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# "Transplanting Receptors from Brains into Oocytes"

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"Transplanting Receptors from Brains into Oocytes"

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The brain is the most complex, and enigmatic, organization in existence. It is made up of myriads of nerve cells that are interconnected by an incalculable number of synapses, and it is safe to say that those features that really make an individual; our thoughts, sensations, love, hate, etc., all depend on the function of these synapses. Because of this, the brain has captivated human endeavor for thousands of years and, in attempting to understand how it works man has used many different methods. Today, we will see one approach that was developed only recently.

The first recorded studies of the brain were carried out in Alexandria, some 5000 years ago. In them, the Alexandrian doctors noted some correlations between skull and brain damage and impairment of movement and speech. This method of studying the brain by simple observation remained in operation for many centuries. It seems that Aristotle and Galen, about 2000 years ago, were the first to use animals, and experimental techniques, to study the nervous system. For many experiments, Galen used pigs, and his many important discoveries and interpretations were accepted without question until the Renaissance.

## The birth of electrophysiology

Although less complicated than humans, Galen's apes and pigs still proved to be difficult to study the structure and function of the nervous system, so other animals were chosen. I could not find who was the first to use frogs as an experimental subject to study the nervous system; but Leonardo da Vinci, in the late 15th century, used frogs to show that mechanical stimulation of the spinal cord caused muscles to contract.

A frog preparation similar to the one used by Leonardo was used by the great Italian scientist that we commemorate today. It is now about 200 years since the days when Luigi Galvani used to spend his afternoons, in the roof of his house, waiting for a storm to break out. Figure 1, taken from Galvani's commentarius (De Viribus Electricitatis in Motu Musculari, 1791) shows the hind quarters of a frog, attached by a wire to a lightning rod. Every time that lightning occurred, the frog muscles contracted: Galvani was indeed lucky that he did not contract himself! This was really the birth of electrophysiology, and I like to think that it was born out of a thunder, whose reverberations are still felt today.

- Figure 1 near here -

I have been following on Galvani's trail, and for many years have worked with frog nerves and muscles. So, why now oocytes? The reason is quite simple. About 30 years ago, Diamond and I had mapped out the distribution of acetylcholine (ACh) sensitivity along foetal muscle fibres of the rat and found that after 17 days of gestation, at a time when nerve-muscle synapses had just been formed

the entire muscle fibre surface had ACh-receptors (Diamond & Miledi, 1959, 1962). I then wondered when ACh-receptors first appeared on the muscle fibre membrane. To examine this question I studied muscles earlier and earlier during development and found the muscles were still sensitive to ACh. As you may imagine, the experiments became increasingly difficult to execute, as I used more and more immature animals, until finally I decided to approach the problem from the other end and asked myself the question: Are oocytes sensitive to ACh? At that time I did not fully anticipate all the consequences that would follow from such a simple question.

To explore this problem it was necessary to choose a suitable system. I was aware that Xenopus oocytes were very convenient, that they were easily available all through the year and that embryologists had already accumulated a great deal of information on their structure, development and changes after fertilization. So, about 20 years ago I performed a few unsuccessful experiments; but it was not until 1976 when Drs. Kiyoshi Kusano, from Chicago, and Jacques Stinnakre, from Paris, joined me in London that we really began to study Xenopus oocytes in earnest.

## Frogs, oocytes and receptors

Presumably most of you have seen a Xenopus, but for those who have not, figure 2 shows two females. It can be seen that Xenopus are quite massive frogs - not unlike Sumo wrestlers in appearance. Comparing Xenopus with the more slender English Rana temporaria frogs that I was used to working with in London, is like comparing a beauty painted by Rubens with one painted by Modigliani.



- Figure 2 near here - - Figure 3 also near here -

The ovary of the adult Xenopus female contains thousands of oocytes, and if we remove a piece of ovary and look at it through a microscope it appears as in figure 3; which shows oocytes in different stages of development: from the very young and transparent ones, to the fully grown oocytes which are about 1.5mm in diameter. We can easily remove the oocytes from the ovary, place them in a chamber, insert two or more microelectrodes, and proceed to apply ACh to see if they are sensitive to it. This is exactly what we did, and to our delight we found that many oocytes responded to ACh (Kusano et al. 1977, 1982).

The response was an oscillatory current, and figure 4 shows one striking example. Without going into detail let me mention briefly some of the main features of these responses. First, they are blocked by atropine and not by curare or α-bungarotoxin, both of which are highly specific antagonists of nicotinic ACh receptors in muscle fibres. Therefore, the responses in the oocytes result from activation of muscarinic, and not nicotinic, ACh-receptors. Second, the oscillatory current is carried mainly by chloride ions, and the onset of the response does not occur until after a very long delay has elapsed. In this particular case the first oscillation only began some 20 seconds after the pulse of ACh was applied. In other cases the delay could be minutes! This is in sharp contrast with similar experiments done earlier by Bernard Katz and myself on muscle fibres. There the stimulus-response delay was measured in microseconds - about a million times shorter!! Because of the very long delay Kusano, Stinnakre and I (1977, 1982) postulated that in the oocyte the ACh did not open the chloride channels directly, but

through the mediation of a substance that had to accumulate inside the oocyte.

- Figure 4 near here -

All that was very nice, because it showed without question that Xenopus oocytes responded to ACh. But, before we could definitely conclude that the oocyte membrane itself had ACh receptors, we had to consider other possibilities.

## Follicles and oocytes.

An oocyte simply plucked from the ovary as is commonly done, is a follicle, and it is not a single cell. It is actually a veritable cellular complex, formed by the oocyte proper, which is surrounded by thousands of follicular cells. This aggregate is in turn surrounded by thousands of epithelial cells. In view of the very long delay between the application of ACh and the onset of the response, it was possible that ACh was not acting directly on the oocyte, but acted instead on any of the surrounding cells. The possibility of an indirect action was even more pertinent in the case of the follicular cells, because they make close contacts with the oocyte. As shown in Figure 5A the surface of the oocyte has many processes, some of which form gap junctions with other processes that arise from the follicular cells (Browne & Werner, 1984; van der Hoef et al. 1984; Limbrick & Miledi unpublished). The follicular cells and the oocyte are electrically coupled through these junctions, and it could be that ACh was combining with receptors situated on the follicular cells and that we were simply recording



the ensuing response at a distance, from within the oocyte.

- Figures 5A and 5B near here -

Fortunately, it is possible to remove the enveloping cells either manually, or enzymatically as in the case illustrated in figure 5B. After treatment with collagenase, the processes of the oocyte were still covered by the fibrous vitelline layer, but nothing else remained. Both follicular and epithelial cells had been removed. Nonetheless, oocytes like the one illustrated gave responses to ACh similar to those obtained with the follicles. Only then, we could definitely conclude that the oocyte membrane itself has muscarinic ACh-receptors which open chloride channels.

## Follicles respond to many neurotransmitters and hormones.

Kusano, Stinnakre and I also found that follicles respond to norepinephrine, dopamine and serotonin, and later on Richard Woodward and I found that some follicles respond to other neurotransmitters and hormones. Table 1 shows a list of some of the agonists that elicit responses in follicles. However, it should be made clear that not every follicle responds to all these substances. Some follicles respond to only a few; and some neurotransmitters like glutamate, GABA and glycine evoke responses which are only a few (<10) nanoamperes in amplitude. The most commonly effective agonists are the top 3 or 4 in each column. Moreover, it is interesting that all the follicles from a given donor tend to be selective for the same agonists. This suggests that the types of receptors expressed in the oocytes are under a genetic control, that

varies among different individuals. One wonders if this control is permanent, or whether oocytes from the same frog manifest different receptors at different times.

- Table 1 near here -

Although a particular follicle does not necessarily have receptors to all the agonists listed in table 1, it is abundantly clear that in some follicles several receptors can coexist. For example figure 6 shows results from a follicle that responded to adenosine, follicle stimulating hormone, norepinephrine and forskolin (an activator of adenylate cyclase). Note that all these substances elicited outward currents, except for ACh which evoked its usual oscillatory inward current. The gonadotropins, VIP, prostaglandins and atral natriuretic factor (ANF) also produced outward currents and all of them are carried mainly by potassium ions. Furthermore, they are all mimicked by forskolin (Fig. 6), and by intra-oocyte injection of cyclic AMP. So we think that all these agonists do not open the $K^+$ channels directly, but via a receptor-channel coupling mechanism that involves cAMP (Woodward & Miledi 1987a,b and unpublished; van Renterghem et al. 1985; Lotan et al. 1985).

The available evidence indicates that the various outward current responses are mediated by specific receptors and, as in the case of the muscarinic ACh-receptor, the question again arises where in the follicle are those receptors, and also where are the $K^+$ channels they operate?

- Figure 6 near here -



## Different location of the two main receptor-channel coupling systems in follicles

To determine where the receptors and channels are located Woodward and I studied follicle-enclosed oocytes and oocytes from which the enveloping cells had been removed. In figure 7 the top traces show the responses obtained in a follicle-enclosed oocyte exposed successively to norepinephrine, adenosine, forskolin and ACh; while the lower records show the effect of these same substances applied to a manually defolliculated oocyte obtained from the same donor. Note that the responses to norepinephrine, adenosine and forskolin have vanished, while the response to ACh was still elicited after removing the follicular cells. Incidentally, the responses to gonadotropins, VIP and prostaglandins were also abolished by defolliculation.

- Figure 7 near here -

All these results may be explained in several ways. However, Woodward and I prefer the scheme illustrated in figure 8, which shows the follicular cells electrically coupled to each other as well as to the oocyte proper. We think that the receptors to catecholamines and hormones, that trigger the outward currents, are in the membrane of the follicular cells (Woodward & Miledi, 1987a,b). When these receptors are activated, for instance by norepinephrine as in the drawing, cyclic AMP is produced inside the follicular cells and this directly, or more probably through the action of protein kinases, opens the $K^+$ channels also located in the

membrane of the follicular cells. When the follicular cells are removed, both the hormone receptors and the $K^+$ channels are eliminated, leaving muscarinic ACh receptors in the oocyte membrane, coupled to chloride channels through a phosphatidyl-inositol system (see below).

Thus it appears that, as far as plasma membrane channels are concerned, the two main receptor-channel coupling systems, cyclic AMP and phosphoinositides, have different intracellular locations in the follicle. The cyclic AMP system is preponderantly located in the follicular cells, while the phosphoinositide system is more abundant in the oocyte.

- Figure 8 near here -

## Voltage-operated membrane channels.

As well as possessing a number of drug-activated currents, oocytes also display a surprising diversity of voltage-activated membrane currents. One of the largest, and most common of these is a transient outward current seen on depolarization to potentials around 0mV (Miledi 1982; Barish 1983). This current arises because the oocyte membrane contains calcium channels which are opened when the membrane is depolarized, causing an influx of calcium into the oocyte. The ensuing rise in intracellular free calcium leads to the activation of calcium-dependent chloride channels (probably the same channels which mediate the ACh response) to give the final outward current response. More recently, Ian Parker and I (Parker & Miledi 1987b; 1988a,b) have re-examined a number of other voltage activated currents that had been observed since we first started working with



oocytes. Some of the currents are illustrated in figure 9. The first two of these are transient sodium and potassium currents, which are activated on depolarization to potentials around 0mV, and which have properties resembling the sodium and potassium currents of nerve cells. The third response is more unusual, being a chloride current which is activated by hyperpolarization to potentials beyond about -100mV. It is not yet clear what role, if any, these currents play in the oocyte. Since some of these currents are not consistently present in the oocytes, it may be that the corresponding membrane channel proteins arise form an apparently random expression of some genes which become important at some later state in the development of the frog.

- Figure 9 near here -

## Transplanting receptors from brain cells into oocytes.

With the variety of receptors and channels that they already have Xenopus oocytes, and follicles, are very suitable subjects for study. However, I thought that their usefulness, as a model system for studies of the nervous system, would be greatly increased if it were possible to induce the oocytes to acquire the neurotransmitter receptors and channels that are so crucial to the functions of the brain.

One obvious advantage in being able to do that is shown in figure 10. On the left is an oocyte and the little speck by its side shows a neuron at the same scale. On the right the same two cells are shown at higher magnification. Because the oocyte is so large and accessible, it allows us to do a variety of

electrophysiological and biochemical experiments that would be very difficult, or nearly impossible, to carry out on the much smaller neuron. Clearly, it would be nice if we could transplant the receptors from the small nerve cells to the oocytes.

- Figure 10 near here -

During the last years my colleagues Katumi Sumikawa, Ian Parker, Cameron Gundersen and I have been doing just that; and by now we have been able to transplant to the oocyte receptors to ACh (both nicotinic and muscarinic) serotonin, dopamine, norepinephrine, glutamate, kainate, aspartate, NMDA, GABA, glycine, substance P and neurotensin. In effect most of the known receptors (Gundersen et al. 1983, 1984a,b,c; Miledi et al. 1982a,b; Sumikawa et al. 1984b; Parker et al. 1986), and other researchers are now adding to this list (Breer & Benke 1986; Cross et al. 1987; Harada et al. 1987; Oron et al. 1987).

This so called transplantation of receptors is not a surgical transplantation, but a biochemical one; and is carried out as is shown diagramatically in figure 11. For example, we take the cerebral cortex from the human brain, homogenize it, and isolate the poly $(A)^+$ messenger RNA. It should be borne in mind that this mRNA is a mixture of many thousands of different messages, some of which carry the instructions for making neurotransmitter receptors. The next step is to inject the foreign mRNA into the oocytes. As was shown first by Gurdon and his colleagues (Gurdon et al. 1971), the foreign mRNA is translated by the oocytes own protein synthesizing machinery. We showed further that the brain mRNA was not only efficiently translated, but that the products of the translation



were also processed, assembled and inserted into the oocyte membrane, where they formed functional receptors. In the example illustrated here activation of serotonin receptors from the human brain, transplanted to the oocyte, caused an oscillatory chloride current similar to that produced by ACh in some native oocytes. This was a pleasant finding. Quite frankly, when the first experiments were being done I thought that after the injection of such crude messengers the oocyte might soon die due to the production of proteases and other unwanted proteins. This was not so, and the oocytes acquired the foreign neurotransmitter receptors, and retained them sometimes for more than two months.

- Figure 11 near here -

Calcium, phosphoinositides and oscillatory currents in oocytes.

The transplantation of receptors and channels into the oocyte membrane is a very powerful and useful technique. Let me just mention briefly some of its potential.

For instance, once we have the receptors in the oocyte membrane we can study the way in which the receptors operate. Take for example the $Cl^-$ current caused by activation of the serotonin receptors. Earlier on we had shown that the native oocyte membrane has $Cl^-$ channels that open whenever the intracellular concentration of calcium ions is increased (Miledi 1982; Miledi & Parker 1984). Therefore, we wondered if the $Cl^-$ channels opened by serotonin were $Ca^{2+}$-dependent, and if so where did the $Ca^{2+}$ come from?

- Figure 12 near here -

As shown in figure 12 the response to serotonin, in an oocyte injected with rat brain mRNA, still occurred when all the $Ca^{2+}$ was withdrawn from the external medium. Moreover, the oscillatory response to serotonin was abolished if the oocyte is injected with EGTA to chelate calcium ions and prevent their intracellular level from rising (Fig. 13). These results indicated clearly that the $Cl^-$ channels are operated by $Ca^{2+}$ ions and that the $Ca^{2+}$ derives from intracellular stores (Parker et al. 1985).

- Figure 13 near here -

The Hokins, Michell, and especially Berridge and their colleagues have shown that some receptors activate phosphoinositide metabolism, leading to the production of inositol trisphosphate ($IP_3$) which then releases calcium (Hokin & Hokin 1953; Michell 1975; Berridge & Irvine 1984). To see if a similar process occurred in the oocytes, Ian Parker and I injected $IP_3$ into oocytes. As shown in figure 14, we found that the $IP_3$ caused an increase in the intracellular level of $Ca^{2+}$ ions - monitored here by the photoprotein aequorin - and produced also an oscillatory $Cl^-$ current, like that produced by serotonin or ACh (Parker & Miledi 1986). Furthermore, Nomura and his colleagues have recently found that both serotonin and ACh cause an increased production of $IP_3$ in oocytes injected with rat brain mRNA (Nomura et al. 1987). Thus we believe that serotonin receptors, through the mediation of a GTP-binding protein causes the production of $IP_3$. This then releases calcium from internal stores, and the calcium finally opens



the Cl-channels.

- Figure 14 near here -

The messenger pathway described above already appears rather complicated, but recent experiments show that it may still be an over-simplified view of the events which mediate the serotonin response. For example, biochemical evidence indicates that $IP_3$ is further phosphorylated inside cells to inositol tetrakisphosphate ($IP_4$) (Berridge 1987). We therefore tried injecting $IP_4$ into oocytes to see if this compound acts as a second messenger and found that, like $IP_3$, it was able to evoke oscillatory chloride currents resulting from a rise in intracellular calcium (figure 15; and see Parker & Miledi 1987a). Interestingly, the characteristics of the currents evoked by $IP_3$ and $IP_4$ were different, suggesting that they might serve different roles as second messengers. $IP_4$ gave almost purely oscillatory responses, while $IP_3$ gave responses comprised of both smooth and oscillatory components (Fig. 15).

- Figure 15 near here -

The characteristics of the phosphoinositide messenger pathway appear to determine many of the properties of the responses to serotonin and other agonists. A striking example of this is seen in the highly non-linear dose response relationship for serotonin, which leads to an almost all-or-nothing membrane current response for doses close to threshold (Gundersen et al. 1983). Recent experiments, using the photolysis of caged $IP_3$ loaded into oocytes to provide a precisely controlled liberation of intracellular $IP_3$,

indicates that this non-linearity probably arises because a threshold level of $IP_3$ is required in order to trigger the liberation of intracellular calcium (Parker 1988). The existence of the non-linearity is likely to have important consequences for the functioning of synapses which employ phosphoinositide signalling, since it may form the bases for a novel form of heterosynaptic facilitation. Thus, we were able to demonstrate in the oocyte (Parker et al. 1987) that large responses were obtained to the simultaneous application of low doses of ACh and serotonin, while the same dose at either agonist alone gave virtually no response (Fig. 16). Another interesting feature of this intracellular signalling system is that, once activated, it leaves membrane changes that greatly outlast the initial surge of current.

- Figure 16 near here -

Non-oscillatory current responses.

As far as I know nobody has yet described a serotonin receptor in the human or rat brain that functions in the same oscillatory way as the serotonin receptors transplanted to the oocyte membrane. Of course, it may be that the oscillatory behavior is peculiar to the oocyte. However, I am inclined to think that sooner or later, someone will discover similar oscillatory currents in nerve cells.

Be that as it may, other receptors transplanted to the oocyte membrane behave very much like the receptors in the membrane of the cells from which the mRNA originated. For example the properties of receptors to GABA, glycine and kainate, as well as the nicotinic acetylcholine receptors transplanted to the oocyte, are similar in



most respects to the properties of the original receptors in brain and muscle cells. Another recent example is the excitatory amino acid receptor activated by N-methyl-D-aspartate: NMDA for short.

A few years ago, we showed that rat brain mRNA induced the appearance of NMDA receptors in Xenopus oocytes (Gundersen et al. 1984a). More recently Philip Ascher and his colleagues showed that glycine potentiates the action of NMDA on cerebral neurons (Johnson & Ascher, 1987; Ascher & Nowak 1987). As shown in figure 17, rat NMDA receptors of the brain transplanted to the oocyte are also greatly potentiated by glycine (see also Verdoorn et al. 1987). Note that application of glycine alone produced a very small response - which in this experiment was undetectable at the amplification used to obtain the record. However, when NMDA was applied together with glycine, a large current was generated. Conversely, the right part of the figure shows that NMDA alone produced no detectable current but, again, a large current was generated when both substances were applied together. Furthermore, Mayer, Westbrook, Ascher and their colleagues, have shown that $Mg^{2+}$ ions block the responses to NMDA in nerve cells (Mayer & Westbrook 1984, 1985; Nowak et al. 1984) and we observed that rat brain NMDA receptors in the oocyte are also inhibited in the presence of $Mg^{2+}$ ions.

- Figure 17 near here -

As well as responding to NMDA, oocytes injected with rat brain mRNA also show small currents in response to aspartate. These responses are also potentiated by glycine, and are blocked by $Mg^{2+}$ (Fig. 18). This suggests that aspartate may act on the same

receptors as NMDA; although we will not be certain about this until the various receptors to excitatory amino acids are cloned.

- Figure 18 near here -

Molecular characterization of channels expressed in oocytes.

Xenopus oocytes also allow us to study the action of neurotransmitter receptors in molecular detail. This is sometimes difficult to do in nerve cells. Consider for instance all the problems encountered if one wishes to study nerve cells of the human brain. Figure 19 shows results obtained from an oocyte that had been injected with human brain mRNA. Thus, this oocyte had been induced to acquire human glycine receptors and, when glycine was applied, it generated a $Cl^-$ current. Note that as the current increased the membrane current noise, shown in the middle trace, also increased. This noise can be analyzed to derive the single channel characteristics (Katz & Miledi 1972, 1973; Anderson & Stevens 1973), and in this way we found that human brain glycine receptors transplanted to Xenopus oocytes open $Cl^-$ channels that have a mean single channel conductance of about 30 pS and remain open, on the average, for about 200 ms (cf. Gundersen et al. 1986).

- Figure 19 near here -

Rat brain mRNA also induces glycine receptors, together with many other types of receptors including those to kainate. When we set out to analyze the properties of the single channels activated



by kainate, we were surprised to find that kainate currents were accompanied by almost no increase in noise fluctuations, despite the fact that glycine currents in the same oocytes showed clear noise (Fig. 20). This suggested that the elementary conductance of the kainate channel must be very small and, indeed, noise analysis gave an estimate of 0.3pS for the single channel conductance; a value one hundred times smaller than for the glycine channel (Miledi et al. 1985). Thus, it is clear that the channel properties are determined largely by the properties of the proteins which form them, and not by the lipid environment in which they reside. Furthermore, the rat brain mRNA appears to express very large numbers of kainate channels because large total currents (a few $\mu$A) could be obtained despite the small single channel current.

- Figure 20 near here -

Xenopus oocytes can also be used to record the single channel currents directly. For example, figure 21 shows results obtained from an oocyte that had been injected with mRNA from both chick brain and denervated cat muscle. This induced the oocyte membrane to acquire chick brain GABA receptors and cat muscle nicotinic ACh-receptors. Single channel currents were then recorded from the same oocyte with either GABA or ACh present in the patch-pipette. Simple comparison of these records shows that the channels activated by GABA and ACh had similar conductances. In contrast the average lifetime of the GABA induced channels was 16msec, while that of the channels induced by ACh was 3msec (Miledi et al. 1983). In these and other characteristics the GABA and glycine receptors in the oocyte behave like those studied in neurons (Barker et al. 1986;

Borman et al. 1987). All this shows that oocytes express faithfully some neurotransmitter receptors and channels, and again shows also that the properties of the receptor-channel complexes depend mainly on the nature of the proteins encoded by the mRNA, and not so much on the cell membrane in which the receptors are embedded.

- Figure 21 near here -

mRNA fractionation.

Most of the experiments considered so far were done with whole mRNA preparations that gave rise to many neurotransmitter receptors and voltage-operated channels. Therefore, to reduce the number of different species of messages contained in the mRNA, the whole mRNA preparations were fractionated by density gradient centrifugation, or by gel electrophoresis. When the individual mRNA fractions were injected into the oocytes, some fractions were found to express only one type of receptor or voltage-operated channel. The fractionation of mRNA offers several advantages. For example, if one wants to study the characteristics of $Na^+$ and $K^+$ channels in nerve, or muscle cells, it is normally necessary to use pharmacological agents to block one type of channel (cf. Hille 1984). However, this is not necessary in oocytes because the mRNAs coding for different channels can be separated, and injected into the oocytes to induce them to acquire one or the other type of channel (Fig. 22). For instance, although whole mRNA from the brain expressed both $Na^+$ and $K^+$ channels, oocytes injected with fraction 7, when depolarized to various levels gave only inward $Na^+$ currents. In contrast, depolarization of oocytes injected with fraction 11 did not elicit



$Na^+$ currents, but generated instead $K^+$ currents (Sumikawa et al 1984a). When first done these experiments indicated that $Na^+$ channels were encoded by a very large mRNA species, a fact that was later on shown more conclusively when the $Na^+$ channel message was cloned and expressed by Numa and his colleagues (Noda et al. 1986).

- Figure 22 near here -

Oocytes as a tool for cloning receptors and channels.

Even though the mRNA fractions still contain many different messages, the partial purification of mRNAs is of great help in trying to understand how the brain works. First, the fractions of mRNA allow us to have oocytes tailor made to contain a given receptor or channel. Perhaps more important, however, is the fact that the fractions are enriched for particular messages; and these fractions, combined with the use of oocytes as a translation system, provide us with a very powerful method for cloning any of the genes encoding the receptors.

The most straightforward way of cloning a receptor involves purifying the receptor protein and determining part of its primary amino acid sequence, so as to be able to produce suitable oligonucleotide probes. Alternatively, one needs to purify, at least partially, the receptor protein and use it to make specific antibodies, which can then be used as probes. However, purifying receptors is frequently a very difficult and laborious task because some receptors are present in the cells only in comparatively small quantities, and because the methods of purification have many

pitfalls.

From the moment we succeeded in expressing neurotransmitter receptors in the oocytes we realized that these could be used to clone receptors, without having first to purify them. Fractionation of mRNA was a further step towards that goal. (Sumikawa et al. 1984a, 1986). For instance, one may take a fraction enriched for the message coding for a receptor or channel, construct a cDNA library, make mRNA from the cloned cDNA and then screen the library for the gene encoding the receptor using functional assays in Xenopus oocytes. The oocytes are very useful for these assays because they are extremely sensitive detectors of mRNA. I believe that it may even be possible to detect the presence in the oocyte of just one or a few transcripts coding for a receptor. For the initial screening, one may pool thousands of clones until a pool is identified that expresses the desired receptor, or voltage-gated membrane channel, in the oocytes. Then that pool is subdivided and tested again until the relevant clone is isolated.

Using Xenopus oocytes in essentially this way Davidson, Lester, Nakanishi and their colleagues, have recently cloned the receptors for serotonin and substance K without having first to purify the receptors (Lubbert et al. 1987; Maru et al. 1987). Furthermore, this approach is not limited only to receptor proteins, but can be applied to any protein whose expression in the oocyte can be readily screened. For example, the Xenopus expression system was recently employed to obtain a clone for the $Na^+$/glucose transporter from mammalian intestine (Hediger et al. 1987).



Antisense RNAs and oocytes.

I should mention that the positive assay - that is expression of functional receptors by synthetic sense mRNA made from the clones demands practically 'full-length' cDNAs. Moreover, if the receptor or channel under investigation requires the presence of two or more different subunits in order to be functional, then selecting the clones by positive assays, although feasible, becomes more complicated and laborious. For these multi-heterosubunit receptors a better approach may be to use the cDNA library to produce antisense RNA, and then inject the antisense RNA together with the whole or fractionated brain mRNA. In this case hybridization of the antisense RNA with the corresponding sense target should inhibit expression of the receptor (reviewed by Green et al. 1986).

That this is indeed what happens is shown in figure 23. The record on the left shows the large response to ACh evoked in an oocyte injected with whole Torpedo electroplaque mRNA; which contained the mRNAs coding for the $\alpha$, $\beta$, $\gamma$ and $\delta$ subunits of the nicotinic ACh-receptor. The expression of functional nicotinic receptors was greatly inhibited when the same whole Torpedo mRNA was co-injected with synthetic $\alpha$-subunit antisense RNA (Fig. 23B). The average response to ACh was reduced to much less than 1%. This inhibition was specific for the ACh receptors, because the antisense RNA did not prevent the expression of the $Cl^-$ channels (Fig. 23C), which are also encoded by mRNA from the electric organ of Torpedo (Sumikawa et al. 1984c). Furthermore, the different antisense RNAs specifically inhibited the translation of the corresponding target subunit RNAs synthesized from cloned cDNAs (Fig. 23D). Thus using the antisense approach it should be possible to identify the clones

encoding the subunits of a heteromeric receptor.

- Figure 23 near here -

Doing neurobiology in oocytes.

Once a receptor mRNA is cloned, be it using oocytes or otherwise, there are a million things that can be done, and here again the oocytes are very useful. For instance one can use the oocytes to study the functional characteristics of selectively mutated receptors, as has been done by Numa, Sakmann and their colleagues (Mishina et al. 1985; Imoto et al. 1986). Or they can be used to study the processes that control the synthesis, assembly and membrane insertion of receptors and channels. Another very important practical application of the Xenopus oocyte system will be in the development and testing of new receptor active drugs that can be used in Medicine - and much more.

Actually there is so much that can be done to study the brain with oocytes, that they are becoming increasingly popular in Neurobiology. A few years ago many journals did not even list Xenopus oocytes in their subject index. They do now and, as shown in figure 24, the number of papers describing research on receptors and channels in Xenopus oocytes is rapidly increasing (in the 5 years before 1977 there were only 2 or 3 papers); and it would be safe to predict that the numbers will grow even more rapidly in the next years.

- Figure 24 near here -



I hope I have been able to convey to you a bit about the great excitement and potential of doing neurobiology in oocytes. However, we must not forget that Xenopus oocytes are only a model system to help us along while we find better ways to tackle the much more complete and intricate human brain. At some stage we must abandon Galvani's trail and return to that of the Alexandrian doctors to see in which way the information gained from oocytes can help us prevent or alleviate some of the many diseases that affect the human brain.

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## Figure Legends

Fig. 1. Galvani's experiments on "atmospheric electricity" conducted during a thunderstorm. Whenever lightning struck, the frog muscles contracted (from Mary A. B. Brazier, 1984).

Fig. 2. Xenopus laevis. Two female frogs. The length of each animal is about 8 inches from head to foot.

Fig. 3. Part of the ovary from a Xenopus showing oocytes at different stages of development.

Fig. 4. Muscarinic response to ACh in a Xenopus oocyte. In this and subsequent figures the traces show membrane current recorded at a clamp potential of -60mV (except where indicated), and downward deflexions correspond to inward currents. At the arrow a pulse of ACh was applied by ionophoresis to the oocyte surface. Calibration bars are 10s and 50nA. (from Miledi et al. 1982)

Fig. 5. Electron-micrographs of Xenopus oocytes. A) section of a follicle showing a follicular cell process meeting one from the oocyte. B) section of a follicle which had been treated with collagenase to remove the cells enveloping the oocyte (A. R. Limbrick & R. Miledi; unpublished)



Fig. 6. Membrane currents recorded from a follicle-enclosed oocyte in response to adenosine, porcine follicle stimulating hormone (pFSH), norepinephrine (NA), forskolin and ACh. Drugs were applied by bath superfusion for the times indicated by the bars. The periodic steps on the trace are currents resulting from voltage pulses (10mV depolarizing) applied to monitor the oocyte's resistance. (R.M. Woodward & R. Miledi 1987a)

Fig. 7. Comparison of drug-evoked responses in a follicle-enclosed oocyte (A), and in an oocyte from which the follicular and other enveloping cells were removed by rolling the oocyte on a poly-L-lysine coated surface (B). (R.M. Woodward & R. Miledi; unpublished data)

Fig. 8. Scheme illustrating the electrical coupling of follicular cells to the oocyte, and the putative locations of neurotransmitter and hormone receptors and membrane channels. AC = adenylate cyclase, PI = phosphoinositidase, R = norepinephrine receptor, M = muscarinic receptor. (R.M. Woodward & R. Miledi)

Fig. 9. Examples of membrane currents evoked by polarization in 'native' Xenopus oocytes. All traces show clamp currents in response to step changes in potential to the voltages indicated, from holding potentials of -100mV (A,B) or -60mV (C). Passive membrane currents were computer-subtracted in (A,B). (From Parker & Miledi 1987b, 1988a,b)

Fig. 10. Sketch illustrating the difference in size and shape between the frog oocyte and the cell body of a typical brain neuron (Pyramidal cell). The width of the neuron is less than one hundredth of that of the oocyte.

Fig. 11. Stages in the 'transplantation' of neurotransmitter receptors into oocytes by the isolation and injection of mRNA from the brain.

Fig. 12. The oscillatory response to serotonin is not abolished by removal of calcium from the external medium. Both traces show responses to serotonin ($5 \times 10^{-7}$M):(a) in normal Ringer; and (b) in Ringer with no added calcium and containing 2mM EGTA and 5 mM $MgCl_2$. Perfusion with the zero calcium Ringer began several minutes before record b. (from Parker et al. 1985)

Fig. 13. Abolition of serotonin-induced oscillatory currents, by intracellular loading with EGTA. a: control record from an oocyte injected with rat brain mRNA. Serotonin ($3 \times 10^{-8}$M) was applied by bath perfusion for the time indicated by the bar. b: record from the same oocyte, but after intracellular loading with EGTA, using ionophoretic pulses of ca.50nA for about 1 h. (from Parker et al. 1985)



Fig. 14. Aequorin light signal and membrane current in response to injection of $IP_3$ into an aequorin-loaded oocyte. The upper trace monitors light emission and the lower trace shows clamp current. Upward deflections correspond to increasing light (i.e. increasing intracellular calcium) and outward membrane current. Injection of $IP_3$ was made at time indicated by the arrowhead. (from Parker & Miledi 1986)

Fig. 15. Membrane currents recorded in a single oocyte in response to intracellular injections of different amounts of $IP_3$ and $IP_4$ (indicated in moles next to each trace). Clamp potential was -50mV, and arrowheads mark the times of injection. (from Parker & Miledi 1987a)

Fig. 16. Potentiation of membrane current response by simultaneous application of ACh ($3x15^{-7}$M) and serotonin ($3x10^{-8}$M) to an oocyte injected with rat brain mRNA. The trace shows membrane currents, and the bars indicate the durations of drug application. ACh gave an inward current of about 5nA and 5HT ($3x10^{-8}$M) previously applied alone gave 10nA. Thus, the combined application of ACh and 5HT elicited a response that was >50 times larger. (R. Miledi, unpublished data)

Fig. 17. Membrane currents in an oocyte injected with rat brain mRNA evoked by the concerted action of NMDA and glycine. NMDA ($10^{-4}$M) and glycine ($10^{-5}$M) were applied by bath perfusion for the times indicated by the bars. Ringer solution contained no magnesium. (R. Miledi, unpublished data)

Fig. 18. Blocking action of $Mg^{2+}$ ions on the current evoked by aspartate plus glycine. Each trace shows currents evoked by aspartate ($10^{-4}$M) plus glycine ($10^{-5}$M). The middle trace was obtained during perfusion with Ringer solution including 0.1mM $Mg^{2+}$. Control records (left and right) were obtained in normal Ringer solution containing no $Mg^{2+}$. (R. Miledi; unpublished data)

Fig. 19. Membrane current and accompanying current fluctuations ('noise') evoked by glycine in an oocyte injected with mRNA from fetal human brain. The upper trace (A) shows the rise in inward current evoked by glycine, monitored on a low recording gain. The same response is shown in (B), recorded at a higher gain and AC-coupled, so as to demonstrate the increase in noise during glycine action. Traces in (C) and (D) were recorded at faster sweep speed and compare the noise levels before (C) and (D) during glycine activation. (C.B. Gundersen, I. Parker & R. Miledi; unpublished data)

Fig. 20. Membrane current noise produced by glycine and kainate in oocytes injected with rat brain mRNA. In each frame the upper trace shows a low gain, DC-coupled record of clamp current, and the lower trace shows the current at high gain and band-pass filtered between 1-50Hz. Clamp potential was -60mV. (I. Parker, C.B. Gundersen & R. Miledi, unpublished)



Fig. 21. Single channel currents induced by GABA (a) and ACh (b) acting on receptors in one oocyte that had been injected with mRNA derived from both chick optic lobe and denervated cat muscle. The records were obtained from membrane patches with seals of > 10 GΩ. Patch pipettes contained $10^{-4}$M GABA in (a), and 2 x $10^{-7}$M ACh plus 5 x $10^{-7}$M atropine in (b). Downward deflexions of the traces correspond to inward currents, and indicate channel openings. Calibration bars apply to all records. The membrane potential across the patch was about -110mV in (a) and -90mV in (b). Records were filtered at 1 kHz (a) and 500 Hz (b). Temperature, 18-20°C. (from Miledi et al. 1983)

Fig. 22. Voltage-activated sodium (A) and potassium (B) currents recorded from two oocytes injected with different fractions of mRNA from chicken optic lobe. (A) Records of $Na^+$ currents in an oocyte injected with fraction 7 mRNA. The membrane potential was held at -100mV and stepped to potentials of (from bottom to top) 0, +10, +20, +30, and +40mV. (B) Records of $K^+$ currents in an oocyte from the same donor injected with mRNA fraction 11. The potential was held at -100mV and stepped to (from bottom to top) -60, -50, -40, -30, -20, -10, 0, and +10mV. (from Sumikawa et al. 1984)

Fig. 23. ACh-activated currents (A,B) and current/voltage relationship of chloride current (C) induced in oocytes by Torpedo mRNA in the presence or absence of antisense RNA from the α-subunit of the AChR. The antisense RNA almost abolished responses to ACh (A,B), but had no effect on the expression of chloride channels (C). D. SDS gel electrophoresis of AChR molecules synthesized in oocytes. A mixture of the four subunit

mRNAs was injected into 10 oocytes with (1) $H_2O$, (2) antisense αRNA, (3) antisense βRNA, (4) antisense γRNA, or (5) antisense δRNA for immunoprecipitation assay. (K. Sumikawa & R. Miledi unpublished)

Fig. 24. Numbers of papers, concerning membrane receptors and channels in Xenopus oocytes, published per year in neurobiological journals.



Table I

Some agonists and hormones that evoke membrane current responses in native Xenopus oocytes.

| | |
|---|---|
| Acetylcholine | Gonadotropins |
| Norepinephrine | VIP |
| Dopamine | Prostaglandins |
| Adenosine | ANF |
| ATP | Oxytocin |
| GABA | Serotonin |
| Glutamate | Substance P |
| Glycine | |

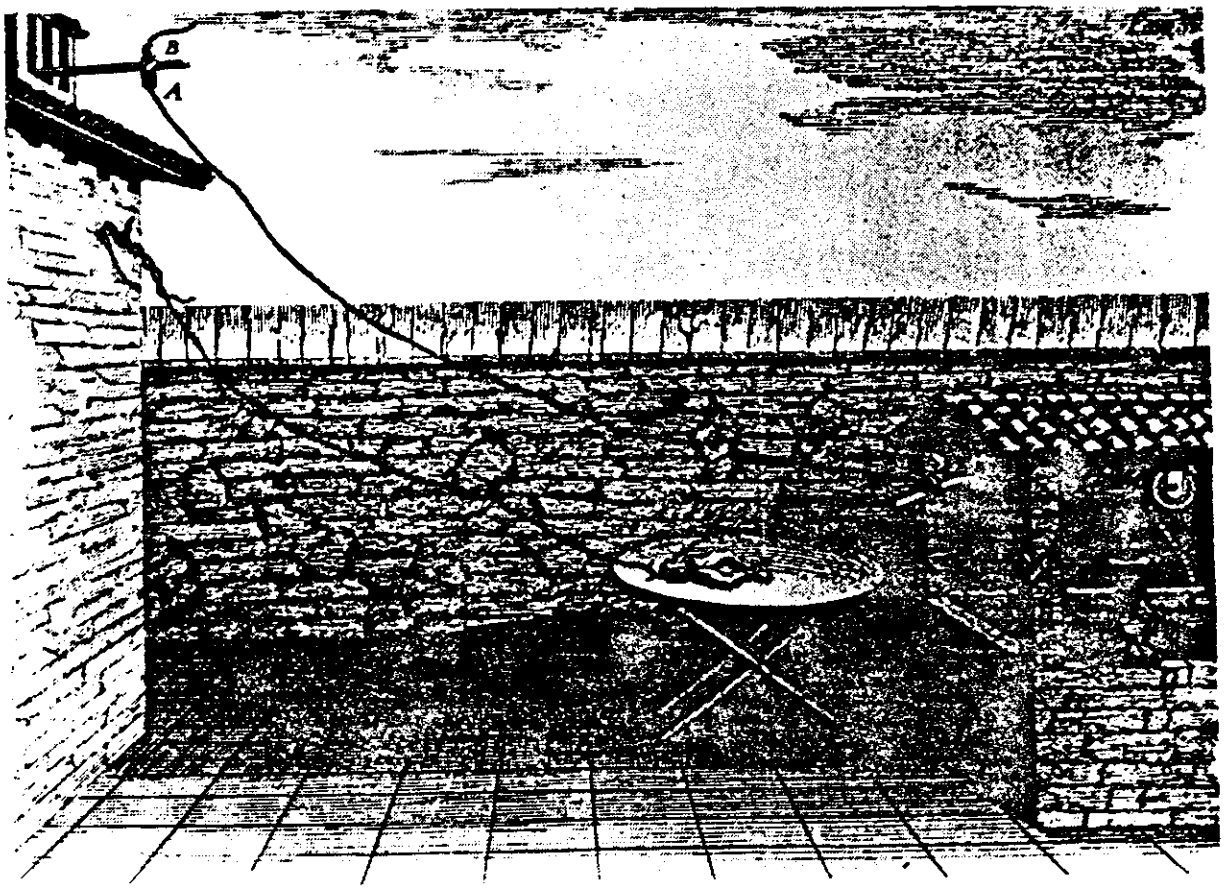
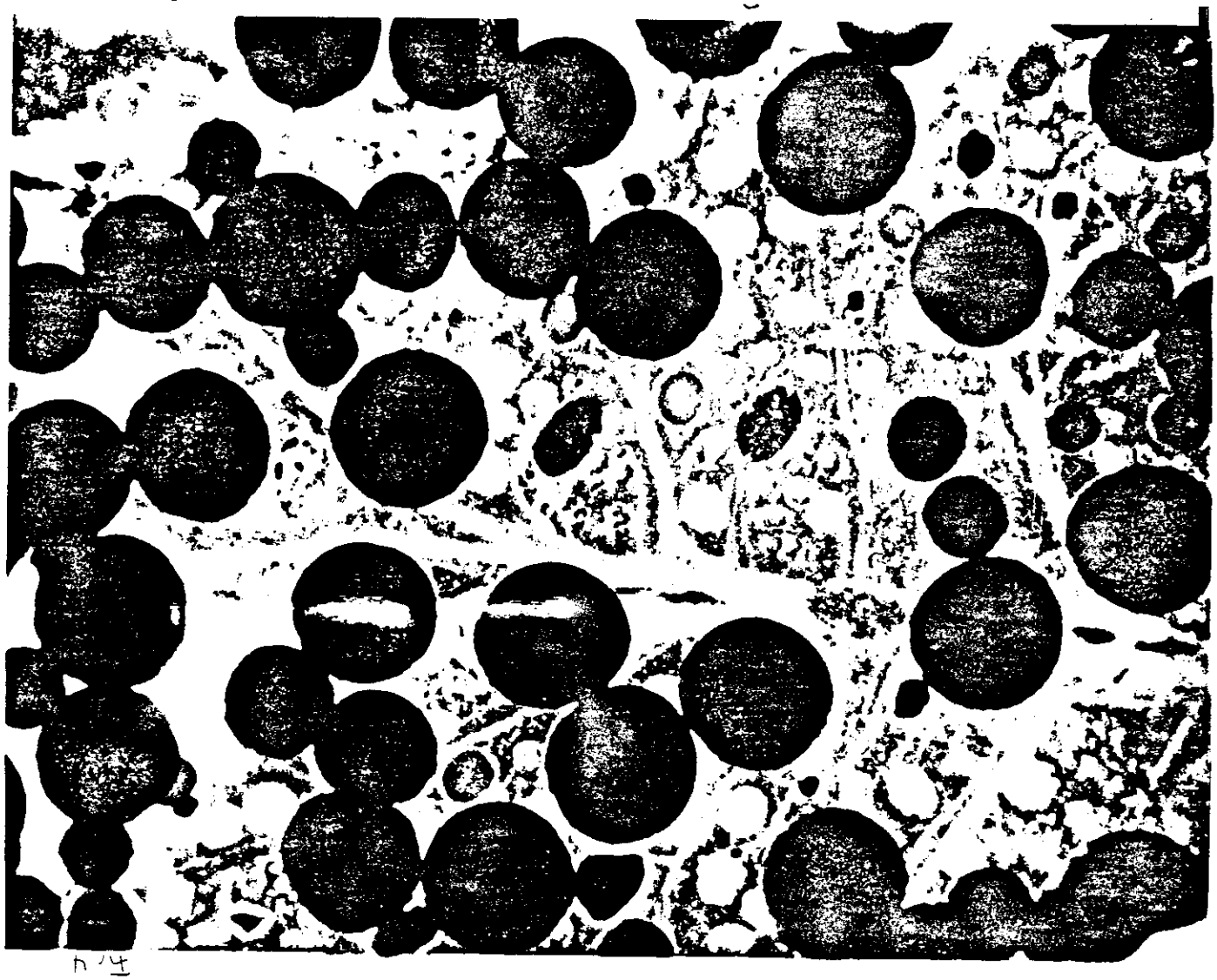
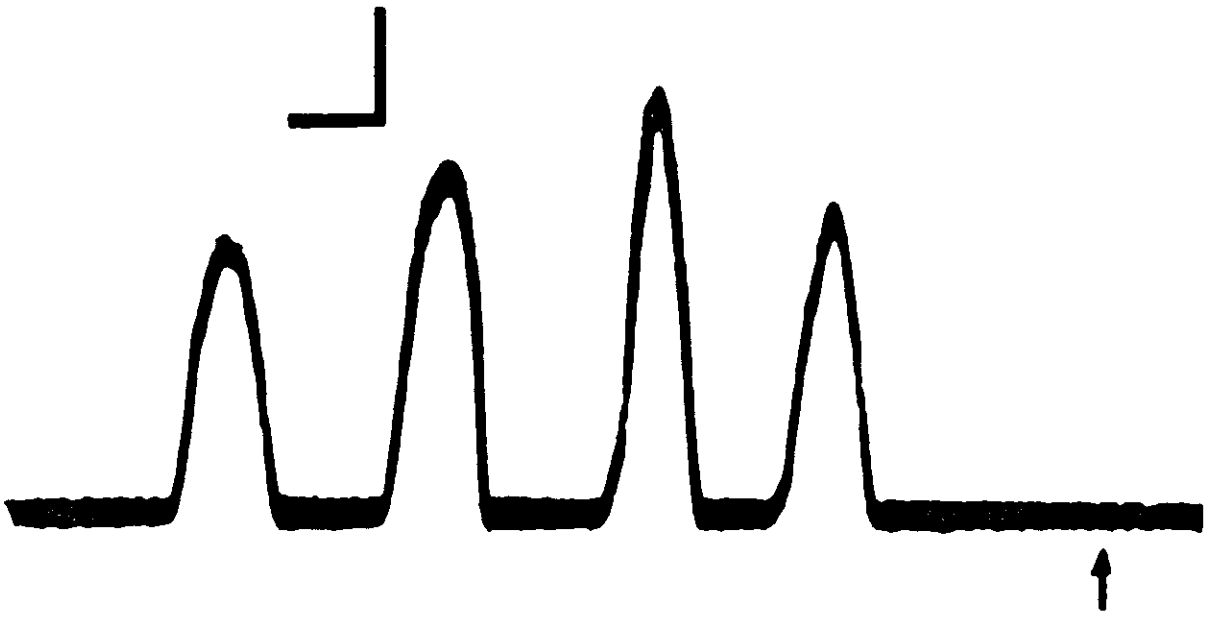
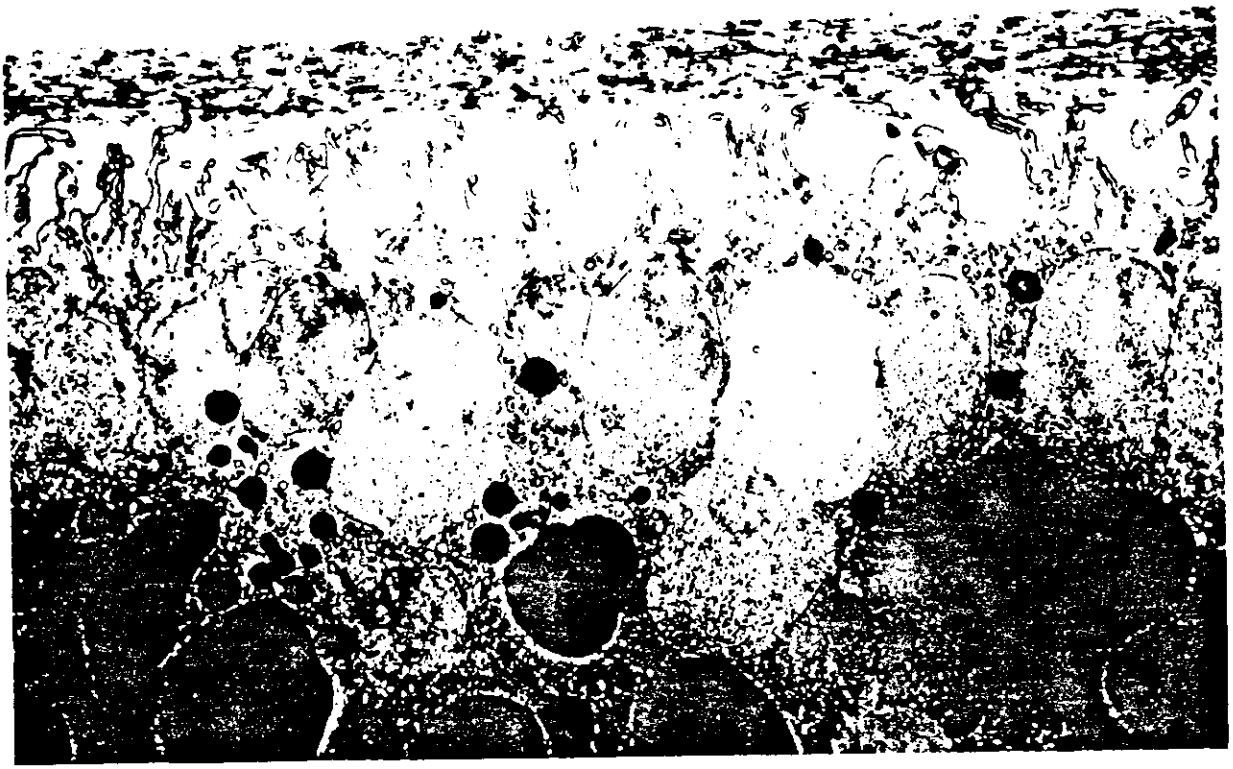



Fig 1







5B

Fig 5B

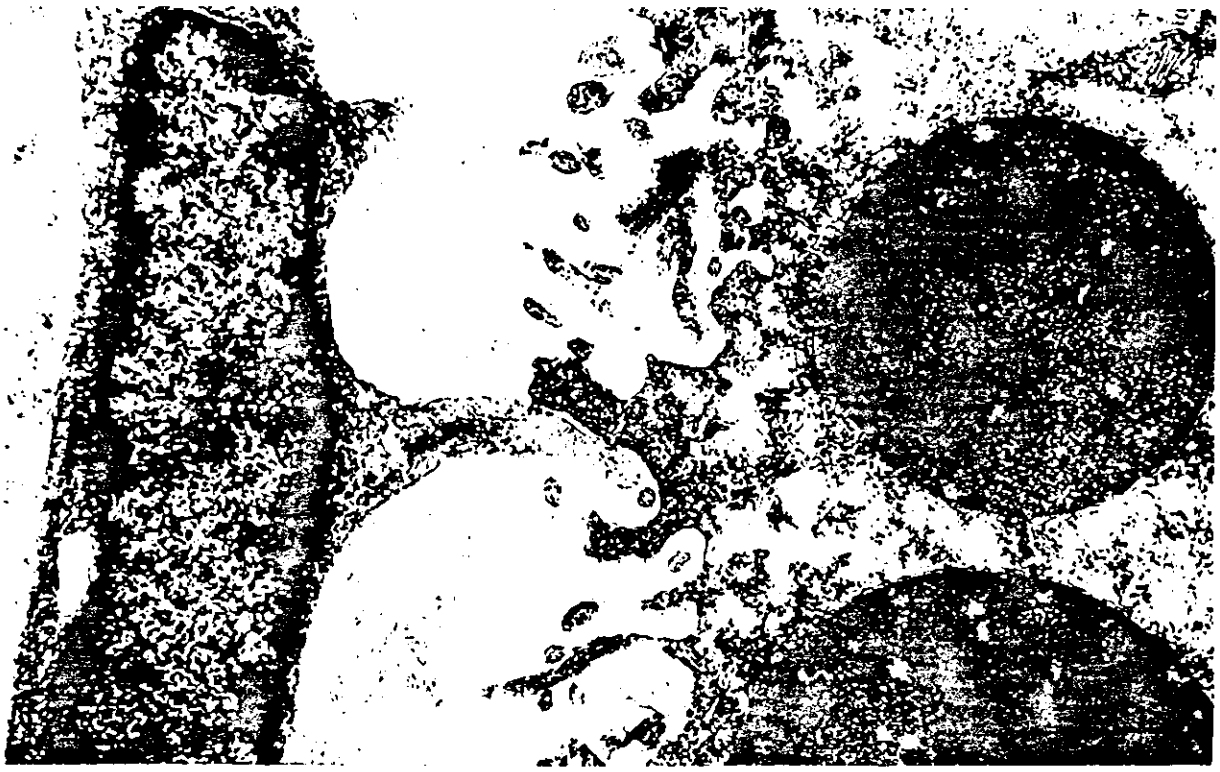
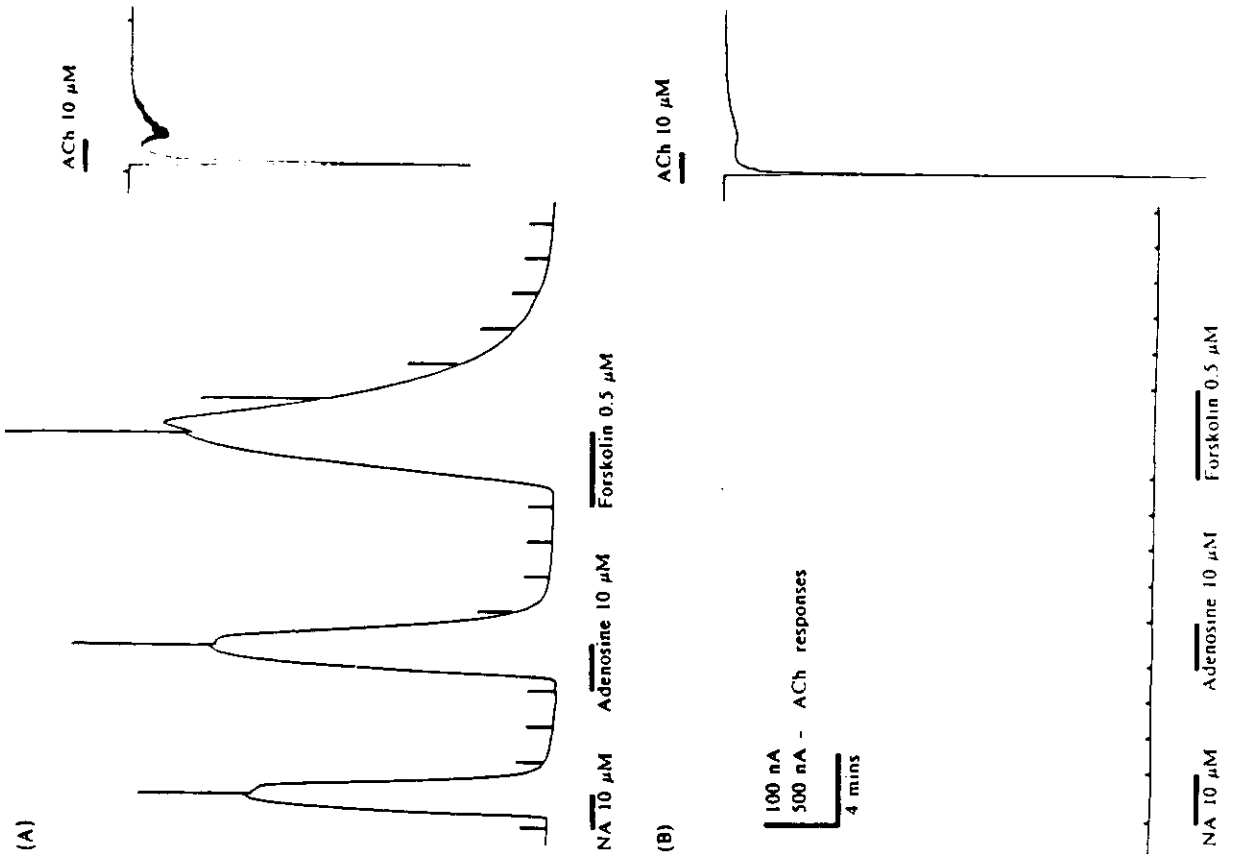
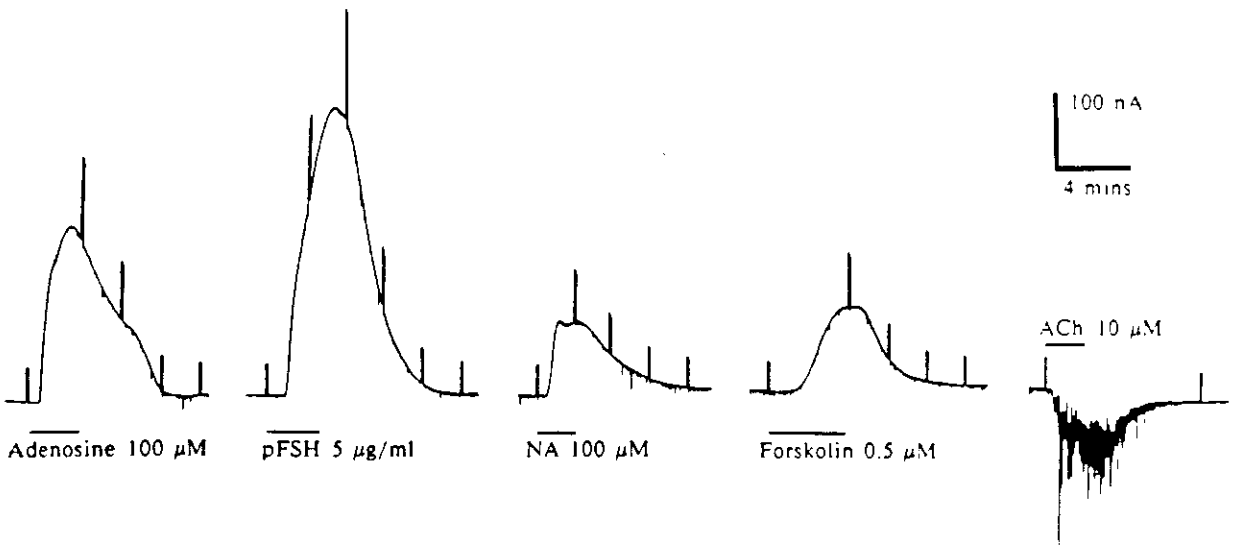


Fig 5

5A

Fig 5A


(A)
ACh 10 μM
NA 10 μM
Adenosine 10 μM
Forskolin 0.5 μM
(B)
ACh 10 μM
100 nA
500 nA - ACh responses
4 mins
NA 10 μM
Adenosine 10 μM
Forskolin 0.5 μM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Adenosine 100 μM
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Forskolin 0.5 μM

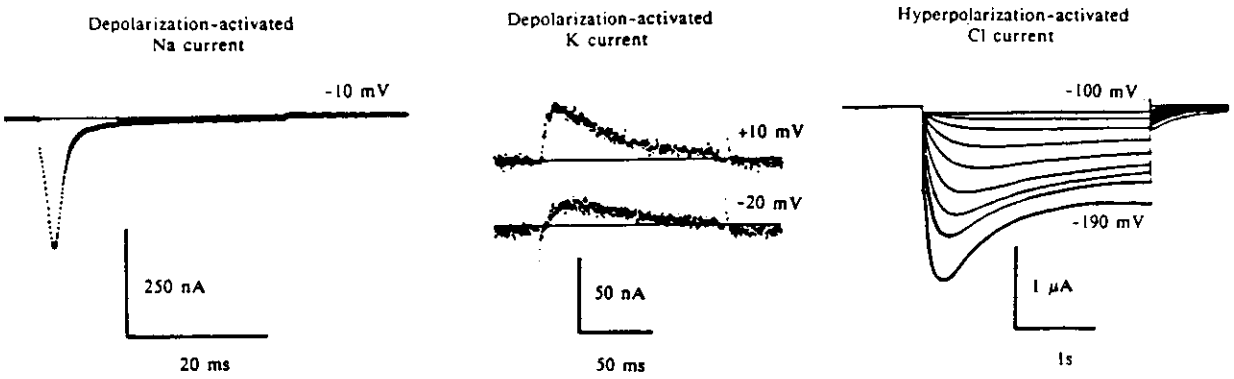



Fig 9

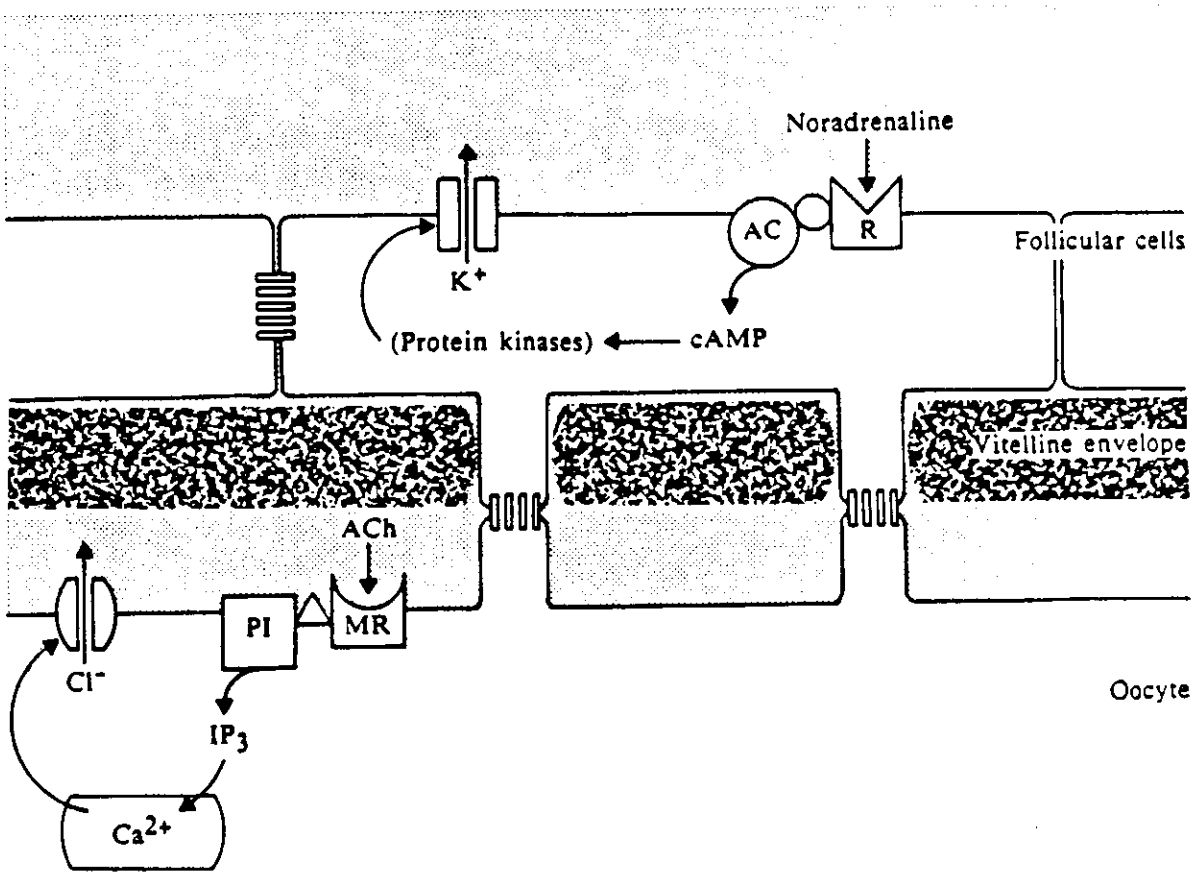
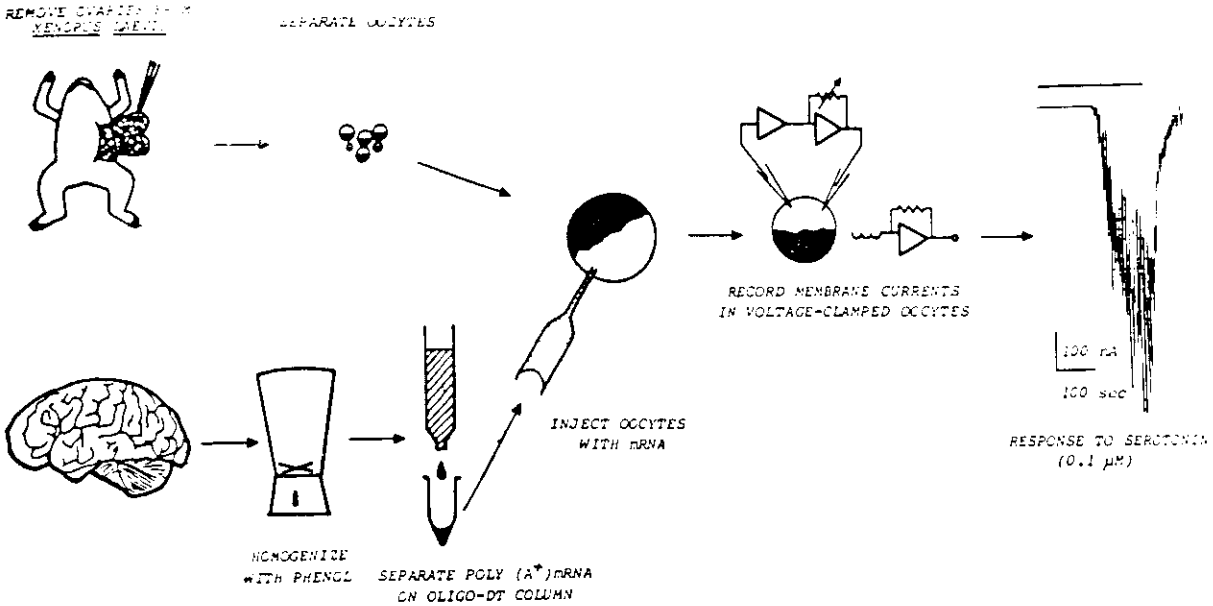
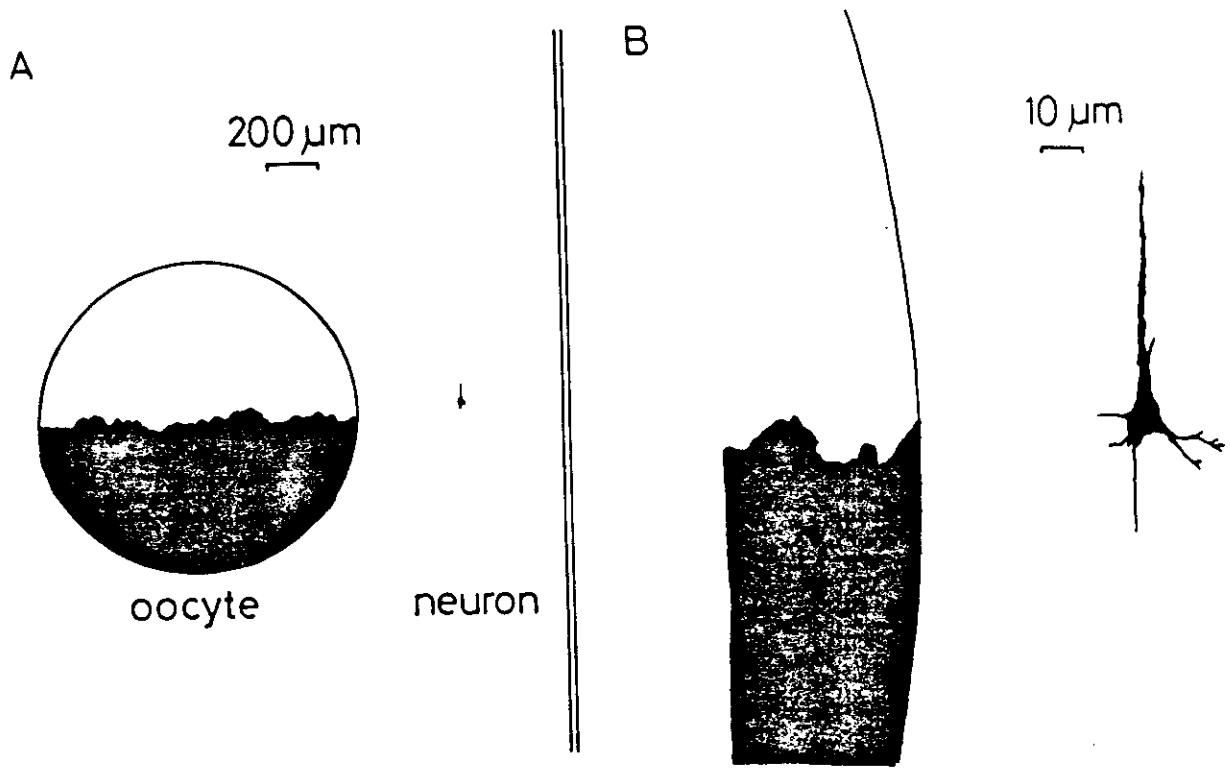



Fig 8

Fig 8


SEPARATE OOCYTES
RECORD MEMBRANE CURRENTS
IN VOLTAGE-CLAMPED OOCYTES
100 nA
100 sec
RESPONSE TO SEROTONIN
(0.1 μM)
INJECT OOCYTES
WITH mRNA
HOMOGENIZE
WITH PHENOL
SEPARATE POLY (A+)mRNA
ON OLIGO-DT COLUMN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200 μm
oocyte
neuron
B
10 μm

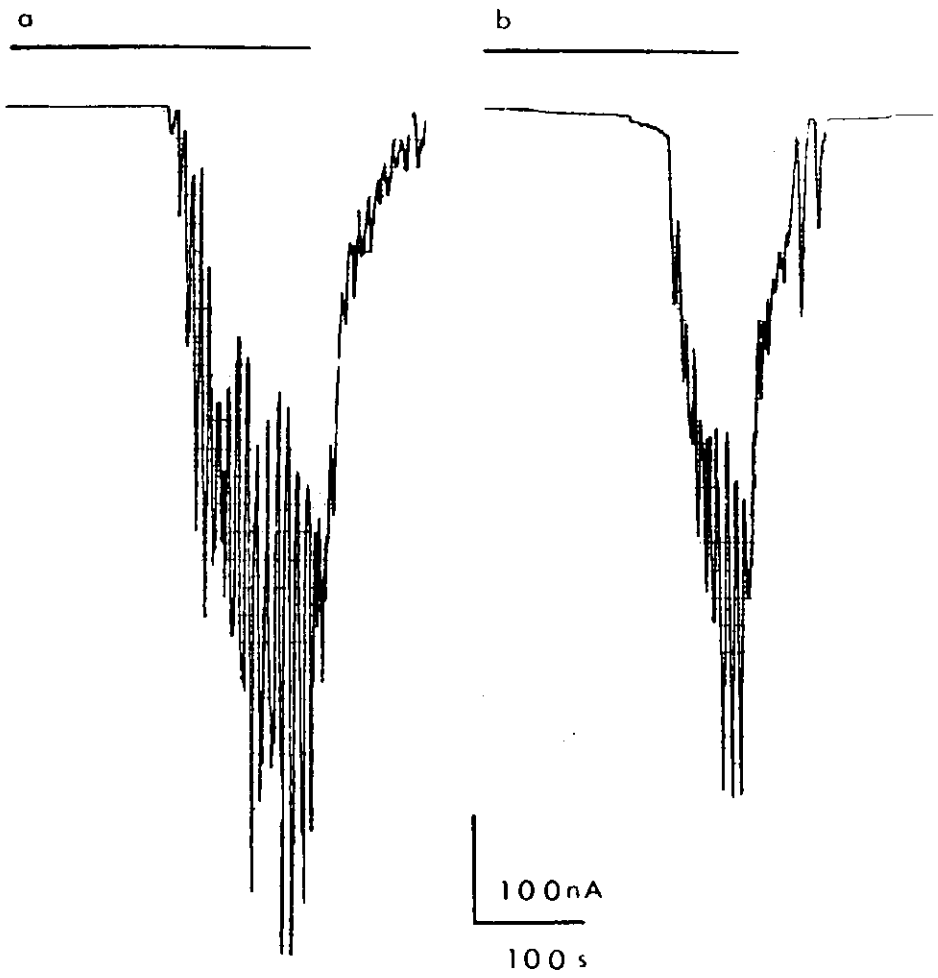



Fig 12

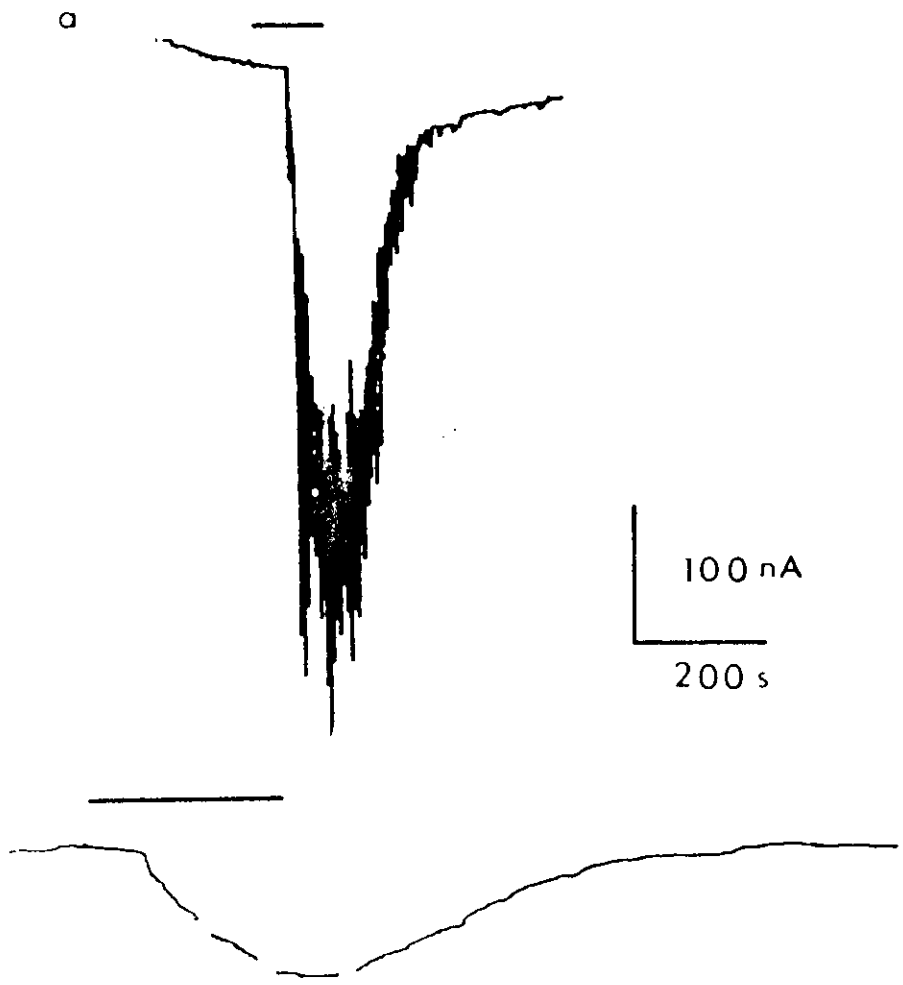
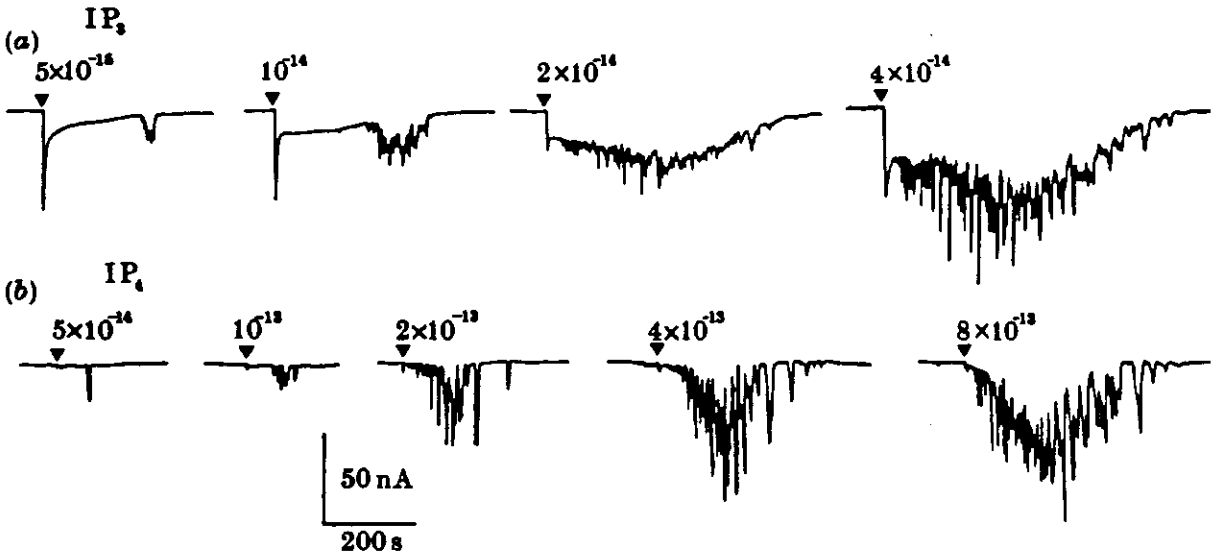
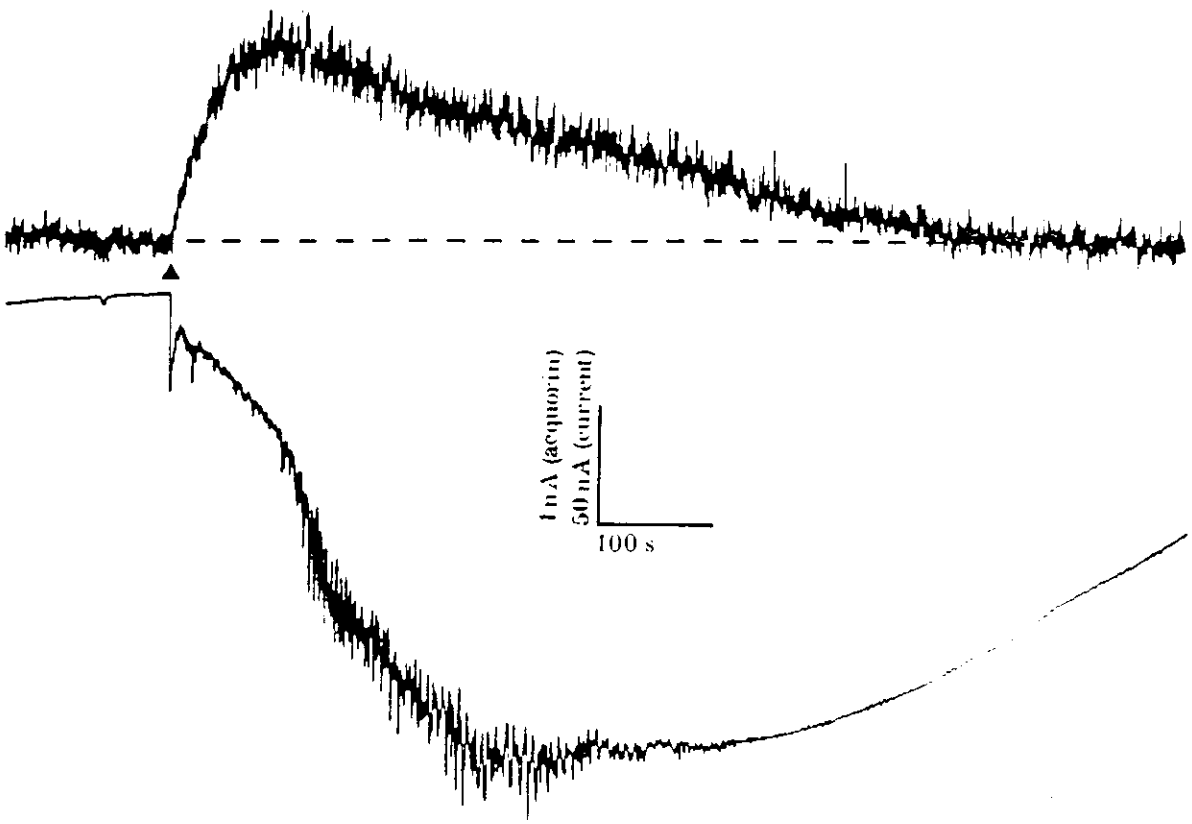
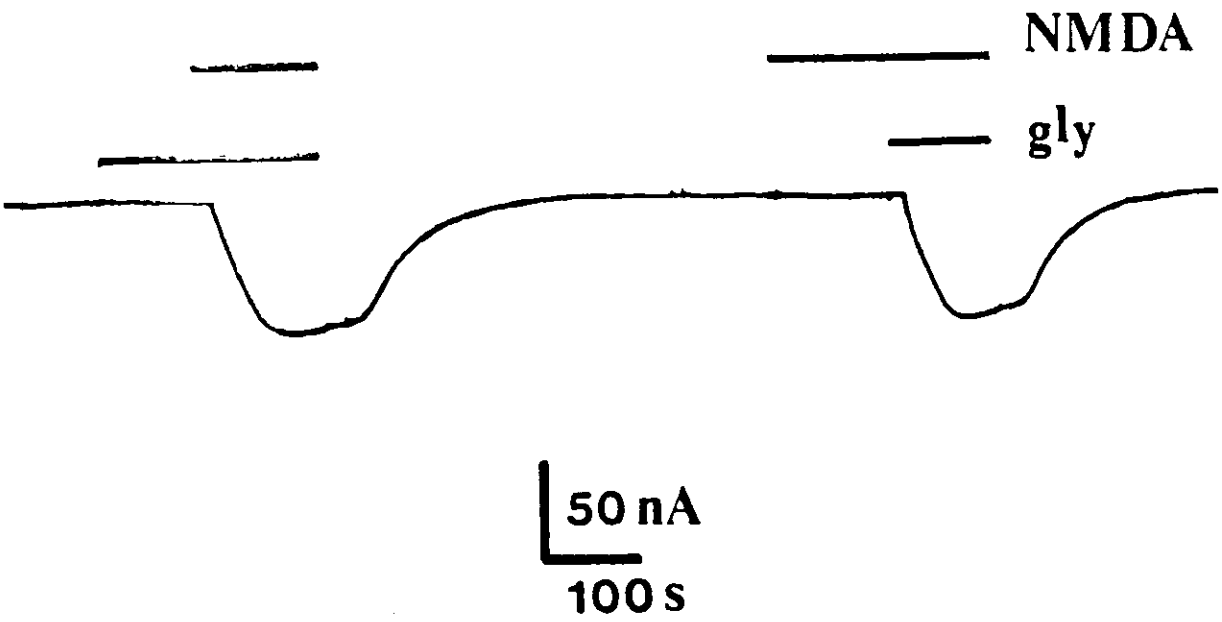
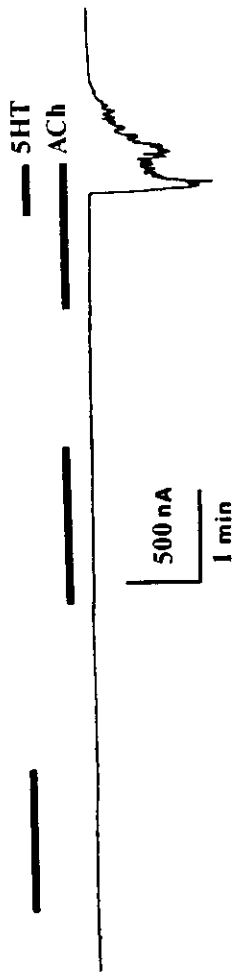


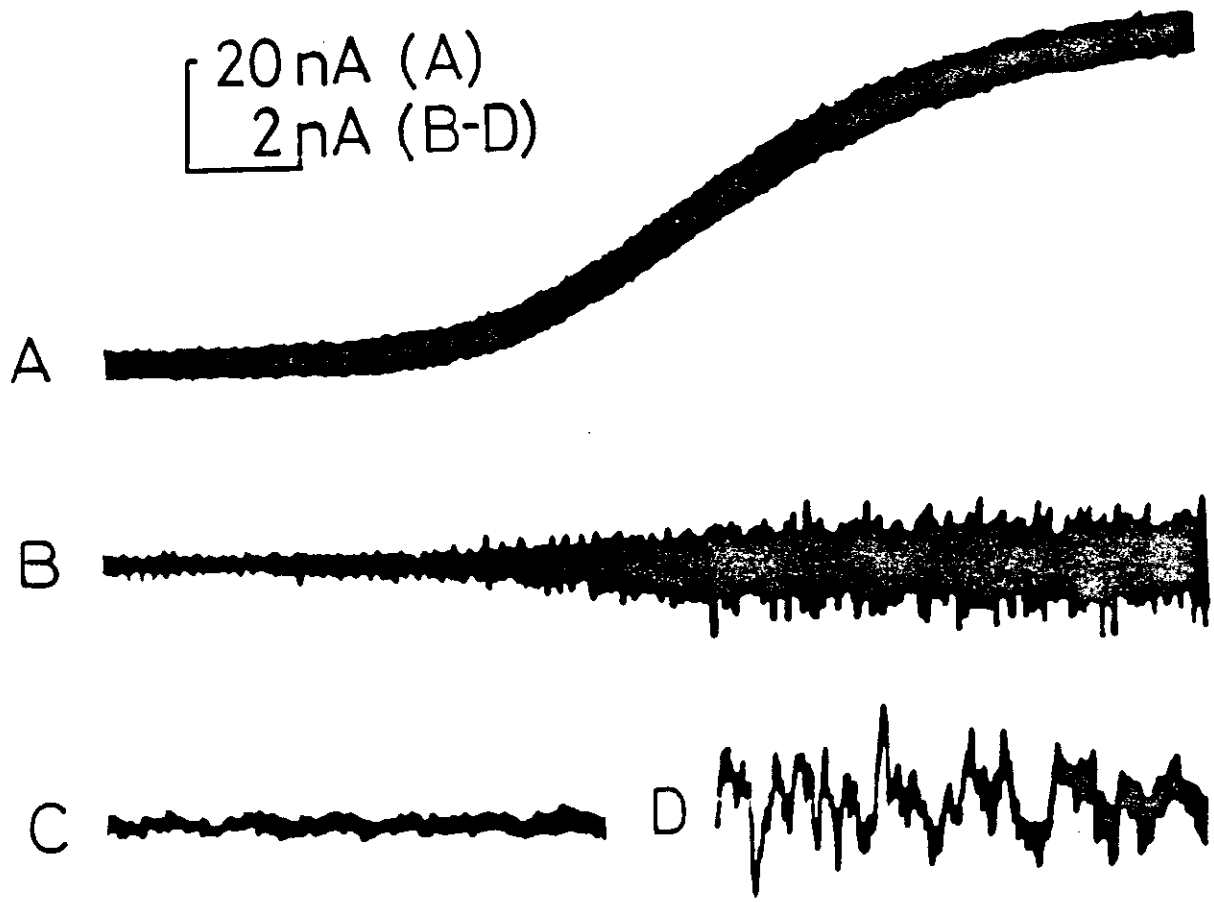

Fig 13

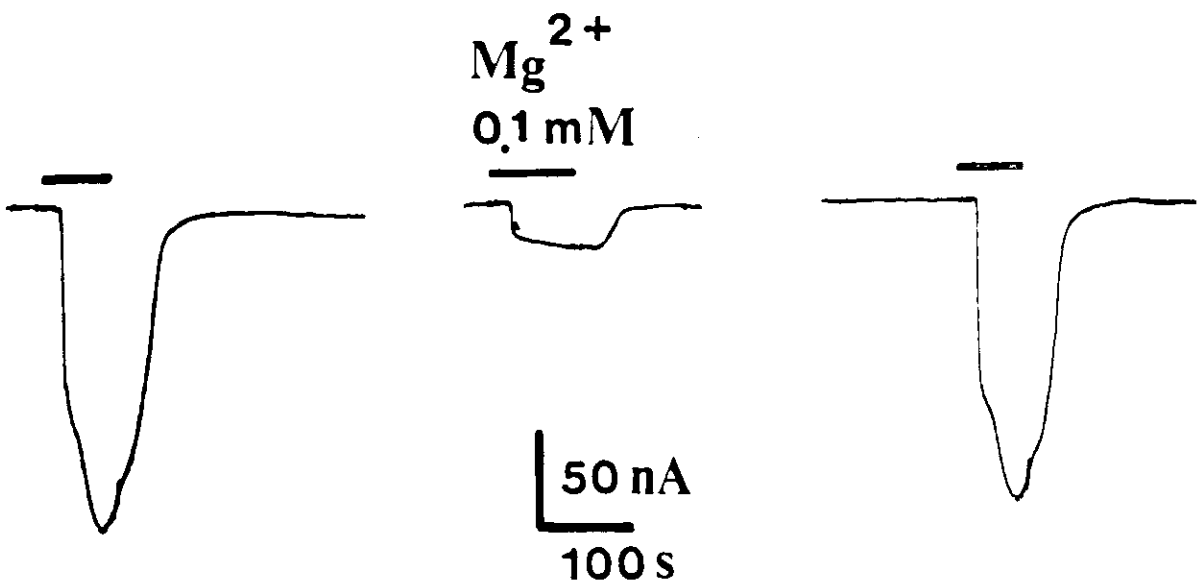

(a)
IP3
5×10-15
10-14
2×10-14
4×10-14
(b)
IP4
5×10-14
10-13
2×10-13
4×10-13
8×10-13
50 nA
200 s

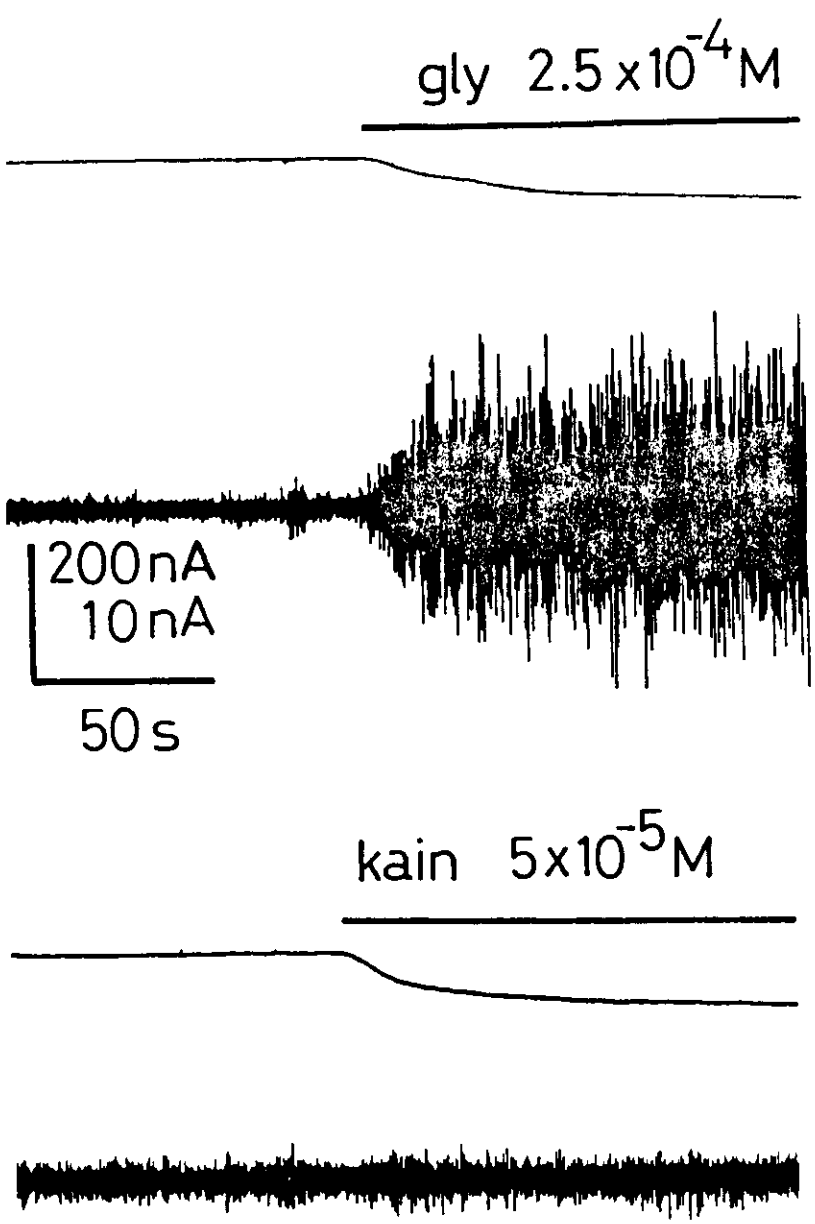

1 nA (aequorin)
50 nA (current)
100 s


NMDA
gly
50 nA
100 s


5HT
ACh
500 nA
1 min


20 nA (A)
2 nA (B-D)
A
B
C
D


Mg 2+
0.1 mM
50 nA
100 s



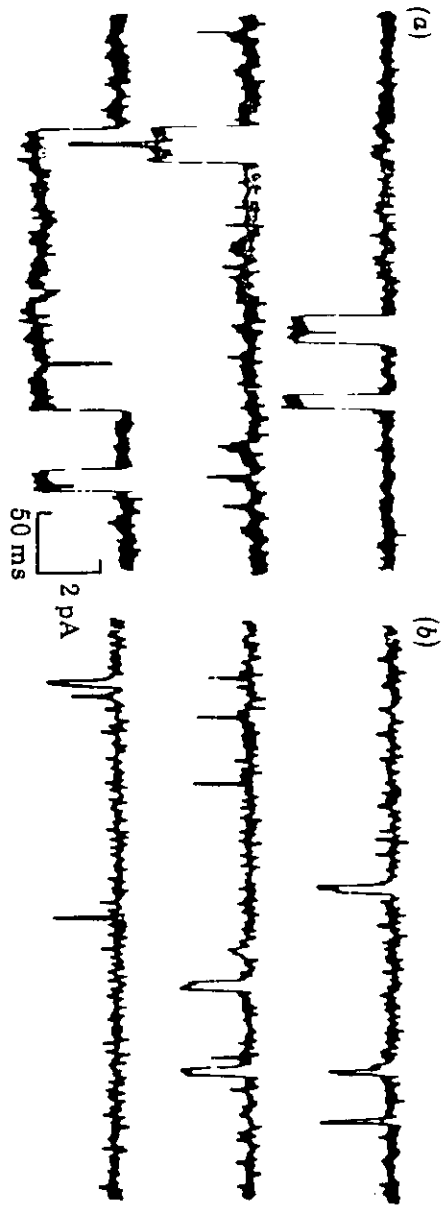
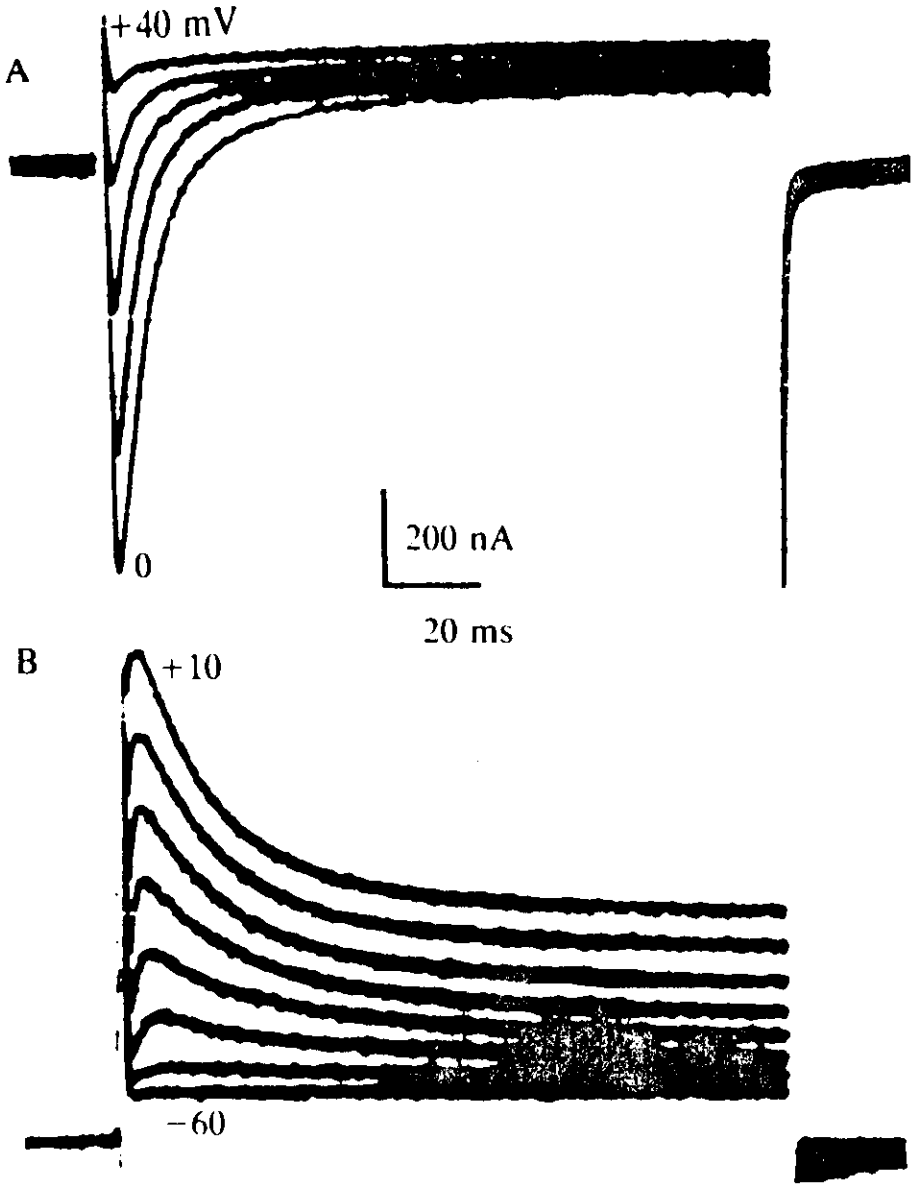
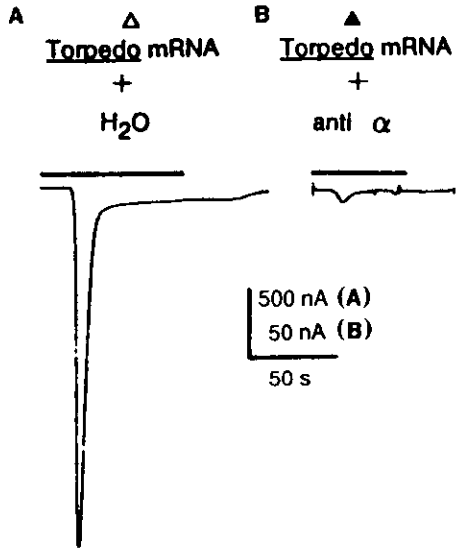
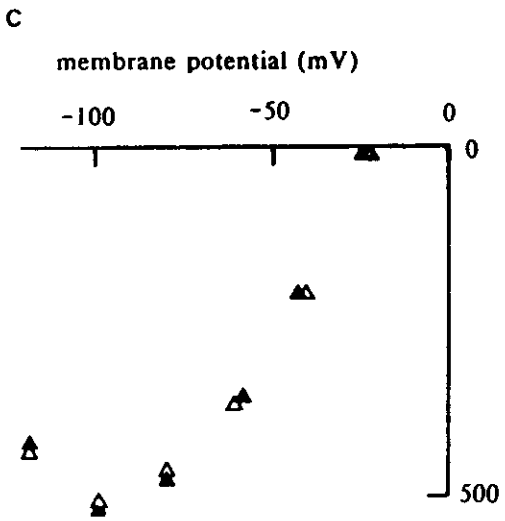




Fig 20


A
+40 mV
0
200 nA
20 ms
B
+10
−60


A
Torpedo mRNA
+
H2O
B
Torpedo mRNA
+
anti α
500 nA (A)
50 nA (B)
50 s


C
membrane potential (mV)
-100
-50
0
0
500
current (nA)

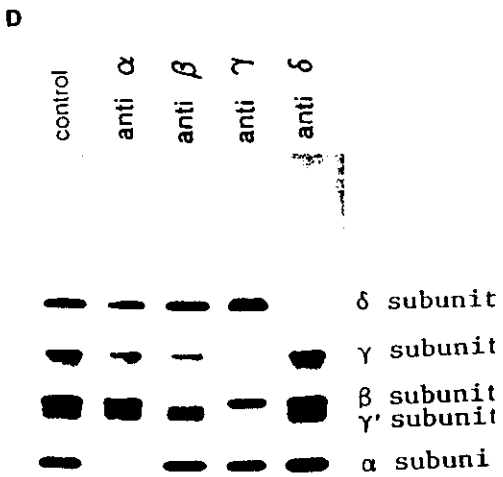
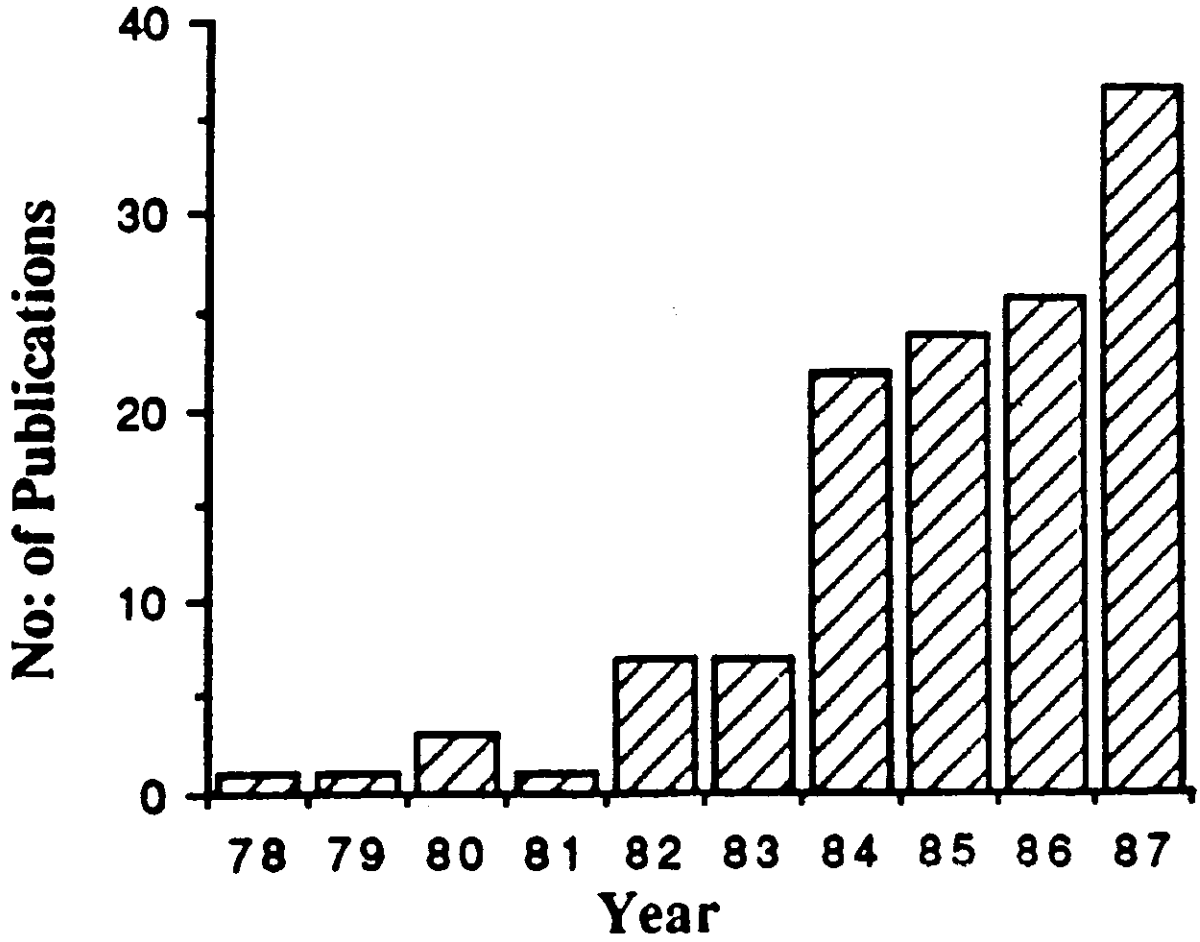

D
control
anti α
anti β
anti γ
anti δ
δ subunit
γ subunit
β subunit
γ' subunit
α subuni

Fig. 23


No: of Publications
40
30
20
10
0
78
79
80
81
82
83
84
85
86
87
Year