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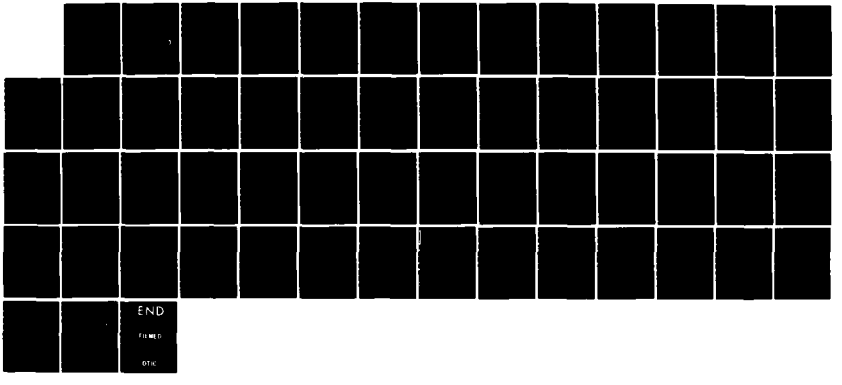

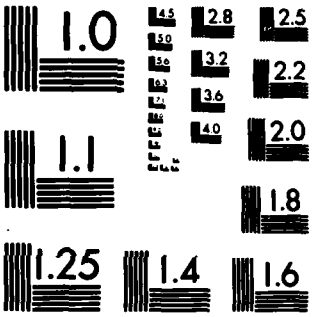

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The effect of anticholinesterase agents on pupillary function and parameters of cholinergic activity were investigated both in vitro and in vivo following topical administration. The study describes changes in three different aspects of cholinergic function: 1) uptake of choline, 2) release of acetylcholine and 3) AChE activity and pupil size. Our results are consistent with the concept of existence of a presynaptic muscarinic autoreceptor which is affected (DFP directly or through acetylcholine). DFP exerts multiple effects on various cholinergic parameters.

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ACUTE EFFECTS OF ANTICHOLINESTERASE AGENTS ON PUPILLARY FUNCTION

SOUTHERN ILLINOIS UNIVERSITY
SCHOOL OF MEDICINE
SPRINGFIELD, IL 62708

Dr. Ezio Giacobini

Controlling Office: USAF Office of Scientific Research/NL
Bolling Air Force Base, D.C. 20332

GIACOBINI, Ezio
472-50-2140

PROGRESS REPORT

(Period September 1, 1983 through March 15, 1984)

General description of the work in progress

The study of the short-term (1 min - 6 hrs) effect of DFP on acetylcholine metabolism and synthesis, pupillary function and acetylcholinesterase inhibition has been completed. A summary of the results obtained have been included in the previous progress report of September 1, 1983. The full paper describing these experiments has been accepted for publication in Neuropnarmacology (see Appendix I).

In the next part of our study we have investigated the kinetics of the high affinity choline uptake (HACU) and low affinity choline uptake (LACU) system in the densely cholinergic innervated albino rat iris. The ionic requirements, temperature sensitivity, and pharmacology of the system were determined. The manuscript describing these results is in preparation.

A summary of these results is reported in the following section. In addition, we have studied the ability of the aging cholinergic synapse to take up choline, release acetylcholine and to deplete and reform synaptic vesicles. A summary of this study is reported here. The results of this study are in publication (see Appendix II).

The results described in the progress report have been communicated at several national and international meetings. The abstracts of the communications are attached (see Appendix III).

Pharmacological and kinetic characterization of choline transport in the rat iris

The pharmacology, ionic dependency and temperature sensitivity of the choline uptake system was determined in irises dissected and preincubated for 10 min at 37°C in a buffer solution containing 1 uM unlabelled choline spiked with 1% ^{3}H-choline. In the pharmacological studies, drugs at various concentrations were included in the second incubation step in the presence of ^{3}H-choline.

Diisopropylfluorophosphate (DFP) was dissolved in propylene glycol which was then added to the uptake buffer. The stability of DFP in the aqueous solution was determined by incubating irises in the solution at various times up to one hour. The irises were then homogenized and acetylcholinesterase (AChE) activity was determined by the rapid method of Johnson and Russell (Analyt. Biochem., 64:229-233, 1975).

A Lineweaver-Burke plot of the kinetics for HACU and LACU is shown in Fig. 1. The K_m for high affinity uptake was 3.23 umoles with a V_{max} of 12.5 pmoles. The low affinity uptake K_m was 68.4 umoles with a V_{max} of 1023.54 pmoles.

High affinity choline uptake was demonstrated to be temperaturd- and sodium-sensitive. Control uptake at 37°C was considered to be equal to 100%. Decreasing the temperature to 20°C and 4°C resulted in a decrease of uptake to

32% and 15% of control, respectively. Sodium sensitivity was determined by substituting equimolar amounts of Na^+ with Li^+. Control uptake in the presence of Na^+ ions was considered to be equal to 100%. Increasing the concentration of Li^+, with a consequent decrease in Na^+, resulted in a decrease in uptake from 72% to 42% of control.

The pharmacology of the HACU system in the rat iris is shown in Fig. 2. Hemicholinium at 10^{-3}M and 10^{-4}M decreased choline uptake to 28% and 44% of control, respectively. Ouabain (10^{-3}M, 10^{-4}M and 10^{-5}M) inhibited choline uptake to 57%, 82% and 88%, respectively. The acetylcholinesterase inhibitors, physostigmine and DFP had similar effects on the HACU system, with DFP demonstrating to be a more potent inhibitor of the uptake. Physostigmine decreased choline uptake to 78% at its highest concentration (10^{-3}M). On the other hand, DFP inhibited choline uptake to 71%, 70% and 78% of control at 10^{-3}M, 10^{-4}M and 10^{-5}M, respectively. Scopolamine, a muscarinic antagonist decreased choline uptake to 82% of control at its highest concentration (10^{-3}M).

Of special interest to this study is the effect of DFP on Ch uptake. We have demonstrated that although this agent's primary role is to inhibit AChE activity, it also decreases Ch uptake and potently inhibits ACh release (Mattio et al., 1984) (attached). These effects can be explained only in part by the increased ACh levels in the tissue.

Aging of cholinergic synapses - uptake and releasing mechanisms

We have made use of the ciliary ganglion-iris preparation of the aging (1.5-9 yrs) chicken as a model of senescent peripheral cholinergic synapses. Neuromuscular junctions in the iris of aging chickens show early (1.5 yrs) morphological signs of damage such as, reduction and polymorphism of synaptic vesicles and increase of neurofilaments and mitochondria. Accumulations of cytoplasmic organelles and lysosomes are seen in the axoplasm of the nerve fiber. At later stages (5-9 yrs), the nerve ending is enveloped by Schwann cells infiltrating and filling the synaptic cleft. Quantitative morphometric changes in the ratio describing the relationship between volumes of terminals and volumes of synaptic vesicles show a progressive decrease in the volume occupied by synaptic vesicles. The ability of the cholinergic synapses to take up ^{3}H-choline and release the formed ^{3}H-acetylcholine (ACh) in response to high K^+-depolarization is impaired at 5 yrs resulting in a significant depletion of the ^{3}H-ACh releasable pool. These experiments seem to point out for the first time a selective functional defect in the cholinergic synapse during aging.

ATTACHMENTS: Appendix I
Appendix II
Appendix III
Figure 1
Figure 2

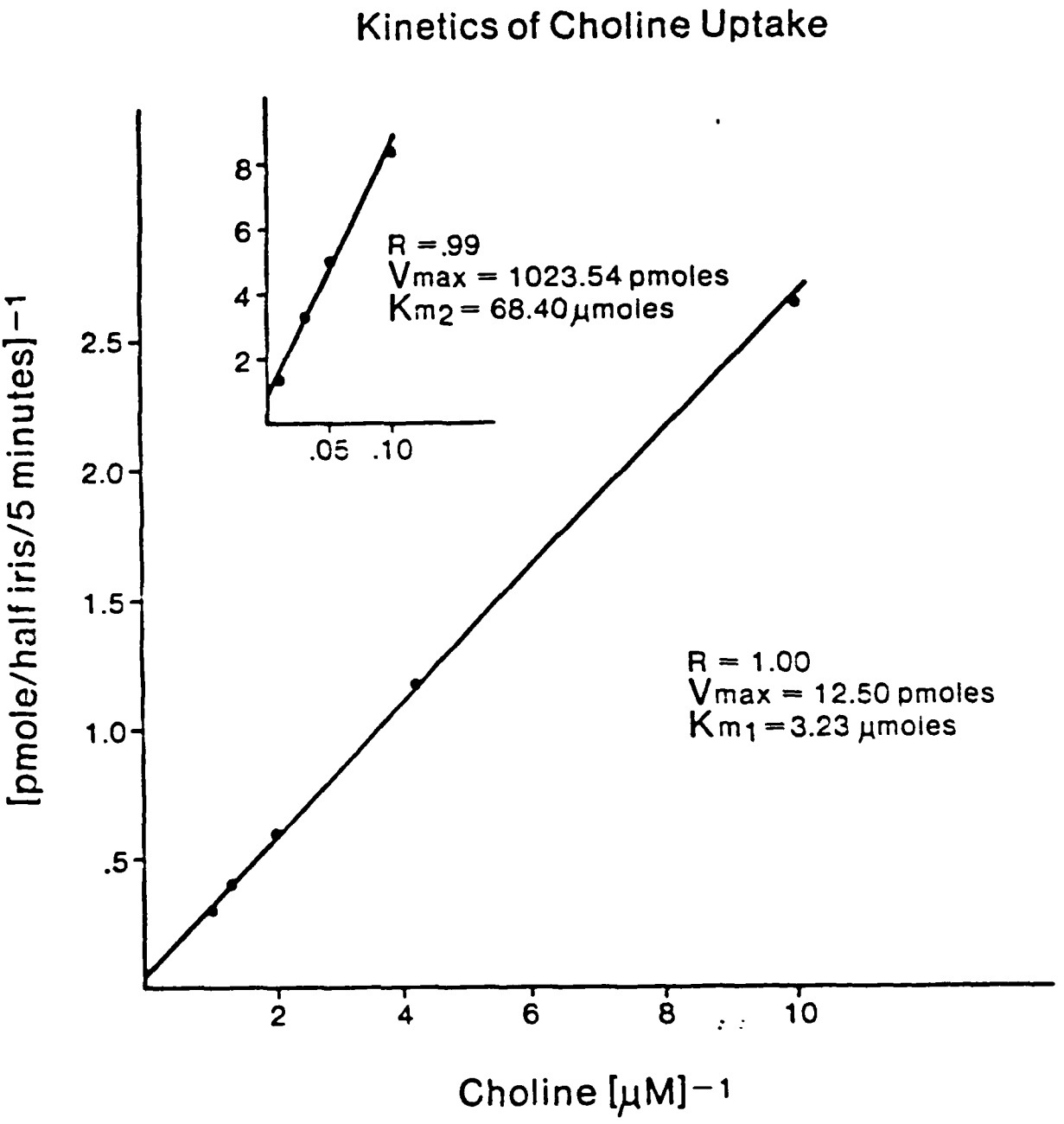

FIGURE 1. Lineweaver-Burke plot of the kinetics for HACU and LACU in rat iris.

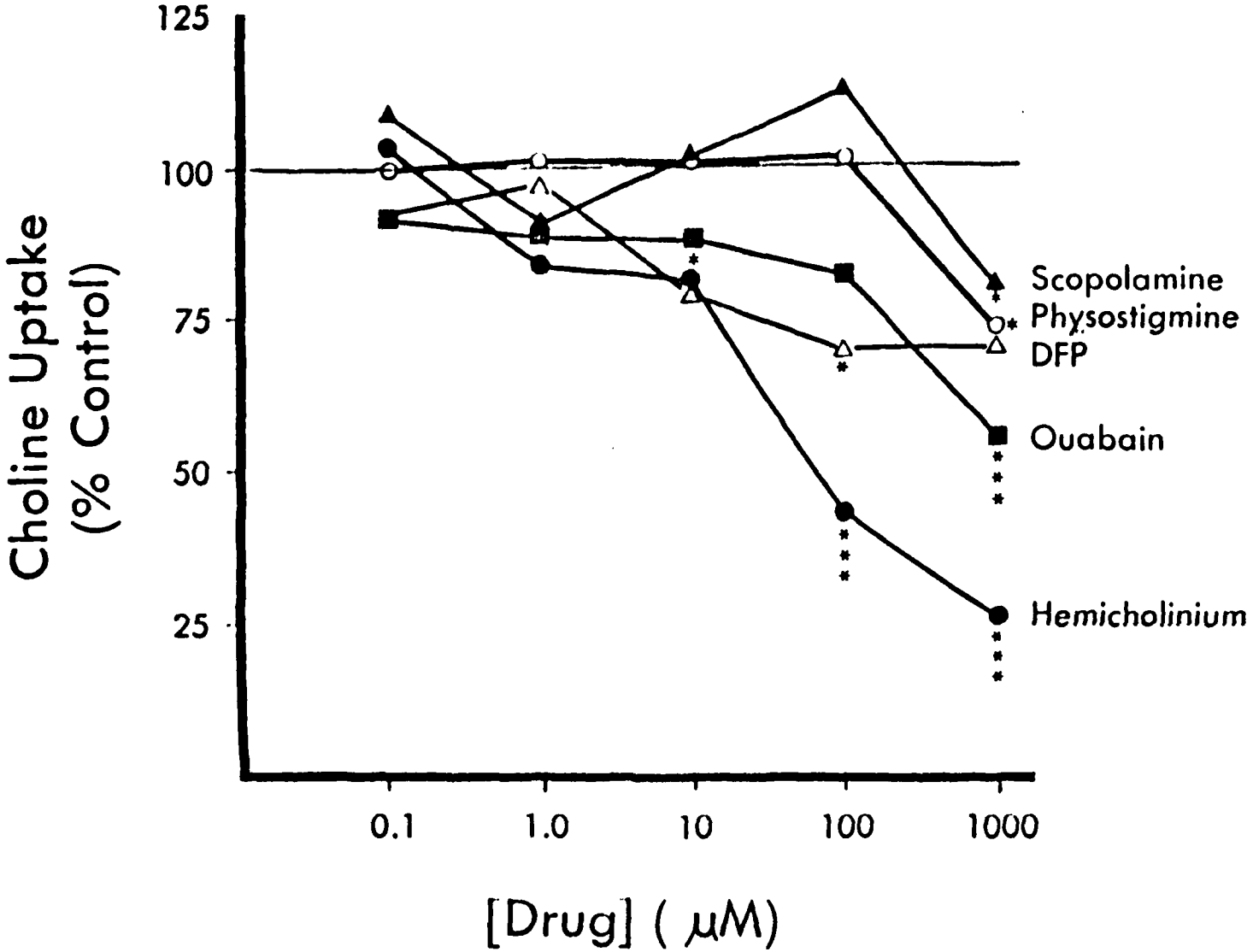

FIGURE 2. Effect of several drugs on the high affinity choline uptake of rat iris.

Accepted for publication in Neuropharmacology, 11/4/83 Appendix I

EFFECTS OF DFP ON IRIDIC ACETYLCHOLINE METABOLISM AND RELEASE AND PUPILLARY FUNCTION IN THE RAT

T.G. Mattio, J.S. Richardson* and E. Giacobini+

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois 62708 U.S.A.

*Departments of Pharmacology and of Psychiatry, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N0W0

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+ To whom reprint requests should be forwarded.

SUMMARY

The time course of the effects of the topical administration of 1 µg of diisopropylfluorophosphate (DFP) onto the cornea of the rat on iris function and acetylcholine (ACh) biochemistry was followed for 6 hrs after the acute inhibition of iris acetylcholinesterase (AChE). Pupil diameter was normal at 1 min but from 5 min to 6 hrs after DFP, pupil size was less than 50% control. As measured by infrared video pupillography, complete miosis occurred within 3.5 to 4.0 min after the application of DFP. From this time point on, up to 6 hrs, a pupillary light reflex could not be elicited.

Acetylcholinesterase activity in the iris was reduced to 36% of control 1 min after application of DFP, decreased to 9% at 5 min and still remained far below control values at 6 hrs. Acetylcholine levels in the iris were increased by 34% 1 min after DFP and by 54% at 5 min. This increase remained stable for 120 min and then started to return to control values but was still 28% above controls at 6 hrs. Choline (Ch) levels in the iris were decreased by 22% 5 min after DFP but quickly returned to normal and were the same as controls at all other time points tested.

Evidence consistent with the presence of muscarinic presynaptic autoreceptors on cholinergic nerve terminals in the rat iris was provided by experiments involving electrically stimulated release of labelled ACh from isolated rat irises. DFP (10^{-4} to 10^{-6}M) and certain concentrations of Ch (10^{-4}M) had an inhibitory effect on ACh release that was blocked by scopolamine (10^{-6}M).

The rat iris is an excellent tissue to use for studying pharmacological agents such as DFP since biochemical parameters can be readily correlated with measurements of physiological function. Moreover, the iris is considerably hardier and longer lasting than a brain slice and morphologically is more homogeneous.

INTRODUCTION

Neurobiologists have used organophosphates as powerful tools to elucidate the role and mechanisms of cholinergic neurotransmission in both the central and peripheral nervous systems. The organophosphate, diisopropylphosphorofluoridate (DFP), is a potent irreversible acetylcholinesterase (AChE) inhibiting agent that phosphorylates the active enzymatic site forming a covalent enzyme-substrate complex (Holmstedt, 1959). In addition to its irreversible nature, DFP occupies a unique position in neuropharmacology due to its relationship to both pesticices and chemical warfare agents.

Acute administration of, or accidental intoxication by, organophosphates, results in neurological symptoms and behavioral effects well-correlated with inhibition of AChE and consequently to the accumulation of acetylcholine (ACh) at receptor sites. Some of these symptoms include marked miosis, oral and nasal secretions, muscular fasciculations, hyperactive reflexes, and mental depression (Sidell, 1974). Most of these symptoms can be alleviated by atropine administration indicating specific cholinergic involvement. This effect of prolonged elevated ACh levels at receptor sites has been extensively studied in brain to determine the effects on acetylcholine biochemistry.

In vivo administration of organophosphates increases ACh levels in brain to a maximum within 5-10 minutes (Mayer and Michalek, 1971; Bignami, Rosik, Michalek, Milosevic and Gatti, 1975; Modak, Stavinoha and Weintraub, 1975). This marked increase has been related to the accumulation of ACh in cholinergic terminals as well as in the synaptic cleft (Modak et al., 1975; Wecker, Mobley and Dettbarn, 1977). With prolonged AChE inhibition (2-3 hrs), ACh levels in brain drop significantly toward normal levels (Mayer and Michalek, 1971; Bignami et al., 1975).

However, intrinsic problems exist in the study of the effects of organophosphates in brain due to the morphological heterogeneity of this organ. Brain preparations are more complex because of the co-existence of cholinergic cell bodies and terminals with a variety of other neurotransmitter cell systems. Therefore, it is advantageous to study the effects of these compounds in a less heterogenous tissue such as the iris. The iris contains a dense plexus of cholinergic nerve terminals whose cell bodies are located at a distance from the organ in the ciliary ganglion. It has long been used in autonomic pharmacological studies and is extremely well-suited for these studies (Giacobini, 1970). It allows sequential analysis of function (miosis or mydriasis) and simultaneous measurement of cholinergic parameters such as ACh synthesis, accumulation, turnover, release and degradation (Giacobini, 1983).

Fonnum, Soli, Opstad, Opsahl and Lund-Karlsen (1981) and Soli, Lund-Karlsen, Opsahl and Fonnum (1980) found a linear correlation between pupil size and AChE inhibition after topical administration of soman or DFP in the guinea pig. However, these investigators did not measure accompanying biochemical changes in ACh and choline (Ch) nor did they analyze the evoked release of ACh from cholinergic terminals. The present study seeks to correlate the levels of ACh, Ch, the stimulated release of ACh, and AChE innibition with the physiological pupillary function as determined by infra-red pupillometry (Murray and Loughnane, 1981) after acute topical administration of DFP to the albino rat eye. The correlation of the biochemical effects of DFP administration with the physiological function of the iris, will give insight on the variety of effects DFP has on both central and peripheral cholinergic systems. Determining the events that occur after DFP administration will lead to a more thorough understanding of cholinergic neurotransmission as well as a background concerning

new approaches to the treatment of organophosphate poisoning. Preliminary reports of these data were given at the Society for Neuroscience (Richardson, Mattio, Bernstein-Goral and Giacobini, 1982; Mattio, Giacobini and Richardson, 1983).

MATERIALS AND METHODS

Animals

The topical application of 1 µg DFP (Sigma) in 5 µl of peanut oil to the cornea of Sprague-Dawley rats (Harlan) was performed with a Hamilton syringe, with vehicle administration (peanut oil) done in parallel. For the biochemical studies, the rats were decapitated at various times after the DFP application, the irises were dissected immediately in ice-cold Elliot's B buffer and a preliminary measurement of pupillary diameter was made using an ocular micrometer (20x magnification). For the pupillography studies, the rats were placed in a restrainer as described below. Irises from drug free rats were used in the release experiments as detailed below.

Acetylcholinesterase Activity

AChE activity was determined in aliquots of iris homogenate using the rapid method of Johnson and Russell (1975). AChE activity was determined at various times after DFP injection in triplicate and compared to oil injected controls. These samples were also washed with equal volumes of chloroform to remove excess DFP and prevent false low AChE activities. Data were analyzed by an unpaired Student's t-test to determine significance.

ACh and Ch Levels

ACh and Ch levels were determined in the iris from 0 to 360 minutes after topical administration of DFP by the radioenzymatic method of McCaman and Stetzler (1977). Briefly, this method uses the enzymatic incorporation of ^{32}P from Y-labelled ATP into the choline molecule to for ^{32}P-phosphocholine. To determine tissue ACh levels, the tissue was preincubated with choline kinase and unlabelled ATP, for 15 min. After this preincubation, the tissue is then incubated with AChE, which hydrolyzes ACh to leave free Ch,

which subsequently is phosphorylated with ^{32}P-ATP. For determination of tissue Ch levels, the ^{32}P-ATP is included in the first incubation and no AChE is added. Assays were carried out in duplicate including internal standards (10 pmoles) with a minimum of eight irises per time point. Samples were washed with equal volumes of chloroform to remove excess, unbound DFP that was found to inhibit the AChE activity added as part of the assay procedure. Data were compared to oil injected controls ran in parallel by an unpaired Student's t-test.

Infra-red Pupillometry

Continuous measurements of pupil area after exposure to DFP were done by using bright-field infra-red illumination as described by Murray and Loughnane (1981). Rats were conditioned to sit in a restrainer that inhibited head movement until they showed little, if any, stress evoked behavior determined by pupillary mydriasis. Normal pupillary light reflexes were determined before and after vehicle application (peanut oil). DFP was then applied topically and pupillary response was recorded until maximal pupillary miosis was determined and no light reflex could be elicited.

Buffer

Elliot's B buffer (Elliot, 1969) containing 122 mM NaCl, 1.3 mM KCl, 1.2 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose in double distilled water, was made fresh every second day and stored at 4°C when not in use. Prior to each experiment, the Elliot's B buffer (EBb) was bubbled with 95% O_2 5% CO_2 until pH 7.4. All buffer was continuously bubbled with 95% O_2 5% CO_2 throughout the perfusion-stimulation periods.

^{3}H-Ch Uptake

In studies involving the labelling of ACh pools by uptake of ^{3}H-Ch, the

uptake parameters were as reported by Richardson, Mattio and Giacobini (submitted). Briefly, irises were dissected and pre-incubated for 10 minutes at 37°C in EBb in a shaking water bath. After pre-incubation, irises were transferred to wells containing 1 μM unlabelled choline spiked with 1% ^{3}H-choline (New England Nuclear 80 Ci/mmole). Tissue was incubated for 5 minutes and then washed in 5 ml of buffer. Irises were then placed in the release chambers and run as explained below.

Perfusion-stimulation Release of ACh

The release of ^{3}H-acetylcholine from the parasympathetic nerve terminals in the iris was determined using a modification of the procedure developed by Potashner (1978, 1979). Each stimulation chamber was a 1 cm diameter plexiglass sleeve into which could be inserted plungers with a flat platinum coil electrode on the end and a perfusion cannula. The plungers were positioned so that the platinum coils were 5 to 6 mm apart which created a stimulation chamber of about .5 ml volume that could be perfused via the cannula. Four such chambers were built into a plexiglass box constructed so that water at a constant temperature (36°C unless stated otherwise) could be circulated around each chamber by a Haake heater circulator.

Each iris was placed on the platinum grid of the lower plunger, the upper plunger was inserted into the sleeve, and EBb with or without drugs, was perfused through the chamber at a rate of 1 ml per minute by a peristaltic pump. The perfusate from each chamber was collected in 1 minute fractions in separate mini vials by using a modified drop guide on a Pharmacia Frac 3000 fraction collector. After 10 minutes of perfusion, each iris received electrical field stimulation for 1 minute from a Grass Model S11 stimulation unit through a capaciter circuit to create a biphasic nearly square wave

stimulus of 20 mA, 50 Hz of 5 ms duration, which was then passed between the platinum coil electrodes and monitored on an oscilloscope. This frequency was observed to give maximal release of ACh under our experimental conditions. The 1 minute stimulation period was repeated every 5 minutes for 20 minutes such that each tissue was stimulated four times and each stimulation was followed by a 4 minute washout period. At the end of the fourth washout period, the radioactivity remaining in each iris was determined by placing the iris in 1 ml of absolute ethanol in an oven at 60°C for at least 1 hour. Two mls of scintillation counting cocktail was added to each perfusate fraction and to the ethanol extract of each iris. The radioactivity in each sample was measured in a scintillation counter (Beckman model LS 5800).

The electrically-stimulated release of ^{3}H-acetylcholine from the rat iris was characterized by studying the temperature and ion dependency of the evoked release. It was determined that in the absence of any cholinesterase inhibiting drugs in the perfusion buffer, the radioactivity appearing in the 1 minute fractions collected during the minute after an electrical stimulation was at least 90% ^{3}H-acetylcholine.

Drugs: The effects of various drugs on the release profile were determined by dissolving the drugs in the EBb used to perfuse the tissues. The stability of the anti-cholinesterase potency of 1 μM DFP in aqueous EBb was established by mixing the DFP in the buffer and adding aliquots of this 1 μM DFP solution to homogenates of rat iris after various time periods up to 40 minutes. AChE activity was then determined 5 minutes after the DFP was added to the homogenate. The DFP did go into solution in the EBb and produced a 75-80% inhibition of iris cholinesterase activity at all time points tested.

Calculations: The radioactivity in each 1 minute sample was converted into a release fraction in which the radioactivity in each sample is expressed as a percentage of the total amount of radioactivity in the tissue at the start of each 1 minute collection period (Potashner, 1978). This total tissue radioactivity is calculated for each 1 minute period by adding the radioactivity in that particular 1 minute sample to the radioactivity in all subsequent samples including the radioactivity remaining in the tissue at the end of the experiment. The release profile is obtained by plotting the release fraction of each 1 minute sample. In the absence of any stimulation, a certain amount of radioactivity is constantly being released, presumably by spontaneous discharge of the neurons. When calculating the release evoked by the electrical stimulation, this spontaneous release must be taken into account. The relationship between the spontaneous release and the stimulation-evoked release is expressed as a release ratio which corrects for changes in spontaneous release. This release ratio is given by the formula $\frac{ER-SR}{SR} \times 100$ where ER=evoked release and SR=spontaneous release.

RESULTS

Pupillometry and Biochemistry

Pupil diameter, measured with an ocular micrometer following dissection, was unchanged at 1 min (Fig. 1). At 5 min pupillary miosis was observed (40% of control) which lasted, with some minor fluctuations for at least 6 hrs. Infra-red pupillometry showed that at 1 min the pupil area was starting to decrease, but was still rather large (Fig. 2). Beyond this point, a steady decline in pupil area was recorded. By 3.5 to 4 minutes, complete miosis was observed and no further pupillary light reflex could be elicited. The iris remained unresponsive for at least 6 hours.

Acetylcholinesterase activity was significantly reduced to 36% of control 1 min after DFP application (Fig. 1). It then further decreased to 8% of control after 5 min and remained decreased recovering to only 20% of control activity after 6 hrs. Acetylcholine levels were significantly increased 34% over control, one min after DFP injection (Fig. 1). By 5 min, ACh levels appeared to peak (54%) and remain relatively constant for 120 min, after which they decreased but were still significantly increased over control (28%) at 6 hrs. Choline levels were unchanged 1 min after DFP injection (Fig. 1). However, by 5 minutes they were decreased to 78% of control. At 15 minutes, Ch returned to control levels and remained unaltered for the duration of the experiment.

Release of ^{3}H-ACh

The electrically stimulated release of ^{3}H-ACh from the rat iris was characterized by determining the appropriate parameters of release. First, it was determined that the tritium released was indeed ^{3}H-ACh. Irises were assayed for ACh and Ch content before and after stimulation (20 mA, 50 Hz,

5 ms). Tissue levels of Ch were unchanged whereas ACh levels were decreased almost three-fold, demonstrating that ^{3}H-ACh was being released (Table I). Temperature and ionic requirements for release were also determined. ^{3}H-Acetylcholine release was completely inhibited at 0°C and decreased to 74% at 23°C when compared to release at 36°C (Table II). The initial release of ^{3}H-ACh at 40°C was unchanged from control, however by the fourth stimulation, the iris was unable to release ^{3}H-ACh at this high temperature. Release of ^{3}H-ACh was also demonstrated to be Na^{+} and Ca^{++} dependent (Table III). Sodium was substituted in the buffer by equimolar amounts of LiCl or by 240 mM sucrose. Both decreased release with LiCl having the most profound effect. Release was demonstrated to be highly dependent on Ca^{++}. When Ca^{++} was omitted from the buffer, evoked release was not seen. These data demonstrate that the release of ^{3}H-ACh from the rat iris, in our system, shows temperature and ionic specificity associated with the in vivo release of neurotransmitters from nerve endings.

The pharmacological analysis of the release of ^{3}H-ACh revealed the presence of a presynaptic muscarinic receptor in the rat iris as it has been described in other tissues including the CNS. When stimulated, this muscarinic autoreceptor inhibits release of ACh (Kilbinger and Kruel, 1981; Marchi, Paudice and Raiteri, 1981). Blocking this presynaptic autoreceptor with scopolamine, a muscarinic antagonist, increases the release of ^{3}H-ACh in a dose-dependent manner (Table IV). Choline (10^{-4} M), which has been shown to act as a muscarinic agonist at high concentrations (Kilbinger and Kruel, 1981), inhibits the evoked release of ^{3}H-ACh from the rat iris (Table IV). Choline (10^{-3} M) increased the spontaneous release of ^{3}H-ACh in our preparation as described by Kilbinger and Kruel (1981). This increase in

spontaneous release of ACh is not understood but appears to be an effect related to the presence of a presynaptic muscarinic receptor. These results are consistent with the hypothesis that a presynaptic muscarinic autoreceptor is present in the rat iris.

Diisopropylphosphorofluoridate, at concentrations where 90% or more of AChE activity was inhibited, decreased the release of ^{3}H-ACh (Figs. 3 and 4). This effect was reversed when scopolamine at 10^{-6}M, a concentration which in itself did not increase release (Table 4), was included in the perfusion buffer. As the inhibition of AChE activity was decreased by lowering the concentrations of DFP, so was the effectiveness of DFP on inhibiting ^{3}H-ACh release. At higher concentrations, 10^{-4}M and 10^{-5}M, DFP increased the spontaneous release of ^{3}H-ACh (Fig. 3). This increased spontaneous release was taken into consideration by expressing the data in a release ratio as described in the methods.

DISCUSSION

Endogenous ACh levels were increased at each time point studied after topical administration of DFP. This increase can easily be explained by the primary effect of DFP on inhibiting AChE hydrolysis of ACh, thus allowing ACh to accumulate in neuronal terminals as well as in the synaptic cleft (Modak et al., 1975; Wecker et al., 1977). These results correlate well, quantitatively and temporally, with the data from other investigators on brain ACh levels after AChE inhibitor administration, depending on dose or route of administration (Mayer and Michalek, 1971; Bignami et al., 1975; Stavinoa, Modak and Weintraub, 1976; Wecker et al., 1977). However, a few hours after the AChE inhibition, ACh levels have been reported to drop significantly toward control levels in brain (Mayer and Michalek, 1971; Bignami et al., 1975), but in the iris, ACh levels are still elevated six hours after AChE inhibition. This apparent discrepancy may be due to the fact that the iris contains only cholinergic terminals in contrast to brain which has both terminals and cell bodies. Also, topical administration of DFP may provide for a longer, more direct exposure of the drug resulting in more prolonged increases in ACh.

A correlation between biochemical effects and physiological function of the iris was made in this study. Pupil diameter recorded by pupillometry was unchanged 1 min after DFP administration but by 5 min pupillary miosis was maximal and a light reflex could not be elicited. Acetylcholinesterase activity at 1 min was decreased but not to its maximal extent as seen at 5 min. It appears that the AChE activity (36%) present at 1 min was sufficient to prevent miosis. However, ACh levels were increased at 1 min but again not to the extent that they were at 5 min. This increase in ACh may be intraneuronal and due to an agonistic effect of DFP on the presynaptic muscarinic receptor

resulting in a decrease in release of ACh, thus increasing intraneuronal ACh stores. An intraneuronal increase in ACh would explain the lack of a miotic effect at this time. Also, it is possible that 1 min of DFP exposure is not sufficient time to observe the miotic effect. However, as determined by infrared pupillometry, the miotic effect elicited by a light flash is extremely quick in a control animal, occurring within 4-8 seconds. It seems probable that 1 min is enough time for the iris musculature to respond to extraneuronal ACh indicating that at this time point the increase in ACh is intraneuronal. By 5 min AChE inhibition was maximal and a buildup of extraneuronal and probably intraneuronal ACh was apparent resulting in continuous stimulation of the iris musculature and complete miosis.

Although the primary direct effect of DFP is to inhibit AChE activity, we observed a decrease in the evoked release of ACh from the nerve terminals in the presence of DFP. This decrease in release can be explained by stimulation of the presynaptic muscarinic receptor we have described in the iris. Extraneuronal ACh would be expected to accumulate and to act on the presynaptic muscarinic autoreceptor to result in a decrease in release. This effect was reversed by scopolamine. However, the possibility that DFP may have a direct effect on the presynaptic muscarinic receptor, especially at the early time point of 1 min, can not be ruled out. It has been demonstrated that brain preparations contain a presynaptic muscarinic receptor that inhibits release of ACh by a negative feedback mechanism (Marchi et al., 1981). These receptors appear to be identical or very similar to peripheral muscarinic receptors (Beld, Van Den Houen, Wouterse and Zegers, 1975; Raiteri, Marchi and Paudice, 1981). However, it remains to be determined whether DFP affects central muscarinic receptors in the same manner as peripheral receptors.

Our study indicates that the rat iris is a good model in which to study the short-term cholinergic effects of anticholinesterase agents and in which to correlate biochemical and physiological parameters with the pharmacological actions of various other agents as well. To our knowledge, this is the first paper to demonstrate the existence of a presynaptic muscarinic autoreceptor in iris tissue and we have shown that this autoreceptor may have a role in regulating ACh release after DFP treatment. Although the primary action of DFP is to inhibit AChE activity, DFP also leads to a decrease in the release of ACh. This secondary action may prove to be an important characteristic of the drug that may help to elucidate the mechanisms of cholinergic neurotransmission and to develop antidotes to preserve iris function following environmental exposure to anticholinesterase agents.

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FIGURE LEGENDS

Fig. 1. The time course of the effect of DFP (topical administration; 5 µg in 5 µl) on AChE, pupil diameter, ACh and Ch levels are demonstrated. Pupil diameter was measured with an ocular micrometer. *** $p < .001$, * $p < .05$.

Fig. 2. Infra-red video pupillogram from a rat after vehicle administration (oil) and DFP. At the points indicated the light was turned on or off. The sharp downward deflections are due to blinking of the eye. Pupillary light reflexes could not be elicited after 3.5 to 4 min.

Fig. 3. Release profiles demonstrating typical release patterns in control (C) and DFP treated tissues. The horizontal bars indicate the 1 min period of electrical stimulation. Note the increase in spontaneous ^{3}H-ACh release at 10^{-4} M and 10^{-5} M DFP (see Results).

Fig. 4. Dose-response relation of the release of ^{3}H-ACh in the presence of various DFP concentrations and DFP plus 10^{-6} M scopolamine. Results were calculated as the release ratio and expressed as a percent of control (control release ratio=222.2) with control equalling 100%. AChE activity in the iris is indicated at the various concentration of DFP. *** $p < .001$, ** $p < .01$.

TABLE I

Acetylcholine and choline levels in irises after ^{3}H-Ch uptake and stimulated release of ^{3}H-ACh.

	Control	After Uptake	After Uptake and Release	% Change
ACh (pmol)/iris ± S.E.	151.0 ± 11.42	645.10 ± 48.76***	223.14 ± 31.8***	289.0
Ch (pmol)/iris ± S.E.	147.6 ± 25.4	176.6 ± 9.76***	174.2 ± 10.3***	1.4

*** $p < .001$ when compared to control levels. % change in release was calculated as the change in tissue levels determined after ^{3}H-Ch uptake and stimulated release.

TABLE II

Effect of temperature on stimulated release of ^{3}H-ACh.

Temperature	Release Ratio ± S.E.	% Release
0°C	98.1 ± 14.3 ***	0
23°C	170.5 ± 12.4**	74.5
37°C	222.2 ± 10.5	100
40°C	230.2 ± 30.2	103.6

Release ratio (evoked-spontaneous/spontaneous x 100) was determined as described in Methods where spontaneous release = 100. *** $p < .001$, ** $p < .01$ when compared to release at 37°C.

TABLE III

Effect of sucrose and LiCl substitution for Na^+ and effect of Ca^{++} free medium on ^3H-ACh release.

Ions	Release Ratio ± S.E.	% Release
Sucrose 240 mM	151.54 ± 13.32***	65.4
LiCl 125 mM	95.9 ± 4.4**	0
-CaCl	111.3 ± 3.0***	9.3
Control	222.2 ± 10.5	100

*** $p < .001$ when compared to controls. Release ratio $= \left(\frac{\text{evoked-spontaneous}}{\text{spontaneous}}\right) \times 100$

TABLE IV

Release of ^{3}H-ACh in the presence of scopolamine or choline.

Conc.	Scopolamine Release Ratio ± S.E.	% Release	Choline Release Ratio ± S.E.	% Release
$10^{-3}M$	-	-	212.2 ± 15	96
$10^{-4}M$	439.6 ± 8.3	188***	164.6 ± 11.9	74***
$10^{-5}M$	329.0 ± 35	141***	220 ± 9.4	99
$10^{-6}M$	232.7 ± 12.1	105	230.5 ± 14.1	104
$10^{-7}M$	211.4 ± 19.4	95	-	-
$10^{-8}M$	217.7 ± 22.6	98	-	-

Values were compared to the control release ratio of 222.2.
Release ratio=$\left(\frac{\text{evoked-spontaneous}}{\text{spontaneous}}\right) \times 100$
***P<.001.

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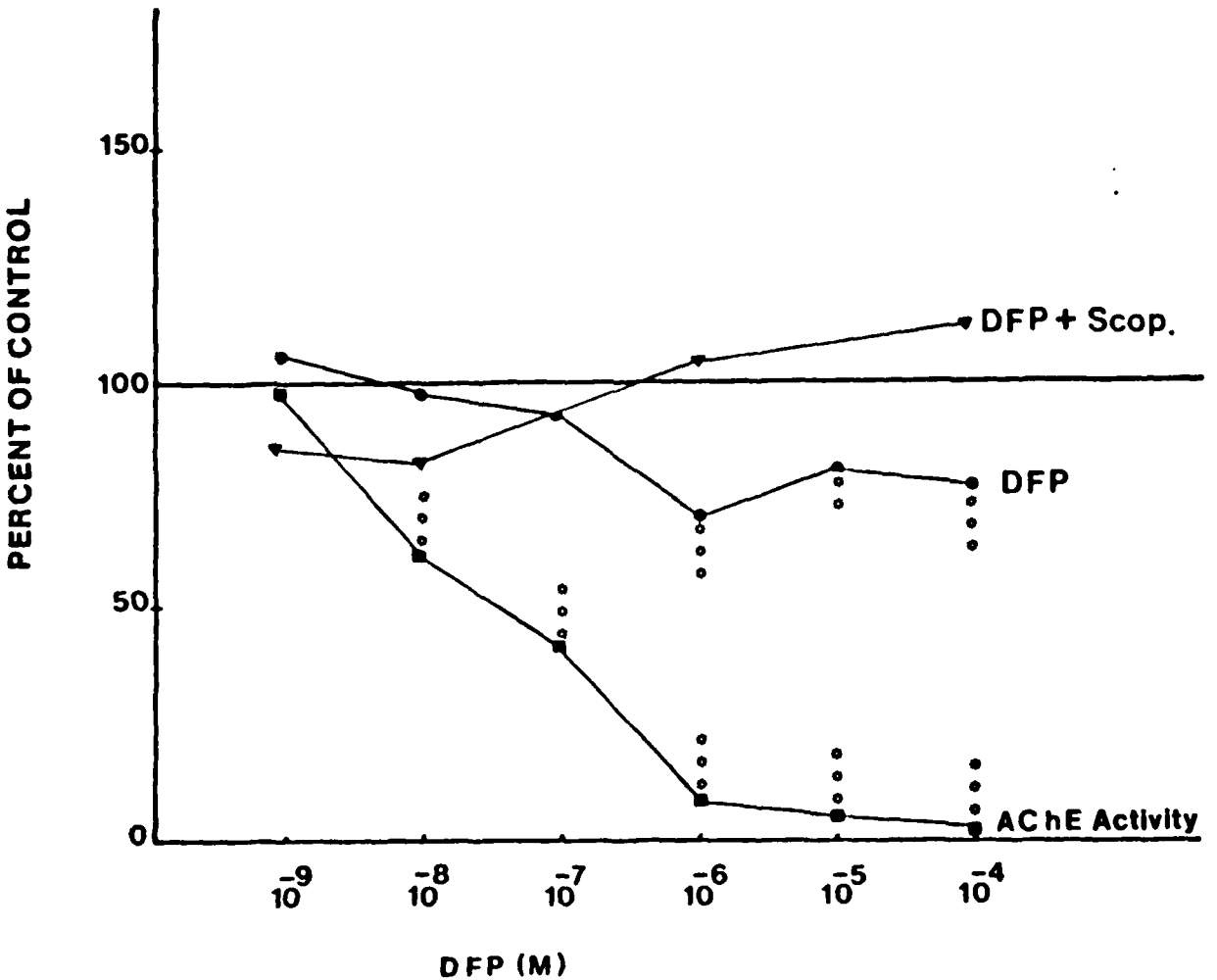
PERCENT OF CONTROL
150
100
50
0
DFP + Scop.
DFP
AChE Activity
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
DFP (M)

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AGING OF CHOLINERGIC SYNAPSES: FICTION OR REALITY?

E. Giacobini, I. Mussini* and T. Mattio

Department of Pharmacology, Southern Illinois University,
School of Medicine, Springfield, Illinois 62708 USA

INTRODUCTION

Cholinergic system and aging of the human brain

The normal process of aging of the human brain seems to be associated with a loss of both cortical and subcortical neurons (Table I). A central problem is whether this decrease in number of neurons is related to neurotransmitter-specific systems or whether it involves unspecifically all neuronal systems. Recent autopsy and biopsy studies in humans (Table I) indicate that aging of the brain is indeed accompanied by a loss of neurons in certain areas, mainly in specific cortical regions. However, no significant changes in cell number are found in other areas, such as brainstem nuclei, cranial nerve nuclei, pons-hypothalamic areas or the medulla oblongata (Table I). A review of the recent morphological and biochemical literature (Table I) suggests that the number of brain regions known to escape neuronal loss in the course of the aging process has grown. Therefore, it seems that in the aging human brain two populations of neurons coexist side by side. One undergoing regressive changes leading to cell death and a second surviving and undergoing continuous and dynamic growth of processes. In pathological conditions, such as in Parkinson and Alzheimer diseases, some decreases are found in corresponding areas but not in other, indicating a variance from normal aging (Table I). The most

*Natl. Res. Coun. Unit for Muscle Biol., Inst. Gen. Pathol., U. Padova, Italy

pronounced difference is seen in the substantia innominata (nucleus basalis of Meinert) of Alzheimer patients, with a 75-80% loss in neurons (2,19,20). It should be noted that in the same nucleus a 66% decrease in cell density is seen also in Parkinson patients (Table I) (1). A decrease in number of cells in Alzheimer brains is seen also in non-cholinergic nuclei such as the locus coeruleus (Table I) (10,18). In spite of the fact that neuronal loss in Parkinson disease may be the same or greater than in Alzheimer disease (Table II), no extensive cortical abnormalities either morphological or neurochemical (ChAc, cholineacetyltransferase activity) have been reported in the former disease (1). A similar situation is found in the locus coeruleus where neuronal loss in Alzheimer brains is not correlated with a reduction in cortical activity of noradrenergic enzymes (10). The mechanism of neuronal loss may be different in the two diseases as suggested by the differences in pathological changes observed in perikarya and neuropil. In fact, there seems to be no apparent relationship between the formation of characteristic neurofibrillary changes in one case and the presence of Lewy bodies and neuroaxonal spheroids in the other. It looks, therefore, unlikely that the two abnormalities might represent different manifestations of the same disease. Recent results on changes in cell density, total number of cells, ChAc and AChE (acetylcholinesterase) activity in the nucleus basalis of human controls and SDAT (senile dementia of Alzheimer type) brains are reported in Table II. It can be seen that all four parameters are strongly affected in SDAT patients, however, contrasting differences between results of various investigators are seen in cell number and cell density (1,11,19,20).

In conclusion, in the case of Alzheimer disease, both morphological and neurochemical results suggest a primary degeneration of cholinergic axons and terminals projecting to cortex and a secondary loss of cholinergic neurons in

TABLE I

CHANGES IN NUMBER OF NEURONS WITH AGE IN NORMAL AND PATHOLOGICAL HUMANS[x)]

	NORMAL		PATHOLOGICAL	
STRUCTURE		Age (years)	PARKINSON	ALZHEIMER
Cerebral cortex (Inf. frontal and sup. temporal)		(90) 45% decr.		17-18% large neurons decr.
Hippocampal cortex		(45-90) 27-80% decr.		47% decr.
Cerebellar cortex (Purkinje cells)		(60-100) 25% decr.		
Parietal and occipital cortex		(80) unchanged		no differ.
Telencephalon (putamen)		decr.		
Subst. nigra	Newborn 400.000 (dopam.)	(60) 250.000	60.000	decreased
Nucleus coeruleus	Young adult 19.000 (norep.)	(60-80) 17.000-11.000		43% decr.
Nucleus bas. Meinert's (subst. innominata)	Young adult 450.00 (cholin.)	(50-66) unchanged(?)	66% decr.	75-80% decr.
Brainstem nuclei (ventral coc., inf. olive, mamm. bodies)		unchanged		
Cranial Nerve Nuclei (trochlear, abducens, facial)		unchanged		unchanged
Pons-hypothalamus		unchanged		
Medulla oblongata		unchanged		

x)For ref. see text.

TABLE II
CHANGES IN CELL DENSITY AND CHOLINERGIC ENZYMES ACTIVITY IN NUCLEUS BASALIS OF CONTROL AND SDAT[x] BRAINS

	CONTROL	SDAT	% DECREASE	AUTHOR/YEAR
CELL NUMBER nr cells/grid	5.9 ± 0.9	1.6 ± 0.3	73	Whitehouse et al., 1981, 1982 (19,20)
total nr cells/ section	344.6 ± 64.6	72.4 ± 14.6	79	Whitehouse et al., 1982(20)
"	--	--	<50	McGeer et al., 1983 (8)
CELL DENSITY mean area per neurone $um^2 \times 10^3$	10	15	33	Candy et al., 1983 (1)
	10	30 [xx]	66[xx]	Candy et al., 1983 (1)
	--	--	33	Perry et al., 1982 (11)
ChAc[xxx] POSITIVE CELLS	104	35	66	Nagai et al., 1983 (9)
(himmunohistochem.)	--	--	<50	McGeer et al., 1983 (8)
ChAc ACTIVITY (nmol/h/mg)	--	--	89	Perry et al., 1982 (14)
	--	--	70	Rossor et al., 1982a(13)
"	196.8 ± 88	13.4 ± 4.4	94	Candy et al., 1983 (1)
"	81.8[xxxx]	25.9[xxxx]	64	Rossor et al., 1980; 1982b (12,14)
AChE ACTIVITY[xxxxx] (histochemistry)	INTENSE STAINING	--	--	Rossor et al., 1982b(14)
(umol/min/mg protein)	676.8 ± 101.2	141.9 ± 33.9	79	Candy et al., 1983 (1)

x) Senile dementia of Alzheimer type
xx) Parkinson patients
xxx) Choline acetyltransferase
xxxx) Medium value
xxxxx) Acetylcholinesterase

subcortical nuclei. Whether the irreversible neuronal loss is large or moderate and its nature remains to be demonstrated by further studies, possibly at the ultrastructural level. Another fundamental question is whether Alzheimer disease is primarily a "cell body" or a "terminal disease" and what is the time course and sequence of the neuronal damage (Table III). Other important questions related brain aging in general and to SDAT in particular are reported in Table III below.

TABLE III

SOME BASIC QUESTIONS WHICH REMAIN UNANSWERED

1. ARE BIOCHEMICAL CHANGES SELECTIVELY LOCALIZED TO CERTAIN BRAIN NUCLEI OR ARE THEY DISTRIBUTED TO ALL CHOLINERGIC SYNAPSES IN THE CNS?

2. ARE CHANGES RELATED TO THE NORMAL CEREBRAL AGING PROCESS, I.E. ARE THEY MECHANISMS OF ENZYMATIC ADAPTATION OR ARE THEY SPECIFIC FOR SENILE DEMENTIA? HOW IMPORTANT IS THE AGE RANGE OF THE CONTROLS? HOW IMPORTANT IS THE SEVERITY OF THE DISEASE?

3. WHICH IS THE PRIMARY TARGET FOR THE CHEMICAL DAMAGE AND THE NEURONAL DEGENERATION? DOES THE AGING PROCESS INVOLVE BOTH PRE- AND POSTSYNAPTIC STRUCTURES? DOES THE PROCESS INVOLVE CHOLINERGIC TERMINALS FIRSTLY AND PERIKARYA SECONDLY?

4. ARE CHOLINERGIC NEURONS IN THE PNS AND CNS EQUALLY AFFECTED?

5. IS THERE A RELATIONSHIP BETWEEN THE REDUCTION IN CHOLINERGIC CORTICAL INNERVATION AND THE PATHOGENESIS OF PLAQUES?

Cholineacetyltransferase is synthesized in the cell body and then transported to the terminal of the cholinergic neurons while acetylcholine can be synthesized at both locations. A defect could occur at both places in developing, adult or aging cholinergic neurons (5). It is, therefore, important to develop models of aging which make it possible to study cell bodies as well as terminals in the same population of cholinergic neurons throughout the entire life of the animal. Such a model is described in the next section.

The avian iris as a model of aging.

Aged chickens are defined as birds of age 36 months or older. However, as in mammals, incipient signs of senescence may appear in the avian nervous system at earlier stages of life. In the chicken, these early signs can be identified morphologically and biochemically in the PNS of the chicken after 24 months of age (5). . . Approximately 90% of adult weight as well as sexual maturity are reached at 200 days (7 months) of age. The rate of growth of the brain is very rapid at first and then gradually decreases as the body weight increases, becoming very small beyond 300 days of age. Among degenerative signs related to old age, the close similarity of spontaneous avian atherosclerosis to the human disease has been recognized since the beginning of the twenthieth century (15,17). A combination of causal factors which remind us of human pathogenesis, such as hemodynamic conditions, blood lipids (including elevated plasma cholesterol) and thrombogenic mechanisms has been demonstrated in avians (17).

In addition, in the chicken as an effect of age, blood pressure increases significantly in males and females from age 10-14 months to age 42-54 months. It is interesting to note that most of the drugs and hormones which are pressor or depressor in mammals have the same effect in birds (17).

Additional advantages in using the avian nervous system as a model of aging are: a) the relatively long life span which allows to observe gradual changes. In rodents, age-related modifications occur almost abruptly at the end of life. b) possibility of studying discrete populations of homogenous (cholinergic) neurons such as in the ciliary ganglion, maintaining a constant number of cells throughout life span. c) possibility of investigating separately, cell body and terminals belonging to the same neuron (ciliary ganglia and iris). d) possibility of parallel physiological and

pharmacological testing of pupillary functions in vivo. e) a good correlation between biochemistry and morphology feasible through morphometric EM studies and quantitative cytochemical analysis (5).

The ciliary ganglion preparation has proved to be a useful and simple neuronal model of cholinergic synapses since its introduction in studies of synaptic development and plasticity (3,4).

In a series of studies which has been recently summarized by Giacobini (5), we have made use of the ciliary ganglion iris preparation of the aging chicken as a model of senescent peripheral cholinergic synapses. In humans, one predictable aging marker has been emphasized: i.e. pupil function as judged by pupil size. Senile miosis, a reduction in pupil size seems to contribute a reliable sign of aging as the diameter of the pupil correlates closely to age for both the dark-adapted and light-adapted eye (7). Seventy-five percent of subjects 55 or older show 3 mm pupil diameter while all those under 55 have pupils at least 4 mm in diameter. In addition, pupillary responses to cholinergic drugs are impaired in the elderly (16).

Based on the studies performed on the iris, an hypothesis of aging of the cholinergic synapse has been suggested (6). This hypothesis contemplates age related changes in carrier-mediated mechanisms and in molecular and physical properties of neuronal membranes leading to a "chemical denervation" of the cholinergic synapse. The concept of neuronal aging as an integration of progressive and regressive cell changes, involving specific membrane mechanisms in selective parts of cholinergic neurons has been previously discussed (5,6). In order to firmly establish the cholinergic hypothesis of neuronal aging in humans, several basic questions remain to be answered (Table III). Experimentally, a close integration of biochemical and ultrastructural data and the development of new tests to detect early signs of pathological aging of the

cholinergic system are necessary. In the next section we will describe an attempt in this direction.

Ability of the aging cholinergic synapse to take up choline, release acetylcholine and to deplete and reform synaptic vesicles.

As illustrated in Fig. 1A, neuromuscular junctions in the iris of an aging chicken (2 year) show polymorphic signs of damage such as, reduction and polymorphism of synaptic vesicles, increase of neurofilaments and mitochondria. Accumulations of cytoplasmic organelles and lysosomes are seen in the axoplasm of the nerve fiber (Fig. 1B). At later stages the nerve ending is enveloped by Schwann cells infiltrating and partially filling the synaptic cleft. Quantitative changes in the ratio describing the relation between volumes of terminals and volumes of synaptic vesicles showing a trend to shift to the left are seen from 4 m to 9 year (Fig. 2). This indicates a progressive decrease in the volume occupied by synaptic vesicles (Fig. 2) and a possible functional deficit. In order to establish the nature of such a deficit, we have examined the ability of cholinergic synapses in the iris at various ages to take up the precursor ^{3}H-choline (Ch) and release the formed ^{3}H-ACh in response to high K^{+} (115 mM) depolarization. We have observed that following release of ACh, exocytosis clearly prevails on endocytosis and a nearly total depletion of vesicles is present (Fig. 3A). This depletion is reversible, as after 60 min incubation in a physiological medium the morphological recovery is almost complete (Fig. 3B). The scheme of a typical experiment is reported in Fig. 4. Changes in endogenous levels of Ch, ACh and PhCh (phosphorylcholine) and in release of ^{3}H-ACh, are expressed as percent differences of levels before and after depletion (A-B) or depletion followed by a period of recovery and a new depletion (A'-B') (Table IV). For simplicity, we shall only comment upon significant percent differences between young (4 m) and old (5 y) animals which represent the extreme ages studied (Table IV). Under the condition of the

Figure 1: Iris muscle of a 2-year-old chicken. The neuromuscular junction (A) shows reduction and polymorphism of synaptic vesicles, increase of microfilaments. (B) Note the accumulation of mitochondria and lysosomes in the axoplasm of a nerve fiber. Calibration bars = 1 μm.

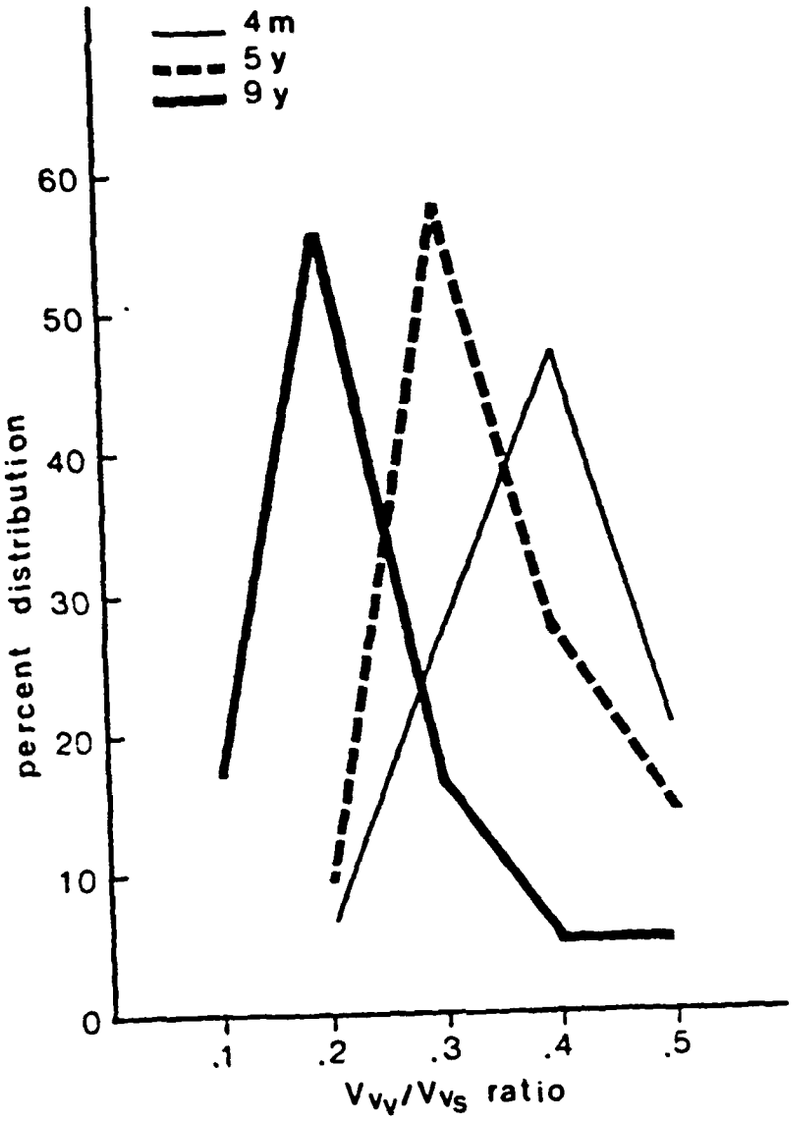

Figure 2: Summary of preliminary results of morphometric analysis of nerve endings and synaptic components in the iris of young adult (4 mo.) and aged (5 and 9 years) chicken. The figure shows the percent distribution of V_{vv}/V_{vs} ratio, where V_{vs} = synaptic bouton volume fraction; V_{vv} = synaptic vesicle volume fraction. The results obtained by the analysis of the iris NMJ of one animal/age group are the average of 15-20 samples/age. As seen from the figure, there is a trend of the peaks to shift to the left from 4 m to 9 y, demonstrating an age-related decrease in the relative volume of synaptic vesicles.

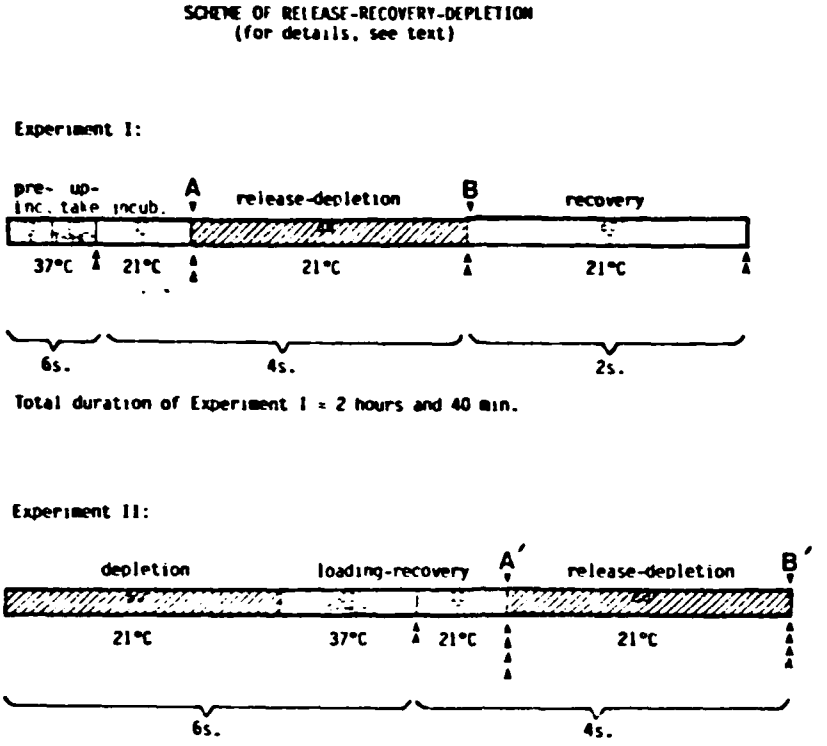

Figure 4: Scheme of Release-Recovery-Depletion

first set of experiments (Exp. I, Fig. 4 and Table IV) endogenous ACh levels are more significantly decreased (56 vs 14%) in the young animals than in the 5 y old, however Ch level declines equally at both ages. The release of ^{3}H-ACh is probably also more pronounced at 4 m (58 vs 35%) than at 5 years. The difference in ^{3}H-Ch and ^{3}H-PhCh are similar at both ages. Such changes suggest that the mechanism of depletion may be more active in young animals.

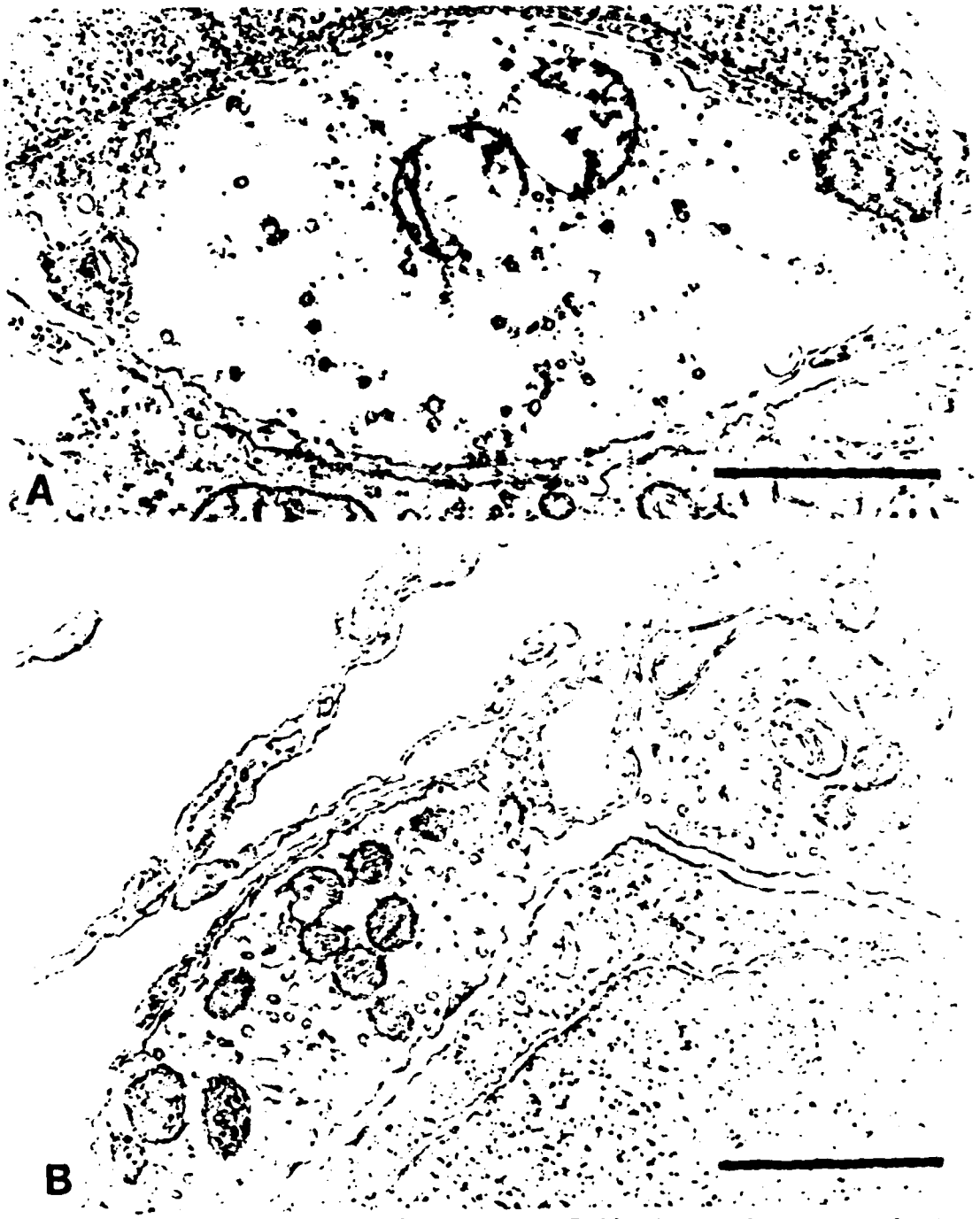

Figure 3: Iris muscle of a 2-month-old chicken. Following prolonged depolarization with high-K^+ concentration (A) the nerve ending appears almost depleted of synaptic vesicles. After reincubation in physiological medium (B) a nearly total retrieval of synaptic vesicles is achieved. Calibration bars = 1 um.

TABLE IV

CHANGES IN CHOLINERGIC PARAMETERS FOLLOWING STIMULATED RELEASE OF ACETYLCHOLINE

EXPERIMENTAL PERIOD	AGE	PER CENT DIFFERENCE				
		ACh	Ch	*ACh	*Ch	*PhCh
A - B	4 m	56↓	32↓	58↓	62↓	25↓
	1.5 y	35↓	28↓	60↓	75↓	20↓
	5 y	14↓	30↓	35↓	69↓	27↓
A'- B'	4 m	37↓	1↑	11↓	19↓	21↓
	1.5 y	21↑	39↓	42↓	66↓	39↓
	5 y	44↓	45↓	33↓	67↓	34↓

The figures represent the mean of at least three analyses performed on a total of five animals.
A-B = First period of release - depletion; A'-B'= Second period of release - depletion; * = labelled compound (see Fig. 4); ACh = acetylcholine; Ch = choline; PhCh = phosphorylcholine

↑ ↓ Arrows indicate positive (increase) or negative (decrease) changes between A and B values (expressed as a percentage of A, starting value) - e.g. 56↓ express a release of 56% of the ACh present at point A. when compared to point B (see Fig. 4)

In the second set of experiments (Exp. II, Fig. 4 and Table IV), on the contrary, endogenous ACh levels are more decreased in the old animals and the percent decrease in Ch levels is also significantly more severe at 5 y (45 vs 1%). The formed ^{3}H-ACh is more depleted at 5 years (33 vs 11%) than at 4 m. This loss is reflected in both Ch and PhCh strong negative changes.

In conclusion, experiments testing the uptake and release capacity of the peripheral cholinergic synapse under acute conditions of stimulation suggest that severe changes may occur at later stages of life. If a sufficiently long period of recovery is allowed, aging iris terminals are still capable of

responding with an adequate depletion of ACh following two subsequent periods of release and reloading. However, under such strenuous conditions both Ch and PhCh pools are significantly depleted. These experiments seem to point out for the first time a specific functional defect in the cholinergic synapse during aging.

ACKNOWLEDGEMENT

The investigations carried out in the author's (E.G.) laboratory were supported by AFOSR Grants 81-9229 and 83-0051; by grants from the Nowatski Eye esearch Foundation and by the E.F. Pearson Foundation. Support from the National Research Council of Italy to the Unit for Muscle Biology (I.M.), Institute of General Pathology, University of Padova, Italy is acknowledged. The authors wish to thank the Department of Pathobiology (Dr. L. Pierro, Chairman), University of Connecticut, Storrs, CT and the University of Connecticut Research Foundation for providing funds and assistance in order to maintain aged birds at their facilities.

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NOTE ADDED AFTER COMPLETION OF MANUSCRIPT

Three laboratories reported independently at this meeting some new results which confirm the loss of cholinergic cells in forebrain structures of human SDAT patients. McGeer et al. reported a decline in cholinergic neurons from 450.000 in the young adult to 50-100.000 in SDAT patients. It should be noted that similar changes in cell number were observed in two non-cholinergic areas (locus coeruleus and substantia migra) by the same authors. According to these authors age-related conditions and senile dementia follow the same pattern, however, in SDAT the insult is more pronounced. According to Bigl et al. (see T. Arendt, V. Bigl, A. Arendt and A. Tennstedt, Acta Neuropathol., 61:101-108, 1983), the neuronal loss is 70% in Alzheimer, 75% in Parkinson (paralysis agitans) and 50% in Korsakoff patients. Etienne et al. found a 60-90% loss in neurons stained with AChE histochemistry and a 60-90% decrease in cell counts. These three recent observations underline the necessity of answering the questions reported in Table III before reaching a final conclusion about SDAT and involvement of the cholinergic system.

Attention: Replace Table I (enclosed) as corrected.

283.11 UTILIZATION OF CHOLINE TRANSPORTED BY SODIUM-DEPENDENT, HIGH-AFFINITY CHOLINE CARRIERS FOR ACETYLCHOLINE SYNTHESIS: COMPARISON OF RAT AND GUINEA-PIG FOREBRAIN SYNAPTOSOMES. R. J. Pylett, T. J. Carlton* and E. M. Colnoun*. Department of Pharmacology, University of Western Ontario, London, Ont., Canada, N6A 5C1.

Controversy exists over the role of choline transported into synaptosomes by sodium-dependent, high-affinity carriers in the synthesis of acetylcholine (ACh). Some of the observed differences could be due to species variability in the parameters measured and the mechanisms involved. Largely, studies have involved the use of rat and guinea-pig brain, species which are known to differ with respect to the molecular forms (pI) of choline acetyltransferase. In the present report, we compare the kinetics of choline transport and the conversion of ^{3}H-choline to ^{3}H-ACh in resting and K$^+$-depolarized synaptosomes prepared from rat and guinea-pig forebrain. Analysis of choline transport over the ^{3}H-choline concentration range 0.1 to 100 μM revealed typical biphasic kinetics with apparent Michaelis constants, K_m, of 2 and 109 μM and V_{max} of 64.3 and 304.6 pmol/mg protein/4 min for rat forebrain synaptosomes; kinetics of transport for choline into synaptosomes prepared from guinea-pig brain did not differ significantly. Following incubation of anticholinesterase-treated synaptosomes with 1 μM choline, conversion of ^{3}H-choline to ^{3}H-ACh was quantitated by preparative HPLC separation of the choline metabolites and liquid scintillation spectrometry. Velocity for choline uptake in rat brain synaptosomes (^{3}H-choline=1 μM) was 20.2 pmol/mg protein/4 min of which 64% was transported by sodium-dependent processes; in comparison, choline transport velocity into guinea-pig synaptosomes was 18.7 pmol/mg protein/4 min, 79% of which was apolijshed in sodium-free (lithium substituted) medium. In regular K$^+$ (5 mM) medium, it was observed that 72% of ^{3}H-choline transported into rat brain synaptosomes by sodium-dependent processes was acetylated, while in guinea-pig only 57% of such choline was metabolized to ACh ($P<0.02$). Following K$^+$ (40 mM)-depolarization, 74.6% of ^{3}H-choline transported by sodium-dependent mechanisms in rat synaptosomes was acetylated, however, in guinea-pig synaptosomes this parameter was significantly increased to 70.3%. The net increase in ^{3}H-choline uptake following K$^+$-depolarization was 142.5% and 13% in rat and guinea-pig, respectively, relative to resting transport. Thus, in guinea-pig synaptosomes the percentage of choline transported via sodium-dependent carriers diverted to ACh synthesis is increased by depolarization of the nerve terminal. These results suggest that there may be differences underlying the coupling of choline transport to the enzymatic acetylation reaction and the utilization of exogenous choline in the synthesis of ACh in synaptosomes from brain of rat and guinea-pig.
(Supported by the Medical Research Council of Canada).

283.12 EFFECTS OF DFP ON THE RELEASE OF ACETYLCHOLINE: ROLE OF A PRESYNAPTIC MUSCARINIC RECEPTOR. T.G. Hattion*, E. Giacobini, J.S. Richardson*. (SPON: C. Su), Dept. Pharmacology, Southern Illinois University School of Medicine, Springfield, IL 62708

The albino rat iris contains a dense plexus of cholinergic nerve terminals whose cell bodies are located in the ciliary ganglion. This structure is a good model for the study of cholinergic function due to its homogeneity. Following characterization of the high affinity choline (Ch) uptake system, the electrically stimulated release of acetylcholine (ACh) was studied. ACh pools were labelled by uptake of ^{3}H-Ch for 10 min (1 uM). The irises were then rinsed and put in a release chamber modified from Potashner (1978). After a 10 min wash the tissue was stimulated by a 50 Hz, 20 mA, 5 ms square wave for 1 min while being superfused by oxygenated Elliots B buffer. The perfusate was collected into scintillation vials, after which 2 ml of cocktail was added and the radioactivity released was determined by liquid scintillation counting. The tritium released was expressed as a percentage of the total tritium present in the tissue at the time of release. We demonstrated that the tritium released was 95-100% ^{3}H-ACh. The release of ACh was found to be Na$^+$, Ca^{++} and temperature dependent. The addition of scopolamine (10^{-4}-10^{-5}M) increased the release of ACh up to 190% while, the addition of choline (10^{-3}M) decreased the release of ACh. This decrease in release was reversed by the addition of 10^{-6}M scopolamine, demonstrating the presence of a presynaptic muscarinic receptor, as has been described in other tissues. The addition of the irreversible cholinesterase inhibitor diisopropyl fluorophosphonate (DFP) (10^{-4}, 10^{-5}, 10^{-6}M) into the superfusion buffer resulted in a significant decrease in the stimulated release of ACh with esterase activity inhibited by more than 90%. DFP at 10^{-7} and 10^{-8}M inhibited esterase activity by 60 and 40%, respectively, but had no effect on the release of ACh. This decrease on the release of ACh was found to be totally reversible with the addition of 10^{-6}M scopolamine into the buffer. The decrease in release of ACh by DFP can be attributed to the accumulation of ACh in the synaptic cleft and consequently its agonistic effect on the presynaptic muscarinic receptor therefore decreasing release of ACh. The muscarinic antagonist scopolamine, at a concentration where itself does not effect release, was able to totally reverse this effect. This data demonstrates the presence of a presynaptic muscarinic receptor on cholinergic terminals in the rat iris and suggests a mechanism by which DFP decreases the release of ACh from cholinergic terminals. (Supported by Air Force grant #81-0229 and 83-0051.)

283.13 SECRETION OF ^{3}H-ACETYLCHOLINE FROM GUINEA-PIG ILEUM MYENTERIC PLEXUS IS ENHANCED BY INHIBITORS OF PHOSPHODIESTERASE. P. Alberts* and B. Sellstrom*. (SPON: P. Greengard). Division of Experimental Medicine, National Defense Research Institute, S-901 82 Umeå, Sweden.

The secretion of acetylcholine (ACh) is regulated by presynaptic muscarinic feedback inhibition. The possible involvement of endogenous cyclic nucleotides in this control was investigated using two inhibitors of phosphodiesterase. The ACh stores of the cholinergic nerves of the myenteric plexus of the guinea-pig ileum longitudinal muscle preparation were labelled with ^{3}H-choline. The preparation was mounted in an organ chamber and superfused with Tyrode solution containing hemicholinium-3 (10^{-5} M) and eserine (10^{-5} M). Stimulation was with trains of 150 shocks (0.5 ms, 120 V) at a low frequency (0.5 Hz). The results are expressed as the evoked fractional secretion of total ^{3}H. Addition of 3-isobutyl-1-methylxanthine (IBMX, 2.25 mM) enhanced the evoked secretion of ^{3}H-ACh by 99 ± 28 % (n=6, p<0.001). From the effects of IBMX (1-5 mM, n=7) the concentration yielding half-maximal enhancement (K_m) was determined to be 2.0 mM. The maximal increase over the control level at infinitely high concentration of IBMX (V_{max}) was estimated to be 190 %. Furthermore, the effects of IBMX (1 or 2 mM) were not altered by atropine (10^{-5} M). A structurally different inhibitor of phosphodiesterase, SQ 20,006, also slightly enhanced the ^{3}H-ACh secretion but within a very narrow concentration range. The secretion was enhanced by 40-110% by SQ 20,006 (0.3-0.5 mM). Above this range the secretion was enhanced drastically, about 10-fold, and was probably not related to the inhibition of phosphodiesterase. The results suggest that endogenous cyclic nucleotides are not involved in muscarinic "autoinhibition" of ^{3}H-ACh secretion in guinea-pig ileum myenteric plexus. However, it is conceivable that adenosine 3',5'-cyclic monophosphate may be involved in the enhancement of evoked ^{3}H-ACh secretion caused by activation of other receptors.

283.14 ACTIVATION OF ACETYLCHOLINE SYNTHESIS IN THE ABSENCE OF RELEASE: DEPENDENCE ON SODIUM, CALCIUM AND THE SODIUM PUMP. R. L. Barnes. Department of Physiology, McGill University, Montreal, Canada H3G 1Y6.

Following a 15 min inhibition of the sodium pump in the cat superior cervical ganglion by perfusion with K-free Locke solution, a 10 min recovery in normal Locke produced a 51% increase in acetylcholine stores. The increase in stores occurred without increase in acetylcholine release. Thus this procedure of pump inhibition followed by recovery selectively activates acetylcholine synthesis. The increase in stores, which occurred entirely during the 10 min recovery period in which the sodium pump was reactivated, represents a rate of synthesis of acetylcholine of 5.1% of stores per min; equal to the maximum rate that can be achieved during high frequency preganglionic nerve stimulation. The increase was not affected by substituting isethionate for chloride in the perfusion fluids. It was prevented by reducing sodium to 25 mM in the K-free Locke and also prevented by omitting calcium from the perfusion fluids. It is concluded that the selective activation of acetylcholine synthesis following the pause in sodium pumping was a direct result of an increased sodium pump rate and an increase in internal calcium in the nerve terminals. It is proposed that similar ionic events produced by repetitive nerve impulses likewise activate acetylcholine synthesis independently of release of transmitter or depletion of stores.

AGING OF CHOLINERGIC SYNAPSES: FICTION OR REALITY? Ezio Giacobini, Southern Illinois University School of Medicine, P.O. Box 3926, Springfield, Illinois 62708 USA

Combined neuropathological and biochemical evidence suggests that a primary degeneration of cholinergic axons projecting to the cortex, and a secondary reduction in number of cholinergic neurons may occur in specific subcortical nuclei (basal forebrain), during pathological aging in humans. The factors inducing such a selective loss in cholinergic function are not known. Quantitative analysis of neuronal population density and biochemistry show that neurons and synapses other than cholinergic may also be affected by the same aging process. Variable data have been reported with regard to the relationship between neuronal losses and cholinergic changes and to the magnitude of the reductions. In order to firmly establish a cholinergic hypothesis of senile dementia, we will first discuss relevant questions such as:

1. Are biochemical changes selectively localized to certain brain nuclei or are they distributed to all cholinergic synapses in the CNS?

2. Are changes related to the normal cerebral aging process, i.e. are they mechanisms of enzymatic adaptation or are they specific for senile dementia? How important is the age range of the controls? How important is the severity of the disease?

3. Which is the primary target for the chemical damage and the neuronal degeneration? Does the aging process involve both pre- and postsynaptic structures? Does the process involve cholinergic terminals firstly and perikarya secondly?

4. Are cholinergic neurons in the PNS and CNS equally affected?

5. Is there a relationship between the reduction in cholinergic cortical innervation and the pathogenesis of plaques?

In the second part of our presentation, a model of peripheral cholinergic aging, the iris, will be introduced. This model allows us to study major cholinergic parameters together with pupillary function. In humans, pupillary size constitutes a predictable marker of age-related pupillary function and senile miosis seems to contribute a reliable sign of aging of the cholinergic innervation of the eye. Observations will be presented which support the view that terminals of cholinergic neurons, particularly in the PNS, represents more vulnerable targets of aging process than cell bodies. Recent attempts to characterize the cholinergic damage to synaptic membrane function will be discussed.

Supported in part by AFOSR grant #83-0051 and Nowatski Eye Fund.

Dynamics of Cholinergic Function Symp.
Oct. 30 thru Nov. 4, 1983
Oglebay, W.Va.

EFFECTS OF DFP ON ACETYLCHOLINE METABOLISM AND RELEASE AND PUPILLARY FUNCTION IN THE RAT. T.G. Mattio, J.S. Richardson and E. Giacobini, Southern Illinois University School of Medicine, P.O. Box 3926, Springfield, Illinois 62708 USA

The effects of acute topical administration of diisopropylphosphorufluoridate (DFP) on cholinergic biochemistry and ACh release were determined and correlated to pupillary function in the rat. DFP (5 ug) reduced acetylcholinesterase (AChE) activity to 36% at 1 min and to 8% after 5 min and remained decreased for up to 6 hrs. Pupillary area was normal at 1 min and by 3.5 to 4 min complete miosis occurred and no light reflex could be elicited for up to 6 hrs. Acetylcholine (ACh) levels were increased 34% at 1 min and by 5 min showed a 54% increase. This increase remained stable for 120 min after which it decreased to 28% at 6 hrs. Choline levels were decreased 22% at 5 min but recovered by 15 min and remained at control levels through all time points studied. The presence of a presynaptic-muscarinic receptor was demonstrated in the iris. The role of this receptor in inhibiting ACh release in the presence of DFP was also determined. DFP shows an inhibitory effect on ACh release which was blocked by scopolamine suggesting that it is mediated through a muscarinic receptor. The rat iris proved to be a good model for studying of AChE agents since biochemical findings are easily correlated to physiological effects on the pupil.

Supported in part by AFOSR grant #83-0051 and Nowatski Eye Fund.

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A SPECIFIC FUNCTIONAL DEFECT OF PERIPHERAL CHOLINERGIC SYNAPSES DURING AGING

Mussini, I.*, Mattio, T.G.*, Giacobini, E. and Richardson, J.S.
Dept. Pharmacol., So. Ill. Univ. Sch. Med., Springfield, IL 62708 USA

Neuromuscular junctions in the iris of aging (2-4 yrs) chicken show polymorphic signs of degeneration such as reduction and polymorphism of synaptic vesicles, increase of neurofilaments and mitochondria. Accumulations of cytoplasmic organelles and lysosomes are seen in the axoplasm of the nerve fiber. At later stages (5-9 yrs) the nerve ending is enveloped by Schwann cells infiltrating and partially filling the synaptic cleft. Quantitative changes in the ratio describing the relation V_{vv}/V_{vs} between volumes of terminals (V_{vv}=synaptic bouton volume fraction) and volumes of synaptic vesicles (V_{vv} = synaptic vesicles volume fraction) show a decrease from .4 to .2 between 4 month and 9 years. This indicates a progressive decrease in the volume occupied by synaptic vesicles and a possible functional deficit. We examined the ability of cholinergic synapses in the iris at various ages to take up the precursor ^{3}H-choline (Ch) and release the formed ^{3}H-acetylcholine (ACh) in response to high K^+ (115 mM) depolarization. We have observed that following release of ACh, exocytosis clearly prevails on endocytosis and a nearly total depletion of vesicles is present. Under acute conditions of stimulated release, aging terminals are still capable of an adequate depletion of ACh. However, under more strenuous conditions of multiple kinds of loading-reloading and release both Ch and phosphorylcholine are significantly depleted. These experiments point out for the first time a specific functional defect in the cholinergic synapse during aging.

(Supported by AFOSR Grants 81-9229; 83-0051, Nowatsky Eye Research Foundation and E.F. Pearson Foundation to E.G.)

A SPECIFIC FUNCTIONAL DEFECT OF PERIPHERAL CHOLINERGIC SYNAPSES DURING AGING

Giacobini, Ezio

So. Ill. Univ. Sch. Med., P.O. Box 3926, Springfield, IL 62708

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THE ROLE OF A PRESYNAPTIC MUSCARINIC AUTORECEPTOR IN ACETYLCHOLINE RELEASE FROM RAT IRIS

Mattio, T.G.*, Giacobini, E. and Richardson, J.S.
Dept. Pharmacol., So. Ill. Univ. Sch. Med., Springfield, IL 62708 USA

The release of acetylcholine (ACh), both in central and peripheral nerve tissues, is controlled by a presynaptic muscarinic autoreceptor that is responsive to exogenous and endogenous muscarinic agents. In the albino rat iris we have demonstrated the presence of a muscarinic autoreceptor and elucidated its role after acetylcholinesterase (AChE) inhibition by diisopropylfluorophosphate (DFP). The electrically stimulated release of ACh (50 Hz, 20 mA, 5 ms square wave) in the rat iris was shown to be temperature, Na^+ and Ca^{++} dependent. Addition of 10^{-4}M and 10^{-5}M scopolamine in the superfusion buffer, increased ACh release by 190 and 150%, respectively. The addition of 10^{-4}, 10^{-5} and 10^{-6}M DFP in the buffer sigificantly decreased the release of ACh and inhibited AChE activity by more than 90%. This inhibition of ACh release was totally reversed by scopolamine (10^{-6}M) indicating the involvement of a muscarinic autoreceptor. The accumulation of ACh in the synaptic cleft after DFP, results in muscarinic activation and a consequent feedback inhibition of ACh release. Further characterization of this autoreceptor and its role after AChE inhibition will be described.

(Supported by Grant AFOSR-83-0051 to E.G.)

Ezio Giacobini

THE ROLE OF A PRESYNAPTIC MUSCARINIC AUTORECEPTOR IN ACETYLCHOLINE RELEASE FROM RAT IRIS

Giacobini, Ezio

So. Ill. Univ. Sch. Med., P.O. Box 3926, Springfield, IL 62708

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J.S. RICHARDSON, T.G. MATTIO* and E. GIACOBINI*
Pharmacology and Psychiatry, University of Saskatchewan, Saskatoon; and Pharmacology, Southern Illinois University, Springfield.

The release of acetylcholine from rat iris is inhibited by a presynaptic muscarinic autoreceptor.

The release of acetylcholine (ACh), both in central and peripheral nerve tissues, seems to be modulated by a presynaptic muscarinic autoreceptor that is responsive to exogeneous muscarinic agents. In the albino rat iris, we have demonstrated the presence of a muscarinic autoreceptor and elucidated its role after aceylcholinesterase (AChE) inhibition by diisopropylfluorophosphate (DFP). The electrically stimulated release of ACh (50 Hz, 20 mA, 5 ms, biphasic square wave) from the rat iris is temperature, Na^+ and Ca^{++} dependent. The addition of 10^{-4} M and 10^{-5} M scopolamine in the superfusion buffer, increased ACh release by 190% and 150%, respectively. In the presence of 10^{-4}, 10^{-5} and 10^{-6} M DFP in the buffer, ACh release was significantly decreased and AChE activity was inhibited by more than 90%. The inhibition of ACh release was totally reversed by scopolamine (10^{-6} M) indicating the involvement of a muscarinic autoreceptor. The accumulation of ACh in the synaptic cleft after DFP, seems to activate the muscarinic autoreceptor and to produce a feedback inhibition of additional ACh release. Since the isolated iris of the rat does not contain nerve cell bodies, this muscarinic autoreceptor would appear to be on presynaptic nerve terminals.
(Supported by USAFOSR).

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THE ROLE OF A PRESYNAPTIC MUSCARINIC AUTORECEPTOR IN ACETYLCHOLINE RELEASE FROM RAT IRIS.

T.G. Mattio, E. Giacobini and J.S. Richardson. Department of Pharmacology, So. Ill. Univ. Scn. Med., Springfield, IL 62708 USA

In the albino rat iris we have demonstrated the presence of a muscarinic autoreceptor and elucidated its role after acetylcholinesterase (AChE) inhibition by diisopropylfluorophosphate (DFP). The electrically stimulated release of ACh (50 Hz, 20 mA, 5 ms square wave) in the rat iris was shown to be temperature, Na^+ and Ca^{++} dependent. Addition of 10^{-4}M and 10^{-5}M scopolamine in the superfusion buffer increased ACh release by 190 and 150%, respectively. The addition of 10^{-4}, 10^{-5} and 10^{-6}M DFP in the buffer sigificantly decreased the release of ACh and inhibited AChE activity by more than 90%. This inhibition of ACh release was totally reversed by scopolamine (10^{-6}M), indicating the involvement of a muscarinic autoreceptor. The accumulation of ACh in the synaptic cleft after DFP, results in muscarinic activation and a consequent feedback inhibition of ACh release. (Supported by Grant AFOSR-83-0051)

To be completed by presenting author:

Giacobini (Last name) E. (Initials) X Eric Giacobini

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GIACOBINI, E., MUSSINI, I. and MATTIO, T.
"Aging of Cholinergic Synapses in the Avian Iris"
Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois 62708

We have made use of the ciliary ganglion-iris preparation of the aging (1.5-9 yrs) chicken as a model of senescent peripheral cholinergic synapses. Neuromuscular junctions in the iris of aging chickens show early (1.5 yrs) morphological signs of damage such as, reduction and polymorphism of synaptic vesicles and increase of neurofilaments and mitochondria. Accumulations of cytoplasmic organelles and lysosomes are seen in the axoplasm of the nerve fiber. At later stages (5-9 yrs), the nerve ending is enveloped by Schwann cells infiltrating and filling the synaptic cleft. Quantitative morphometric changes in the ratio describing the relationship between volumes of terminals and volumes of synaptic vesicles show a progressive decrease in the volume occupied by synaptic vesicles. The ability of the cholinergic synapses to take up ^{3}H-choline and release the formed ^{3}H-acetylcholine (ACh) in response to high K^{+}-depolarization is impaired at 5 yrs resulting in a significant depletion of the ^{3}H-ACh releasable pool. These experiments seem to point out for the first time a selective functional defect in the cholinergic synapse during aging. (Supported by AFOSR Grant NL-144 and by Nowatski Eye Research Fund to E.G.)

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AGING OF CHOLINERGIC SYNAPSES IN THE AVIAN IRIS

Giacobini, E., *Mussini, I. and Mattio, T. Dept. Pharmacology, Southern Ill. Univ. Sch. Med., Springfield, Ill. 62708 and *C.S. Biol. Fisiopat. Musc., Ist. Patologia Generale, Università di Padova, 35100 Padova, Italy

We have made use of the ciliary ganglion-iris preparation of the aging (1.5-9 yrs) chicken as a model of senescent peripheral cholinergic synapses. Neuromuscular junctions in the iris of aging chickens show early (1.5 yrs) morphological signs of damage such as, reduction and polymorphism of synaptic vesicles and increase of neurofilaments and mitochondria. Accumulations of cytoplasmic organelles and lysosomes are seen in the axoplasm of the nerve fiber. At later stages (5-9 yrs), the nerve ending is enveloped by Schwann cells infiltrating and filling the synaptic cleft. Quantitative morphometric changes in the ratio describing the relationship between volumes of terminals and volumes of synaptic vesicles show a progressive decrease in the volume occupied by synaptic vesicles. The ability of the cholinergic synapses to take up ^{3}H-choline and release the formed ^{3}H-acetylcholine (ACh) in response to high K^{+}-depolarization is impaired at 5 yrs resulting in a significant depletion of the ^{3}H-ACh releasable pool. These experiments seem to point out for the first time a selective functional defect in the cholinergic synapse during aging. (Supported by AFOSR Grant NL-144 and by Nowatski Eye Research Fund to E.G.)

ILING ADDRESS OF THE AUTHOR WHO WILL
SENT THE PAPER
ase print or type Provide full name rather than initials)
. Ezio Giacobini
partment of Pharmacology
. Ill. Univ. Sch. Med.
). Box 3926
ringfield, IL 62708 USA

ne: 217/785-2185

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