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MODIFICATION OF ^{3}H-γ-AMINOBUTYRIC ACID (^{3}H-GABA) UPTAKE AND RELEASE BY ACETYLCHOLINE (ACh) IN SYNAPTOSOMAL FRACTIONS OF RABBIT RETINA

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ABSTRACT

In retinal outer plexiform layer (OPL) synaptosomal fraction (P_1), photoreceptor terminals have pre-and postsynaptic elements of the triad attached. In retinal inner plexiform layer (IPL) synaptosomal fraction (P_2), serial and reciprocal synapses are abundant. Thus, OPL and IPL fractions obtained from the retina are highly suitable for a direct, biochemical analysis of presynaptic receptor mechanism. In these studies, modifications in uptake and release of a given neurotransmitter were monitored after exposure to various concentrations of other neurotransmitters. One of the most pronounced effects was the stimulation of ^{3}H-γ-aminobutyric acid (^{3}H-GABA) uptake and release in OPL synaptosomes by acetyl choline (Ach). Aspartate (Asp) and dopamine (DA) did not modify ^{3}H-GABA uptake of IPL and OPL, and ACh did not change the ^{3}H-GABA uptake of IPL synaptosomes. Furthermore, the increase in ^{3}H-GABA uptake of OPL caused by ACh was mediated by an increase in the maximal velocity, Vmax, with no change in the affinity (K_T) of the uptake system for GABA. ^{3}H-GABA uptake of OPL was also increased by preincubation with choline (Ch), a precursor of ACh ; by neostigmine, an acetylcholinesterase (AChE) inhibitor ; and by nicotine and 1, 1-dimethyl-4-phenylpiperazinium (DMPP), nicotinic cholinergic receptor agonists.

The effect of Ch preincubation on the increase in OPL synaptosomal uptake of ^{3}H-GABA was blocked by hemicholinium-3 (HC-3), hexamethonium, mecamylamine, d-tubocurarine, (-) scopolamine and atropine. The concentrations of the nicotinic cholinergic receptor blockers needed to inhibit cholinergic stimulation by 50 % (IC_{50}) were lower than those needed for the muscarinic blockers.

P_1 Ch preincubation and the addition of ACh into the perfusion medium also enhanced Ca^{++} dependent ^{3}H-GABA release from the OPL synaptosomes.

These data suggest that the relatively small GABA ergic system present in the

OPL of rabbit retina is sensitive to cholinergic stimulation. Furthermore, the stimulation of the GABA system is mediated mainly via nicotinic ganglionic-like receptors which are apparently located on the presynaptic GABAergic terminal itself. These GABAergic terminals may be horizontal cell neurites associated with cone triads.

Introduction

In retina, both acetylcholine (ACh) and Ɣ- aminobutyric acid (GABA) systems are found in amacrine cells in the inner plexiform layer (IPL)[1-6]. In the outer plexiform layer (OPL), processes from some cone horizontal cells have been shown to be GABA-ergic (goldfish[7-8]) while some photoreceptor cells may be cholinergic (turtle[8,9]). In 1975, Lam concluded that GABAergic horizontal cells are associated specifically with red sensitive cones in goldfish, turtles and chicks[8]. Marc *et al.*[7], has shown that the dendrites of GABAergic horizontal cells form triad complexes with red cones in goldfish retina. Information concerning cholinergic photoreceptor terminals and GABAergic horizontal cell dendrites in rabbit retina, however, is very limited. Glutamate (Glu) and aspartate (Asp) have also been suggested as putative neurotransmitters for photo-receptor terminals, though there is only limited electrophysiological evidence to support this hypothesis[10]. Gerschenfeld and Piccolino[11] found that atropine (a muscarinic cholinergic blocker) can block transmission between some photoreceptors and horizontal cells in turtle retina. This added further support to the theory that ACh may be a putative neurotransmitter for some photoreceptor terminals.

Redburn's isolation technique, developed in 1977[12], now makes it possible to separate large retina[6] synaptosomes, P_1, mainly from photoreceptor terminals of OPL, from small synaptosomes, P_2 arising in the inner plexiform layer (IPL). The electron microscopic analysis demonstrates a considerable number of synapto-synaptic synapto-some complexes in both P_1 and P_2 retinal fractions. This suggests that the retina contains many presynaptic receptors and that retinal synaptosomal fractions contain "intact" reciprocal and serial synaptic terminals whose synaptic activity can be studied directly *in vitro*.

A series of experiments were carried out in order to determine if functional presynaptic receptors were present in synaptosomal fraction from outer and inner plexi-form layer of rabbit retina. It was reasoned that if presynaptic receptors in synaptosomes were activated by adding exogenous neurotransmitters, then the resulting synaptosomal depolarization or hyperpolarization should lead to an alteration in the uptake or release of the native neurotransmitter of the affected terminals. A group of four putative

neurotransmitters were studied : GABA, ACh, DA and Asp. The uptake systems of each neurotransmitter and Ch (instead of ACh) was assessed in the absence and presence of the other three compounds. Only a few specific interactions were observed. Among these was the stimulation of P_1 retinal ^{3}H-GABA uptake by ACh. This observation led to the hypothesis that some photoreceptor terminals (in the P_1 or OPL retinal fraction) of rabbit retina may be cholinergic and may synapse upon GABAergic horizontal cell neurites. Subsequent experiments were designed to give a detailed investigation of the neurotransmitter systems in the P_1 synaptosomal population. The study included an investigation of the pharmacological properties of cholinergic receptors which mediated cholinergic stimulation of the GABA uptake system of nerve terminals in P_1.

The results obtained from this investigation show interactions and physiological modifications of the cholinergic and GABAergic systems in P_1. These observations strengthen the hypothesis that there are synaptosynaptic interactions in the retina which may be involved in the visual processing mechanism.

Materials and Methods

Retina tissue was obtained from adult, male New Zealand white rabbits. Synaptosomal fractions of outer plexiform layer (OPL, P_1) and of inner plexiform layer (IPL, P_2) were prepared using technique of Redburn[12] Study of uptake and/or release of ^{3}H-Ch or ^{3}H-Ach, ^{3}H-GABA, ^{3}H-DA, ^{14}C-Asp by retinal homogenate, P_1 and P_2 fractions were carried out using technique of Simon and Kuhar[13] and Levy and coworker[14] respectively. Detail procedure of the study of uptake and release of radioactive neurotransmitters has been described previously[15] Observing interaction of one neurotransmitter (NT) upon the uptake of other radioactive labeled NT by retinal synaptosomal fractions, cold NT were simultaneuously added with radioactive NT except otherwise stated in the results. In studying effects of one NT on the release of other NT, labeled NT was incubated with retinal synaptosomal fraction as usual. In the Ca^{++} stimulation for NT release, cold NT at appropriate concentrations were simultaneously perfused, except, otherwise stated. Addition technical detail will be included in figure legend of each set of data. The data obtained in the experiments was averaged and the mean $\pm$ standard error of mean ($X \pm$ S.E.M.) are presented. All comparisons of data were done using student's paired t-test.

Linear regression analysis has been used to determine the slope and the y-intercept in the Lineweaver-Burk analysis of kinetic data, and for direct calculation of the K_T (Michaelis constant for transport) and the maximal velocity (V_{max}) of uptake.

Logarithmic probability paper was used to plot and extrapolate the IC_{50} values for inhibitor drugs and EC_{50} values for stimulatory drugs. These values represent the molar concentrations of those drugs which required to cause a half maximal inhibition (IC_{50}) or excitation (EC_{50}).

Results

Interactions Between Cholinergic and GABAergic Systems

Synaptosomal preparations offer many advantages in the study of neuronal uptake systems. Among these advantages are the presence of minimal diffusion barriers and intact neuronal circuitry in a subcellular fraction which is highly enriched in presynaptic terminals. Both of these synaptosomal characteristics obviate many of the complicating factors present in studies which use more intact preparations. Redburn's techniques[12] permit the fractionation and separation of two distinct synaptosomal populations, P_1 and P_2, from the retina. In the present study, retinal synaptosomal fractions are screened for possible interactions between the neuronal uptake systems mediated by presynaptic receptors located on synaptosynaptic synapses. The results obtained have made it possible to localize the specific cellular sites of these interactions. Table 1 indicates those interactions which were observed. It can be seen that exogenous ACh (10 μM) significantly reduced ^{3}H-Ch uptake in both the P_1 and P_2 retinal fractions. In contrast ACh increased the uptake of ^{14}C-Asp by P_2 and ^{3}H-GABA by P_1 (Also Fig. 1). Combinations of the ligands marked by (-) produced no significant changes in uptake. GABA and Asp (10 μM) specifically decreased Ch uptake in P_2, while DA had no significant effect on the uptake of ^{3}H-Ch, ^{14}C-Asp or ^{3}H-GABA in any retinal fraction studied. However, the effect of cholinergic stimulation on increase of ^{3}H-GABA uptake in retinal P_1 fraction was chosen for more detailed study.

A. Effects of ACh on ^{3}H-GABA Uptake in P_1 and P_2 Retinal Fractions.

Figure 1 demonstrates the results obtained during a for minute incubation interval when a 100 fold range of concentrations of unlabeled ACh were added to each subcellular fraction in the presence of 0.5 μM ^{3}H-GABA at 37 ℃ and 4 ℃. The ACh stimulated increase in ^{3}H-GABA uptake, by P_1, was concentration-dependent with an EC_{50} of 0.80 ± 0.37 μM (Table 3). There was no significant change observed in the P_2 uptake (Fig. 1). Examination of the P_1 kinetics parameters of ^{3}H-GABA uptake in the presence or absence of ACh (10 μM) (Fig. 2) revealed that ACh increased the uptake Vmax but did not significantly alter the K_T.

B. Effects of Ch Preincubation on ^{3}H-GABA Uptake in P_1

Masland and Livingstone[16] have demonstrated that Ch taken up by rabbit

retina is used for ACh synthesis. High affinity Ch uptake can be blocked by hemicholinium-3 (HC-3), thus reducing ACh synthesis[1]. The effects of HC-3 and/or Ch preincubation on ^{3}H-GABA uptake in the P_1 fraction were further investigated to demonstrate the involvement of cholinergic modifications.

Figure 3. shows that when the P_1 fraction was preincubated with several concentrations of Ch for 10 minutes, the subsequent ^{3}H-GABA uptake was increased in a concentration-dependent manner. Subsequent statistical analysis has shown that the variation observed in this set of data was much less than that observed in the presence of ACh. The concentrations of ACh and Ch which increased the P_1 ^{3}H-GABA uptake by 50 % of its maximal effect (EC_{50}, calculated on logarithmic probability paper) are given in Table 3. The calculations show that the EC_{50} of ACh and Ch preincubation (10 min.) were not significantly different.

To determine whether or not the effects of Ch preincubation on subsequent P_1 ^{3}H-GABA uptake was due to an increase in Ch uptake, HC-3 was simultaneously mixed with Ch and preincubated before ^{3}H-GABA was added. Figure 4 shows that the effects of Ch preincubation were blocked by HC-3 when it was added simultaneously with Ch. HC-3 alone, however, did not change ^{3}H-GABA uptake in the P_1 fraction.

C. Effects of Neostigmine Methylsulfate on P_1 ^{3}H-GABA Uptake.

Because neostigmine was routinely added to all assaying mediums, it was necessary to determine its effect on the uptake of ^{3}H-GABA by the P_1 system. This determination may also give additional evidence for the involvement of the cholinergic system P_1 in the modification of ^{3}H-GABA uptake. Figure 5 shows that neostigmine alone (10 μM) increased ^{3}H-GABA uptake in the P_1 fraction. This effect could be blocked by adding (-) scopolamine (100 μM), a muscarinic cholinergic receptor blocker.

D. Effects of Ach Receptor Blockers on Ch Preincubation Stimulation of P_1 ^{3}H-GABA Uptake.

To clarify whether or not the effects of Ch preincubation on P_1 ^{3}H-GABA uptake are mediated by cholinergic receptor mechanisms, several cholinergic receptor blockers were added to the assay system. The Ch (preincubation) stimulation of P_1 ^{3}H-GABA uptake could be inhibited by the muscarinic cholinergic receptor blockers, atropine and (-) scopolamine, by ganglionic nicotinic cholinergic receptor blockers, hexamethonium and mecamylamine, and by a skeletal muscle nicotinic cholinergic receptor blocker, d-tubocurarine. Only data of atropine, d-tubocurarine are shown in Fig 6, whereas IC_{50} of all cholinergic receptor blockers used are shown in Table 2.

The potency of the inhibitors differed, as evidence by the difference in the concentrations of cholinergic receptor blockers needed to achieve 50 % of maximal inhibition (IC_{50}). Table 2 shows that the IC_{50} for nicotinic receptor blockers are less than those

HOT LIGAND (0.5 μM)	^{3}H - Ch			^{14}C - Asp			^{3}H - DA			^{3}H - GABA		
Cold ligand (0.1 - 10.0 μM)	Hg	P_1	P_2	Hg	P_1	P_2	Hg	P_1	P_2	Hg	P_1	P_2
Ach	-	↓ 82.0 ± 7.0 %*	↓ 83.0 ± 4.0 %*	-	-	↑ 170.0 ± 35.0%*	-	-	-	-	↑ 161.0 ± 28.0%*	-
Asp	-	-	↓ 79.0 ± 6.0 %*				-	-	-	-	-	-
DA	-	-	-	-	-	-				-	-	-
GABA		-	↓ 79.0 ± 6.0 %*	-	-	-	-	-	-			

Table 1. Preliminary experiments screening the effects of unlabeled putative neurotransmitters on the uptake of ^{3}H - Ch, ^{14}C - Asp, ^{3}H - GABA, ^{3}H - DA. Retinal fractions (Homogenate, Hg, P_1, P_2) were mixed with Ringers' buffer containing labeled ligands in the absence (control) or presence of unlabeled putative neurotransmitters (0.1 - 10.00 μM) at 37 and 4° C. At 4 minutes after incubation the incubants were filtered into GF/A Glass fiber filters fixed in the filter box. Filters were rinsed with 8 mls Ringers' buffer, digested with 1 % sodium dodecyl sulfate (S.D.S.) for 3 - 5 hours, counting solution was then added, and the samples were counted. The high affinity uptake of the labeled ligands was plotted as a function of the unlabeled uptative neurotransmitter concentrations. In this table only the values of statistically significant (*) modifications of the uptake at 10 μM concentrations of the unlabeled ligands are shown as percentages of the controls. ↑ designates increase, - designates no change in uptake, ↓ designates decrease.

TABLE 2. INHIBITION OF CHOLINERGIC STIMULATED ^3H - GABA UPTAKE BY CHOLINERGIC ANTAGOISTS IN THE P_1 FRACTION. THE IC_{50} VALUES WERE EXTRAPOLATED USING LOGARITHMIC PROBABILITY PAPER.

	IC_{50} (μM) X ± S.E.M.	N
Hexamethonium	1.2 ± 0.2	5
Mecamylamine	4.5 ± 0.8	5
d - Tubocurarine	5.5 ± 1.1	5
(-) scopolamine	25.0 ± 4.4	5
Atropine	124.0 ± 10.6	5

TABLE 3. STIMULATION OF P_1 ^3H - GABA UPTAKE BY Ach, CHOLINERGIC AGONISTS, AND CHOLINE PREINCUBATION.

	EC_{50} (μM) X ± S.E.M.	N
ACh	0.80 ± 0.37	4
Nicotine	2.18 ± 0.14	4
DMPP	1.54 ± 0.22	5
Ch preincubation	0.52 ± 0.10	3

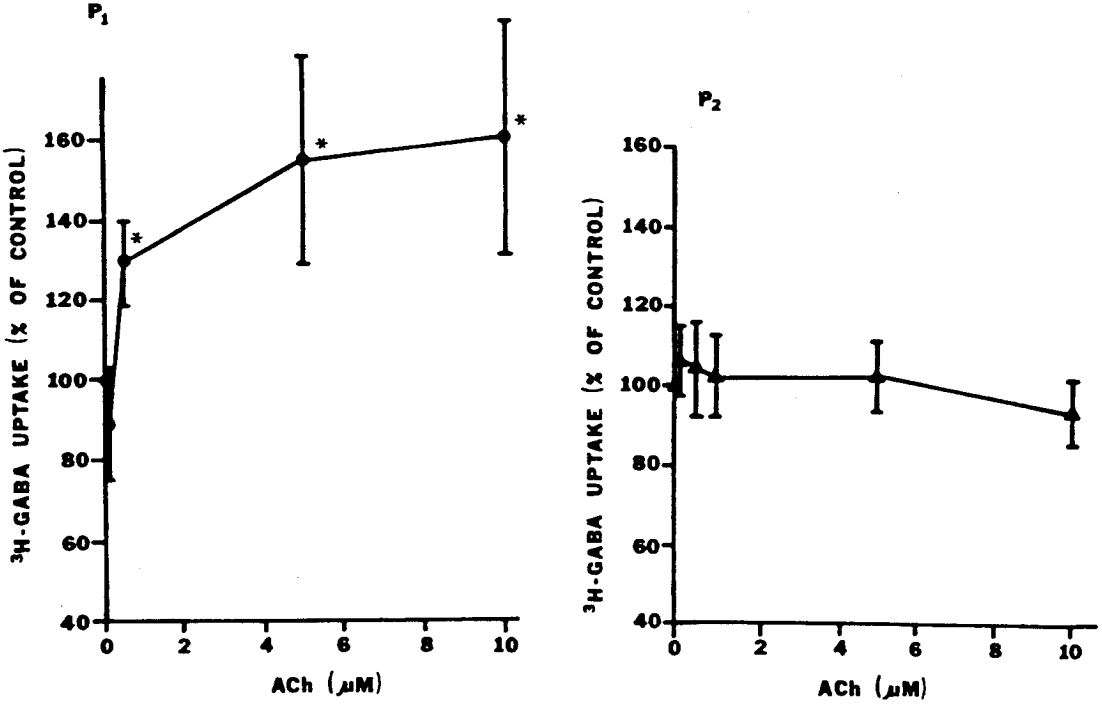

Figure 1. A concentration - response curve showing the effect of ACh on ^3H - GABA uptake of P_1 and P_2 retinal synaptosomal fractions. The concentration of ^3H - GABA in the incubation was kept at 0.5 μM, and the ACh simultaneously incubated was between 0.1 and 10 μM.

* = $P<0.05$, designates a significant difference between ^3H - GABA uptake at 0 μM ACh and other ACh concentrations. (N = 6, each N = a pool of 4 eyes)

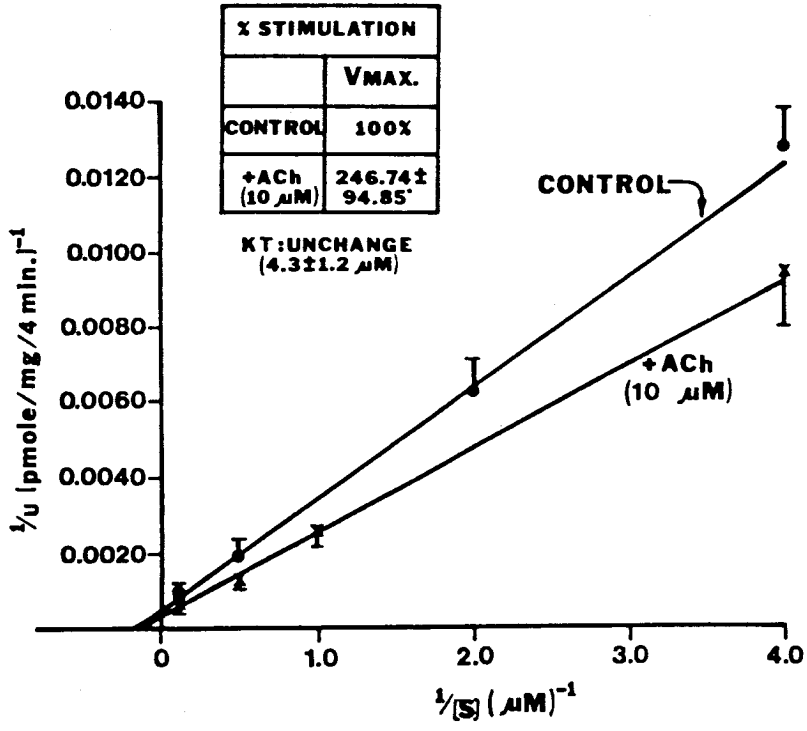

Figure 2. Kinetic analysis (Lineweaver - Burk plot) of P_1 ^3H - GABA uptake in the presence or absence of ACh. It can be seen that ACh significantly increases the V_{max} of P_1 ^3H - GABA uptake while the K_T is not significantly altered. ACh concentration used was 10 μM. Concentrations of ^3H - GABA were between 0.25 to 6 μM.

* = $P<0.05$

(N = 3, each N = a pool of 4 eyes)

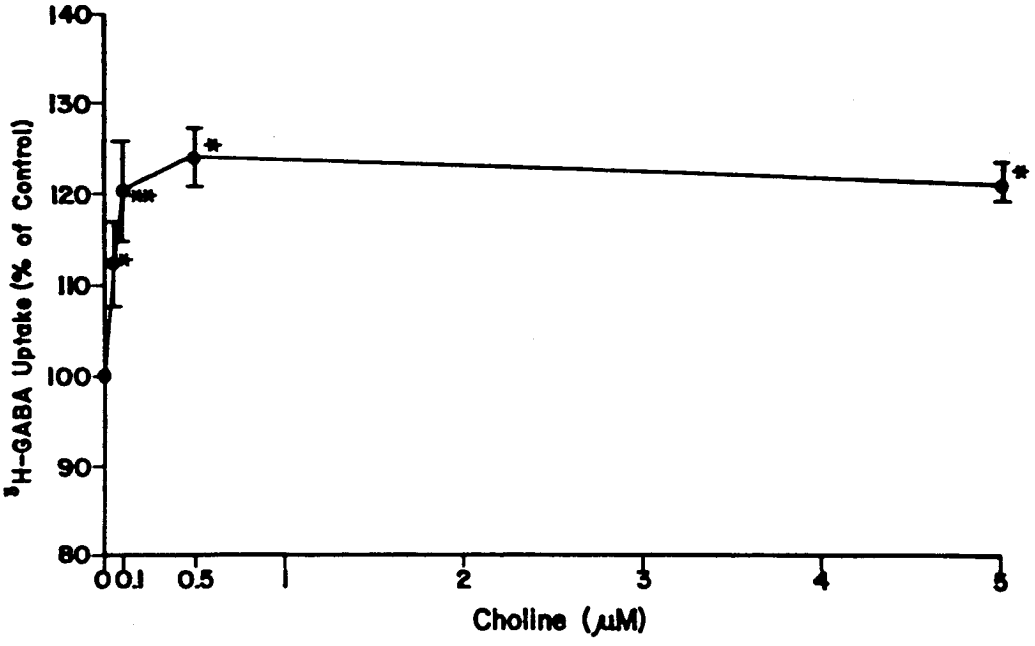

Figure 3. Concentration - response curve of P_1 ^3H - GABA uptake subsequent to P_1 Ch preincubation. Rabbit retina P_1 fraction was preincubated with Ch (0.1 to 10 μM) for 10 minutes, then incubated with ^3H - GABA (0.5 μM) for 4 minutes, trapped on GF/A filter, washed, solubilized and counted. ^3H - GABA uptake was calculated as % of control (0 μM Ch preincubation). Paired t-tests were done comparing the p moles of ^3H - GABA taken up in the presence and absence of Ch preincubation.

* = $P<0.05$, ** = $P<0.01$ (N = 6, each N = a pool of 4 eyes)

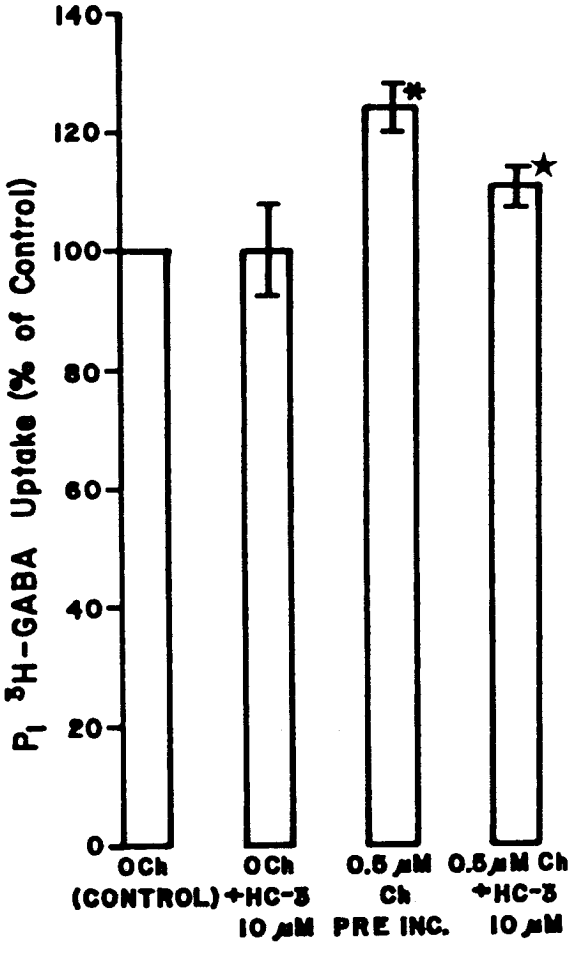

Figure 4. Effect of hemicholinium - 3 (HC - 3) on P_1 ^{3}H - GABA uptake obtained subsequent to Ch preincubation. Ch concentration was 0.5 µM (10 min.), HC - 3 : 10 µM (10 min.), and ^{3}H - GABA : 0.5 µM (4 min.).
* designates $P<0.05$ compared with control (0 Ch and 0 HC - 3). * signifies $P<0.05$ compared with Ch preincubated P_1 ^{3}H - GABA uptake.
(N = 6, each N = a pool of 2 eyes)

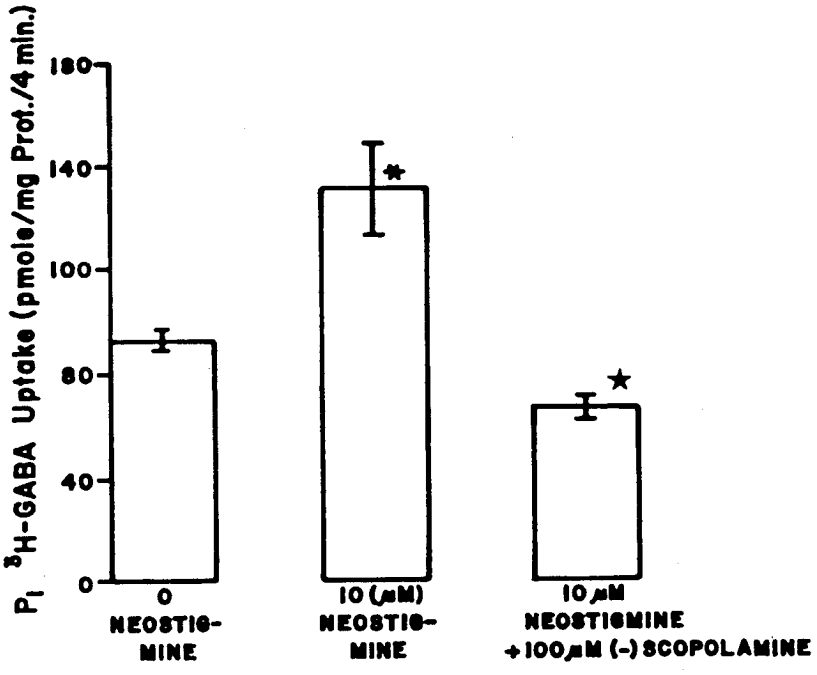

Figure 5. Effects of neostigmine and (-) scopolamine on P_1 ^3H - GABA uptake. Neostigmine concentration used was 10 μM; ^3H - GABA concentration was 0.5 μM.

* = $P<0.05$, a comparison of P_1 ^3H - GABA with and without neostigmine (control).

* = $P<0.05$, comparison of P_1 ^3H - GABA uptake in 10 μM neostigmine with and without (-) scopolamine (10 μM.) (N = 4, each N = a pool of 8 eyes)

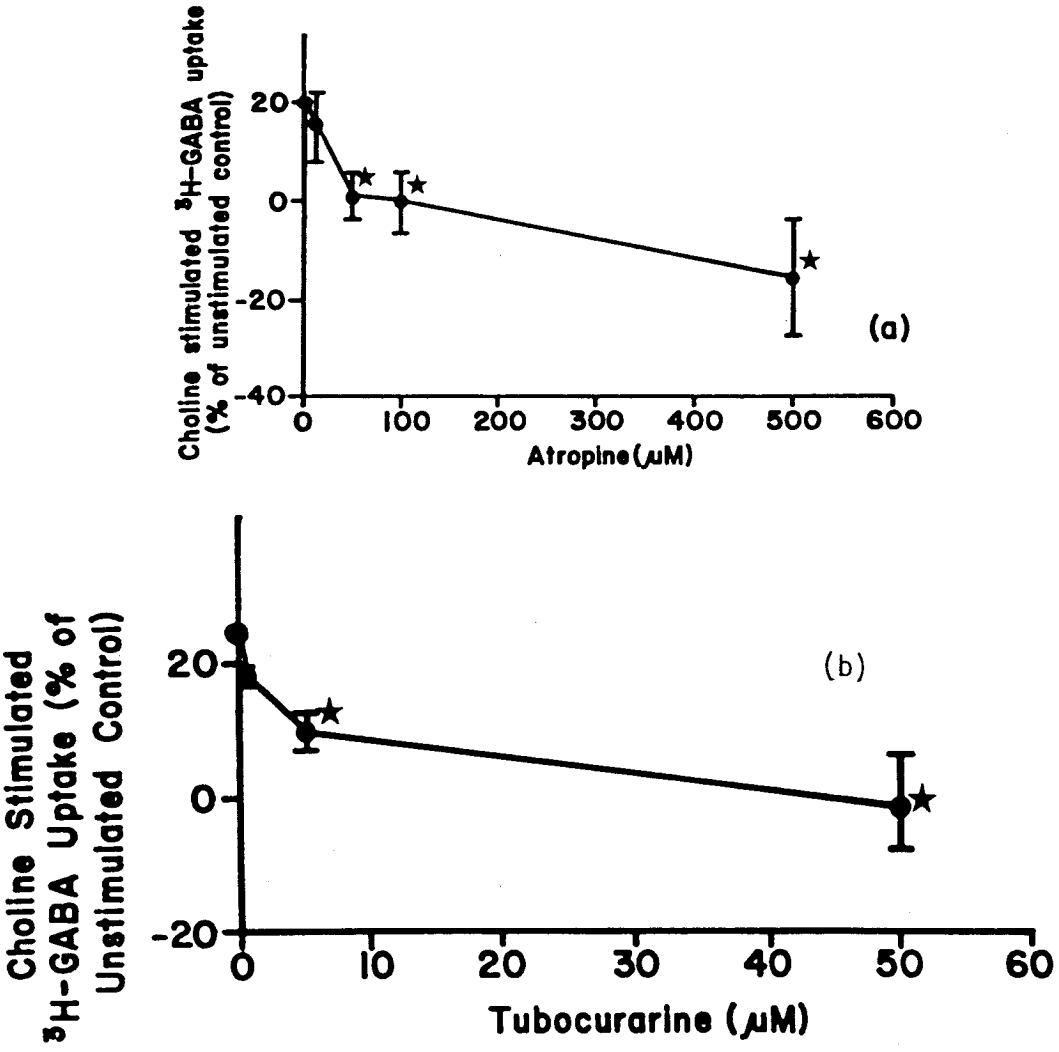

Figure 6. Effects of muscarinic and nictotinic cholinergic receptor antagonists on cholinergic stimulation of P_1 ^3H - GABA uptake by Ch (0.5 μM) preincubation. Atropine or d - tubocurarine of varying concentrations were simultaneously incubated with ^3H - GABA (0.5 μM) after a 10 minute incubation period with Ch (0.5 μM). Data were calculated as a percentage of the values obtained from the control P_1 ^3H - GABA uptake (without Ch preincubation). Similar experiments using (-) scopolamine, hexamethonium, mecamylamine were also done.

* signifies $P<0.05$, a comparison between P_1 ^3H - GABA uptake at 0.5 μM Ch preincubation, alone or drug treated. (N = 5, each N = a pool of 8 eyes).

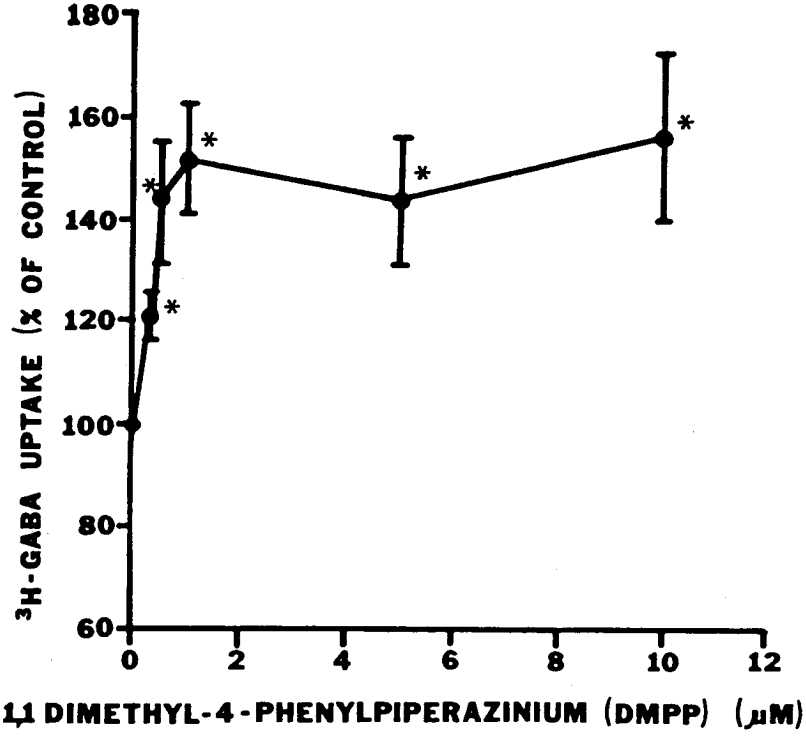

Figure 7. Effect of 1, 1 - Dimethyl - 4 - phenylpiperazinium (DMPP) on P_1 ^{3}H - GABA uptake. ^{3}H - GABA (0.5 μM) and several concentrations of DMPP were simultaneously incubated with the P_1 fraction of retina for 4 minutes. Uptake was calculated as a percentage of the ^{3}H - GABA uptake at 0 μM of DMPP (control). Concentration at 50 % of maximal response (EC_{50}) was 1.54 ± 0.22 μM. Similar experiment using nicotine was also done.

* signifies significant difference between drug treated ^{3}H - GABA uptake and that of control or $P<0.05$. (N = 6, each N = a pool of 2 eyes).

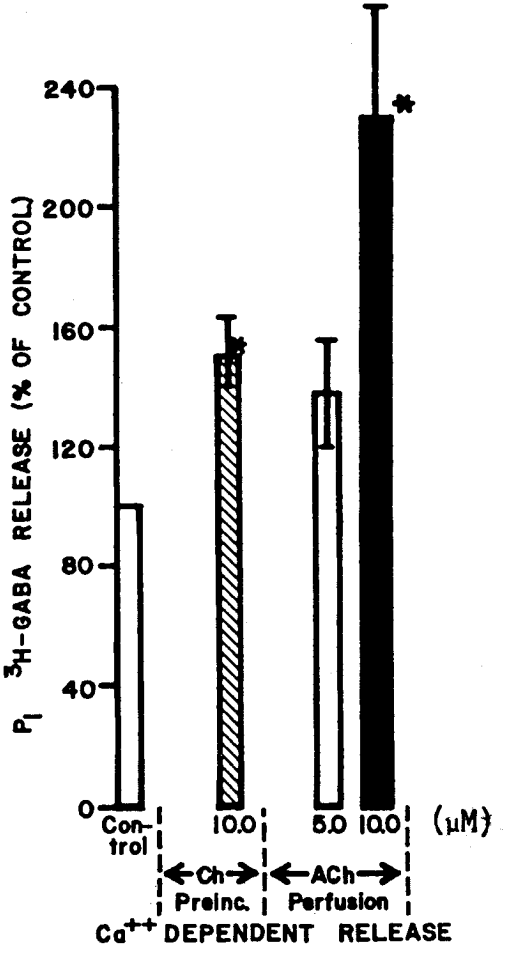

Figure 8. The effects of Ch (10 μM), preincubation for 10 minutes, or ACh (5.0 and 10 μM) perfusion on Ca^{++} dependent, K^+ stimulated release of ^3H - GABA from the P_1 fraction of rabbit retina. Details of calculations of Ca^{++} dependent - K^+ stimulated release are previously described [15]. The high $K^+ + Ca^{++}$ stimulated release in the presence of ACh or Ch preincubation, were calculated as percentages of the values obtained for the release by normal High $K^+ + Ca^{++}$ stimulating conditions (controls).

* = $P<0.05$, indicates significant differences between Ca^{++} dependent release in the controls and release in the presence of ACh or Ch preincubation. (N = 5, each N = a pool of 4 eyes).

for muscarinic receptor blockers.

E. Effects of Cholinergic Agonists on P_1 ^3H - GABA Uptake.

The effects of two nicotinic agonists were also tested in the incubating system. Nicotine and 1, 1 - dimethyl - 4 - phenylpiperazinium (DMPP), two ganglionic nicotinic cholinergic agonists, increased P_1 ^3H - GABA uptake in a concentration - dependent manner. However, only data of DMPP are shown in Fig 7. Table 3 shows that the EC_{50} of DMPP is slightly lower than that for nicotine.

F. Effects of Ch Preincubation and ACh on ^3H - GABA Release from P_1.

It has already been shown that stimulation of the cholinergic system increased the P_1 ^3H - GABA uptake. It was of interest, therefore, to determine what effect cholinergic stimulation had on P_1 ^3H - GABA release. The experiments (Fig. 8) showed that ACh perfusion or preincubating P_1 with choline increased the Ca^{++} dependent K^+ stimulated release of ^3H - GABA from P_1.

Discussion

A series of experiments were carried out in order to determine if functional presynaptic receptors were present in synaptosomal fractions from outer and inner plexiform layers (OPL and IPL) of rabbit retina. In was reasoned that if presynaptic receptors in synaptosomes were activated by adding exogenous neurotransmitter (NT), then the resulting synaptosomal depolarization or hyperpolarization should lead to an alteration in the uptake or release of the native NT of the affected terminal. A group of our putative NT were studied : GABA, ACh, DA and Asp. The uptake systems of each NT and Ch (instead of ACh) were assessed in the absence and presence of the other three compounds (Table 1). One striking effect was noted and analyzed in greater detail. It was observed that ACh increased the ^3H-GABA uptake in P_1, but did not do so in P_2 (Fig. 1). The specific modification of the P_1 ^3H-GABA uptake by ACh may be related to a synaptosynaptic interaction of serial synapse involving a cholinergic terminal which synapses on a GABAergic terminal[9,17]. Kinetic analysis of the effect of ACh on P_1 ^3H-GABA uptake (Fig. 2), shows that the K_T was not changed, but that the Vmax was enhanced. This suggests that there was no direct effect of ACh on the affinity of the carrier sites for ^3H-GABA. The increase in Vmax may indicate that the actions of ACh lead to an increase in the number or activity of transport sites.

P_1 ^3H - GABA uptake was also increased by preincubating the P_1 fraction with Ch (Fig. 3). When hemicholinium - 3 (HC - 3) (a blocker of Ch uptake) was simultaneously incubated with Ch, it blocked the increase of P_1 ^3H-GABA uptake brought

about by Ch preincubation (Fig. 4). Exogenous Ch which remains extracellular has no effect. The stimulation is seen only when the exogenous Ch is taken up. Most of the Ch taken up by synaptosomal fractions is synthesized into ACh (unpublished data of Redburn). Furthermore, Baughman and Bader[1] report an inhibition of ACh synthesis from Ch incubation by HC - 3. Thus, it would appear that the stimulation observed after addition of exogenous Ch is mediated via ACh synthesized intra - synaptosomally from the added Ch. This speculation is also suported by data in Table 3, which shows that the EC_{50} of Ch (preincubated 10 minutes) is approximately the same as that of exogenous ACh in stimulating ^3H - GABA uptake by P_1.

Ch's enchancement of P_1 ^3H - GABA uptake can be blocked by both muscarinic and nicotinic cholinergic receptor antagonists (Fig. 6). In addition, the nicotinic agonists, nicotine and 1, 1 - dimethyl - 4 - phenylpiperazinium (DMPP)[18] affect ^3H - GABA uptake in the same manner as Ch preincubation (Fig. 7 Table 3). This also implies that the increase in P_1 ^3H - GABA uptake due to Ch preincubation is attributable to an increase in endogenous ACh synthesis and release. Masland and Mills[19] have shown that under similar incubation conditions (with 10 μM physostigmine), most of the Ch uptake up by rabbit retina is used for Ach synthesis. The accumulated ACh is subsequently stored in a releasable pool. The small remaining portion is used in the formation of phosphorylcholine and phosphatidyl choline.

Neostigmine, an acetylcholinesterase inhibitor[20], in the absence of added ACh or Ch, caused an increase in P_1 ^3H - GABA uptake. Its actions were blocked by (-) scopolamine, a muscarinic cholinergic receptor blocker (Fig. 5). Such findings indicate that endogenous ACh in the P_1 retinal fraction, spontaneously released, can stimulate the uptake of ^3H - GABA.

Stimulation of P_1 ^3H - GABA uptake by P_1 Ch preincubation may be mediated by nicotinic receptors, muscarinic receptors, or both (Fig. 6, 7 and Table 2). However, the concentrations of muscarinic cholinergic receptor blockers, atropine and scopolamine, needed to inhibit 50 % of the cholinergic stimulation of retinal P_1 ^3H-GABA uptake (IC_{50}) are higher than those needed by nicotinic receptor blockers (d - tubocurarine, mecamylamine, hexamethonium).

ACh or Ch preincubation not only enhances the P_1 ^3H - GABA uptake, but also results in an increase in potassium stimulated, calcium dependent release (Fig. 8). It is not known if cholinergic stimulation leads to an increased release which then activates the uptake system or whether the initial effect is to stimulate uptake which subsequently leads to increased release. However, it is clear that cholinergic stimulation would lead to an increase in GABA turnover (via increased uptake and release) in affected nerve terminals.

Several sets of anatomical, electrophysiological and biochemical data suggest the presence of a cholinergic and a GABAergic system in outerplexiform layer. Lam[8] has shown that the cone photoreceptor terminals of many vertebrate retinas (skate, goldfish, furtle) can synthesize Ach from Ch. In 1977, Gerschenfeld and Piccolino[11] found that acetylcholinesterase inhibitors (e.g., eserine, neostigmine) increased synaptic transmission between photoreceptor and horizontal cells, whereas atropine block the transmission. The high density of nicotinic receptors located in the outer plexiform layer, as well as in the inner plexiform layer (rabbit[3]), indicate that there is cholinergic transmission between photoreceptors and horizontal neurites (at outer plexiform layer). In addition, it has been found that some horizontal cells contain acetylcholinesterase (rabbit[21]). Horizontal cells in cone rich retina can take up ^3H - GABA (goldfish[5]; frog[22]; gecko and turtle[8]; pigeon and chicken[23])). In 1978, Marc *et al*[7]., demonstrated that the H_1 horizontal cells in red sensitive cones of goldfish retina have a high affinity uptake for ^3H - GABA. Electron microscopic analysis has shown that rabbit retina has a considerable number of cone photoreceptor cells[24,25]. If ACh is associated with certain cone terminals and GABA with certain cone horizontal cells in every species of verterbrate, then it can be assumed that the P_1 retinal fraction of rabbit retina would contain some cone synaptosomes of that type. This speculation is supported by the recent findings that, compared to the P_2 retinal fraction, the P_1 fraction has a small but significant uptake of ^3H - Ch and ^3H - GABA[15]. Furthermore, synaptic connections between photoreceptor terminals and horizontal neurites have been demonstrated electron microscopically (rabbit[24]) and electrophysiologically (dogfish[26]; turtle[27]; toad[28];). The work of Redburn and Thomas[33] clearly showed synaptic connections between triad complexes with photoreceptor terminals in P_1 retinal fraction.

Physiological evidence to differentiate among the specific types of cholinergic receptors in retina suggest that nicotinic receptors represent the more dominant type. Ames and Pollen[29] and Masland and Ames[16] found that low concentrations of the hexamethonium, d - tubocurarine, and DMPP blocked specific electrophysiological responses in ganglion cell which are presumably cholinergic. Atropine concentrations needed to block the same phenomena to the same degree were, comparatively, much higher. Nicotine gave an opposite effect to that of the cholinergic blockers and the concentrations needed were very low (rabbit[1,30]). These responses, although measured in the ganglion cell, may reflect the polysynaptic cholinergic activity in both plexiform layers. These findings confirm the dominance of nicotinic ACh receptors in the rabbit retina.

The inner plexiform layer receives a large input of nerve terminals from amacrine cells (rabbit[24]). There are also dyad synapses between bipolar, amacrine and ganglion neurites. Contacts between amacrine cells and between amacrine and bipolar cells are

cells are also seen. Some of these synapses are serial; others are reciprocal. All amacrine cells do not have the same neurotransmitter. There are cholinergic amacrine cells (rabbit[2,19]), and GABAergic ones (rabbit[31]; chicken[23]), as well as other types. In spite of the presence of GABA and ACh system in the inner plexiform layer, the treatment of P_2 fractions with cholinergic agents (e.g., ACh, Ch preincubation) does not affect ^3H - GABA uptake. This may indicate that synaptosynaptic interactions between cholinergic and GABAergic terminals are not the same in P_2 as in P_1. There may be very few cholinergic amacrine terminals which make synaptosynaptic contacts upon GABAergic amacrine terminals in the inner plexiform layer. Alternatively, such connections may exist, but the modification of GABAergic terminals by ACh may not be reflected in an increase in ^3H - GABA uptake (e.g., only release may be increased).

There are, however, several reports which suggest that neither ACh or GABA are neurotransmitters in the outer plexiform layer of rabbit retina. Masland and Mills[19] showed autoradiographically that the majority of the label seen in the outer plexiform layer after ^3H - Ch incubation was not associated with ACh, but rather with phosphatidyl choline. Glutamic acid decarboxylase (GAD), a synthesis enzyme for GABA, activity in the outer plexiform layer is present, but in much lower concentrations than inner plexiform layer[32]. Horseradish peroxidase (HRP) labeled antibodies for GAD show minimal reactivity in outer plexiform layer of rabbit (Chris Brandon, personal communication). These data are in obvious conflict with the reports presented here, which clearly show the existance of relatively small, but significant, cholinergic and GABAergic systems in the outer plexiform layer synaptosomal fraction. One possible explanation is that the outer plexiform layer synaptosomal fraction is contaminated with synaptosomes from the inner plexiform layer. However, the GABAergic system in outer plexiform layer fraction is sensitive to cholinergic stimulation; the inner plexiform layer synaptosomal GABA system is not. Another suggestion is that autoradiographic and horseradish peroxidase (HRP) labelling techniques are not suitable for localizing relatively minor transmitter components. For example, if GABAergic horizontal cells contact only red sensitive cones in rabbit (as has been shown in goldfish[5]), then only a very small proportion of horizontal cells would be GABAergic. The total number of cones in rabbit is less than 5 % (Redburn, personal communication); the number of red - sensitive cones would be even less. In morphological analyses, such a minor component might be easily missed because of sampling problems. In this case, the biochemical analysis of uptake and release is perhaps, a more sensitive and quantitative method, as compared to morphological methods and thus allows the measurement of the relatively minor GABAergic and cholinergic systems in the outer plexiform layer of rabbit.

In conclusion, ACh was shown to increase ^3H - GABA uptake in the P_1 fraction.

The stimulation was specific for ACh among all other neurotransmitters tested and it was specific for the P_1 fraction. The increase is caused by enhancement of the Vmax of the P_1 ^3H - GABA uptake and not by changing the K_T. This indicates that ACh's modification of the uptake system takes place without changing the affinity of GABA carriers, but instead by increasing the number of active uptake sites. Subsequent experiments have shown that the ACh effect appears to be mediated by a nicotinic cholinergic receptor mechanism. ^3H - GABA uptake can also be increased in the P_1 fraction by Ch preincubation, AChE inhibitors (neostigmine), nicotine and 1, 1 - dimethyl - 4 -phenyl-piperazinium (DMPP) incubation. The stimulation of P_1 ^3H - GABA uptake by choline preincubation can be blocked by nicotinic and, to a lesser extent, by muscarinic cholinergic receptor blockers. The IC_{50} of nitotinic cholinergic receptor blockers, however, is much lower than that of muscarinic cholinergic blockers.

The data obtained in this study imply the existence in retina of presynaptic cholinergic nerve terminals, which synapse upon GABAergic terminals, i.e., synaptosynaptic complexes. The cholinergic input to the GABAergic system appears to be excitatory and is present only in the outer plexiform layer.

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บทคัดย่อ

รายงานการวิจัยนี้ ได้นำเทคนิคแยกกลุ่มของปลายประสาทเพล็กซิฟอร์มชั้นนอกของเรตินา (Outer plexiform layer, OPL) ซึ่งเป็นปลายประสาทของโฟโตรีเซ็บเตอร์เซลล์จากกลุ่มของปลายประสาทเพล็กซิฟอร์มชั้นในของเรตินา (Inner plexiform layer, IPL) มาใช้เพื่อศึกษาผลของนิวโรทรานสมิตเตอร์ 4 ตัว ที่ความเข้มข้นต่าง ๆ กันต่อการดูดซึมและการปล่อยนิวโรทรานสมิตเตอร์ที่มีกัมมันตภาพรังสี โดยกลุ่มปลายประสาทจาก OPL และ IPL ของเรตินา พบว่าอะซีทิลโคลีนสามารถกระตุ้นการดูดซึมและการปล่อยแกมม่าอะมิโนบูทีริคแอซิดจากกลุ่มประสาทที่ได้จาก OPL แต่ไม่มีผลต่อปลายประสาทจาก IPL การกระตุ้นการดูดซึมแกมม่าอะมิโนบูทีริคแอซิดของกลุ่มปลายประสาทจาก OPL โดยอะซีทิลโคลีนทำโดยการเพิ่มอัตราความเร็วสูงสุด (V_{max}) ของการดูดซึมแต่ค่าคงที่ไมเคลลิส (K_T) ของการดูดซึมไม่เปลี่ยนแปลง การดูดซึมแกมม่าอะมิโนบูทีริคแอซิดโดยกลุ่มปลายประสาท OPL ยังถูกกระตุ้นได้โดยการใส่โคลีนซึ่งเป็นตัวต้นกำเนิดของอะซีทิลโคลีนก่อนหน้าการทดสอบการดูดซึมแกมม่าอะมิโนบูทีริคแอซิด นีโอสติกมีน (ซึ่งยับยั้งการทำลายอะซีทิลโคลีน) และนิโคติน, และไดเมทิลเฟนิลปีเปอราซีเนียม (DMPP) ก็สามารถกระตุ้นการดูดซึมของแกมม่าอะมิโนบูทีริคแอซิดดังกล่าวได้

การกระตุ้นการดูดซึมของแกมม่าอะมิโนบูทีริคแอซิดโดยกลุ่มประสาทจาก OPL โดยการใส่โคลีนล่วงหน้าถูกยับยั้งได้โดยเฮมิโคลีเนียม, เฮกซาเมโทเนียม, เมคามิลลามีน, ดี - ทิวโบคิวรารีน, (-) สโคโปลามีน, และอะโทรปีน ความเข้มข้นของสารเหล่านี้ที่ทำให้เกิดการยับยั้ง 50 % ของการยับยั้งสูงสุด (IC_{50}) ของสารกลุ่มนิคโคตินิคต่ำกว่าของสารกลุ่มมัสคารินิค

การใส่โคลีนล่วงหน้าและการใส่อะซิทิลโคลีนสามารถกระตุ้นการปล่อยแกมม่าอะมิโนบูทีริคแอซิดชนิดที่ใช้แคลเซียมไอออนโดยกลุ่มปลายประสาท OPL อีกด้วย

ข้อมูลที่ได้บ่งชี้ว่าอาจมีปลายประสาทที่ใช้แกมม่าอะมิโนบูทีริคแอซิดเป็นนิวโรทรานสมิตเตอร์ (สารส่งสัญญาณประสาท) ในกลุ่มปลายประสาทจากเพล็คซีฟอร์มชั้นนอก (OPL) ของเรตินาหกระต่าย ปลายประสาทนี้ไวต่อการกระตุ้นโดยอะซีทิลโคลีน การกระตุ้นนี้อาศัยรีเซ็ปเตอร์ประเภทนิคโคตินิคซึ่งอาจอยู่ที่ปลายประสาทที่มีแกมม่าอะมิโนบูทีริคแอซิดดังกล่าว ปลายประสาทพวกนี้อาจเป็นแขนงของฮอริซอนทัลเซลล์ที่ไปต่อโยงกับปลายประสาทของโฟโตรีเซ็ปเตอร์เซลล์ประเภทที่เรียกว่า โคน