the position of the Renshaw cells led Willis and Willis (1966) and Willis et al (1969) to conclude the maximum negativity for the RFP generated by antidromic stimulation of the ventral spinal roots, to be the ventral-most portion of Rexed's Lamina VII. The pathway of the antidromic volley being from ventral roots to the motor nucleus and then the Renshaw cell via the axon collateral of the motor neurone. These authors demonstrated the major sink of the RFP to be in ventral lamina VII with a source in mid lamina IX. From the data of Eccles et al (1954) in experiments with RFP, they hypothesised the Renshaw cell population to be centered medial to the motor nucleus with axons projecting dorso-lateral into the motor nucleus. Also described in the potential from the antidromic stimulation was the initial M or motoneurone potential evoked by activation of the motoneurone pools. Our findings corroberate those in the literature and further show that the amplitude of the RFP is a function of the intensity of stimulation which is supported by observations of increased duration of response in RUP with increasing intensity of stimulation. (cf. Fig 13).

The RUP response to repetitive antidromic ventral root stimulation (20 Hz) shows (A) (i) a depression of response during stimulation, (ii) a depression of response after stimulation (PTD), (iii) a delay in response recovery, and (iv) PTP after which responses return to pre-repetitive stimulus values; or shows (B) (i) a depression of response during stimulation, (ii) no PTD, and (iii) PTP without a delay after which responses return to pre-repetitive stimulus values; and lastly shows (C) (i) an incomplete depression of response during repetitive stimulation, (ii) no PTD, and (iii) little to no PTP (cf. Figs. 14-16). The mechanism underlying these responses is at present unknown and future studies with the suitable antagonists to known Renshaw cell receptors will be carried out, via intravenous as well as iontophoretic application.

On the other hand, RFP (cf. Fig. 17 have responded in much the same manner as the RUP during and subsequent to repetitive antidromic stimulation of the appropriate ventral roots (20 Hz). These potentials will also be profiled pharmacologically to help elucidate their mechanism. While admittedly the RFP in the spinal cord are complex, they are much less complicated than those found in the brain and therefore, correlations between RUP and RFP might be more amenable to analysis in this system compared to the difficulties encountered by such analysis in the brain.

Finally, while increased duration of RUP was not observed in cats treated chronically with DFP (cf Fig. 18), amplitudes of RFP were increased in amplitude. This latter finding cannot be explained as the result of an increase in the duration of RUP discharges and at present awaits further analysis.

BEHAVIOURAL:

The behavioral data is consistent with data in the literature for exposure to DFP (cf. Cholinesterases and Anticholineserase Agents In: Handb. d. Exp. Pharmak. XV G.B.Koelle Editor Springer-Verlag Berlin 1964). However, of interest are the responses to chronic low dose (sub LD50) exposure. Morphological changes occur in a dose dependent manner (vide

supra). Behavior, as measured through gross observations, appears to be consistent with the rate of accumulation of an amount of DFP reflecting an LD50. That is to say that if the rate of accumulation is slow, the LD50 dose may be exceeded without marked indications of organophosphate intoxication consistent with such a dose. Recovery from these effects has not been followed and will be the object of future studies.

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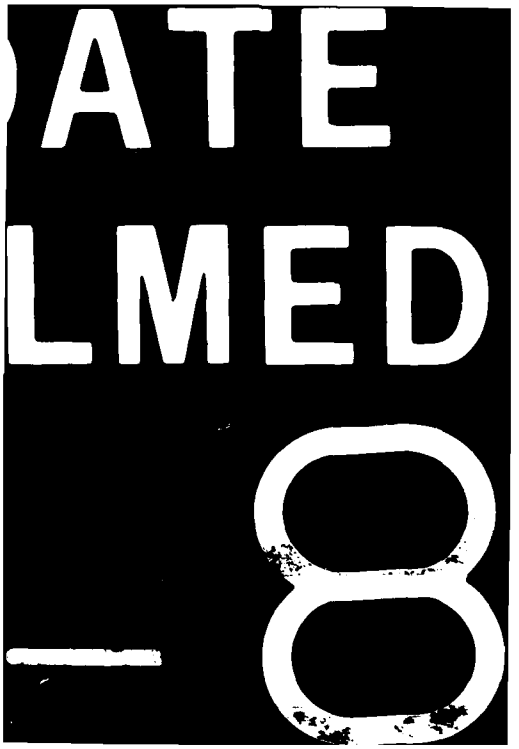
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