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Xenopus oocytes as a tool for molecular cloning of the genes coding for neurotransmitter receptors and voltage-operated channels

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Application of the methods of molecular genetics to the nicotinic acetylcholine receptor (AChR) has given a deep insight into the structure of this multi-subunit receptor (Noda et al., 1982, 1983a, b; Devillers-Thiery et al., 1983; Claudio et al., 1982; Sumikawa et al. 1982, 1983; Mishina et al., 1984, 1985; White et al., 1985). There is every reason to suppose that these techniques will prove equally as powerful for study of the many different neurotransmitter receptors in the brain, providing that it is first possible to isolate the genes coding for the receptors.

Well established methods are available for the construction of clone libraries containing cDNA copies of mRNA (Maniatis et al., 1982). Thus, in principle, any gene coding for the neurotransmitter receptors or channels can be isolated from such a library, if a method for screening is available. Conventional techniques for cloning involve first the purification of the desired protein, and partial determination of its amino acid sequence. This is then used to prepare probes, such as antibodies or oligonucleotides, which are used to screen the libraries for their receptor or channel genes.

However, the low abundance of many neurotransmitter receptors and ion channels in the brain makes difficult the preparation of suitable probes. Instead, we are using a novel approach to screen a cDNA library for the presence of receptor or channel genes. This involves the translation of mRNA in *Xenoopus* oocytes followed by electrophysiological recording from the oocytes. *Xenopus* oocytes have been shown to be a highly efficient translation system of microinjected heterologous mRNA, and are able to faithfully execute posttranslational processes such as glycosylation, sequestration and secretion of appropriate products (Gurdon et al., 1971; Lane, 1983). Moreover, we have used this system to translate mRNAs coding for neurotransmitter receptors. For example, oocytes injected with poly(A)$^+$ mRNA extracted from *Torpedo* electric organ (the richest known source of the nicotinic AChR) efficiently assembled multi-subunit AChR which showed properties characteristic of the native AChR (Sumikawa et al., 1981). Furthermore, experiments using electrophysiological techniques revealed that the foreign receptor subunits are inserted into the oocyte's surface membrane where they form functional receptors (Barnard et al., 1982). We have also shown that the oocyte system could be used to monitor the purification of mRNA which was subsequently used to clone the gene coding for the α subunit of the AChR (Sumikawa et al. 1982; Sumikawa et al., 1983).

To see if the oocyte membrane was also able to acquire *de novo* some of the tansmitter

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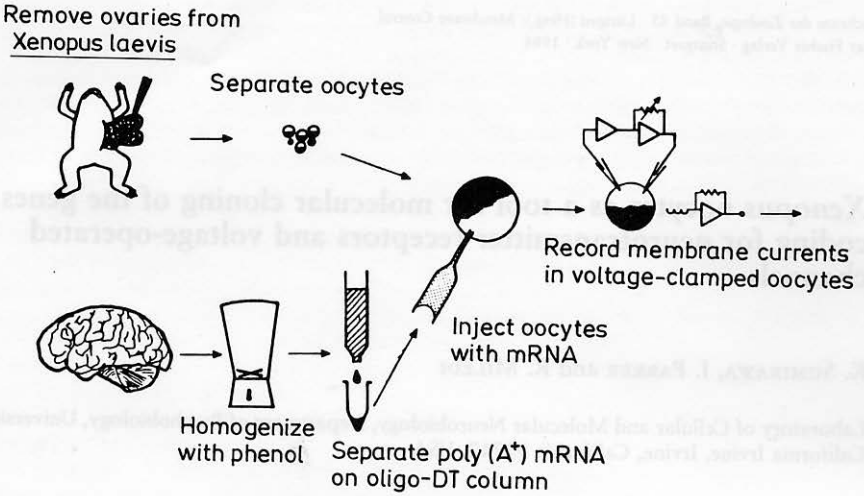

Fig. 1: Stages in the ‹transplantation› of neurotransmitter receptors into oocytes by the isolation and injection of mRNA from brain.

receptors which operate in the brain, we injected the oocytes with brain mRNA. The method used is shown diagramatically in Fig. 1. Essentially, poly $(A)^+$mRNA was extracted from the brain and injected into *Xenopus* oocytes. We, and subsequently others, found that the foreign mRNA was translated by the oocytes own protein synthesizing machinery, and that the products were correctly processed and incorporated into the oocyte membrane where they formed receptor-channel complexes that functioned like those in the cells from which the mRNA was extracted. Using this method oocytes have been induced to acquire most of the known receptors to transmitters, as well as the voltage operated channels (Miledi et al., 1982, 1983; Gundersen et al., 1983a, b, 1984a–c; Sumikawa et al., 1984a, b; Houamed et al., 1984). Thus, *Xenopus* oocytes provide us with a very sensitive and specific detector of mRNAs coding for neurotransmitter receptors and voltage operated channels and can, therefore, help us to purify these mRNAs. These can then be used for the construction of cDNA libraries as well as for screening for the genes coding for these proteins. We described here the initial step in our cloning strategy for neurotransmitter receptors and voltage operated-channels, that is the purification of mRNAs coding for these proteins.

Partial purification of mRNA coding for voltage-operated sodium and potassium channels

Xenopus oocytes that have been injected with whole poly$(A)^+$mRNA extracted from chick, rat or human brain acquire large numbers of Na^+ and K^+ channels, which are activated when the oocyte membrane is depolarized (Gundersen et al., 1983b, 1984c). The content of specific mRNAs encoding voltage-operated channels can be assayed by measuring the maximal current elicited when the membrane of the poly$(A)^+$mRNA injected oocytes is depolarized. As Fig. 2 shows, the amplitude of the membrane current induced by membrane depolarization varies with the amount of mRNA injected and is

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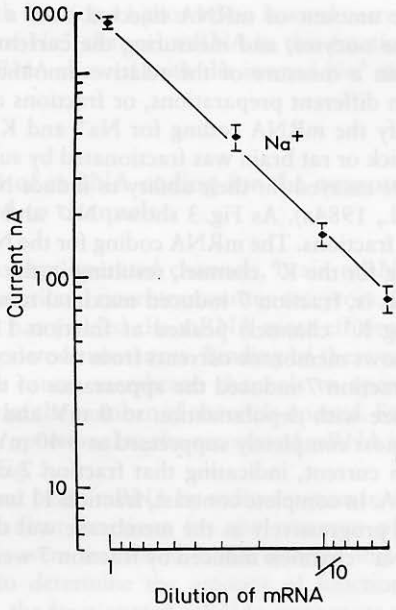

Fig. 2: Relation between amount of mRNA injected and mean size of voltage-operated sodium current, in oocytes injected with chick brain mRNA. Sizes of sodium currents were measured with depolarization to −10 mV.

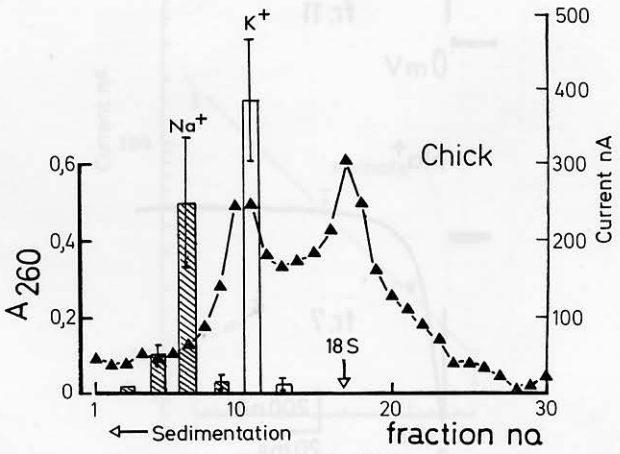

Fig. 3: Sedimentation profile of poly(A)$^+$ mRNA and profiles of voltage-activated responses induced by mRNA fractions derived from chick brain. Open columns represent the size of potassium currents induced by the corresponding fractions, shaded columns the size of sodium currents, and triangles the optical density of the messenger fractions at 260 nm.

tively, it may be that the mRNAs coding for proteins that constitute the second messenger system and the Cl^- channels exist in different sizes and are sedimented in many fractions, including fractions 9 to 13, which are the ones that led to expression of receptors which trigger oscillatory Cl^- currents.

There are several ways in which one can go forward towards the cloning of receptors and channels without their prior purification. For instance, the sucrose gradient fraction that best expresses a particular receptor or channel in the oocytes will be subjected to a second fractionation procedure, and the most purified mRNA fraction will be used to construct a cDNA library. The cDNA clones can then be screened by a hybridization-selected translation method (PARNES et al., 1981), except that the mRNA hybridized to cDNA clones will be released, and then assayed by translation in the oocytes and subsequent electrophysiological recording from the oocytes. The receptor or channel cDNA clones will be identified because the mRNA released from them should induce the appearance of receptors or channels in the oocytes. If a functional receptor or channel consists of a single subunit or several identical subunits, screening should be relatively straightforward. On the other hand if the functional receptor is made by different subunits coded by mRNAs of very different sizes the screening will be more difficult, but not impossible. If oscillatory responses arise through a separate transmitter binding protein, a Cl^- channel and a second messenger system, then the oocytes may be a very good system for the study of the corresponding receptors, because the native (non-injected) oocytes already contain the Cl^- channel and the second messenger system to which the transmitter binding protein could link up, resulting in expression of functional responses to glutamate, ACh, serotonin, dopamine or norepinephrine.

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directly proportional to the amount of mRNA injected over a large range. Thus, by translating the mRNA in the oocytes, and measuring the current elicited by membrane depolarization we can obtain a measure of the relative amount of a voltage-operated channel mRNA contained in different preparations, or fractions of mRNA.

In order to partially purify the mRNA coding for Na^+ and K^+ channels, total poly-$(A)^+$mRNA derived from chick or rat brain was fractionated by sucrose gradient centrifugation and the fractions were assayed for their ability to induce Na^+ and K^+ channels in the oocytes (Sumikawa et al., 1984a). As Fig. 3 shows, Na^+ and K^+ channels were synthesized by different mRNA fractions. The mRNA coding for the Na^+ channel sedimented much faster than that coding for the K^+ channel, resulting in the complete separation of the two mRNA species. That is, fraction 7 induced maximal numbers of Na^+ channels, while the mRNA expressing K^+ channels peaked at fraction 11. Sample currents are illustrated in Fig. 4, which shows membrane currents from two oocytes injected with either fraction 7 or fraction 11. Fraction 7 induced the appearance of the inward Na^+ current alone, which increased in size with depolarization to 0 mV and then declined at more positive potentials, being almost completely suppressed at +40 mV. Even at this potential there was no fast potassium current, indicating that fraction 7 contained no detectable amount of K^+ channel mRNA. In complete contrast, fraction 11 induced only the outward K^+ current, which increased progressively as the membrane was depolarized. At about 0 mV, at which potential the Na^+ channels induced by fraction 7 were maximally activated,

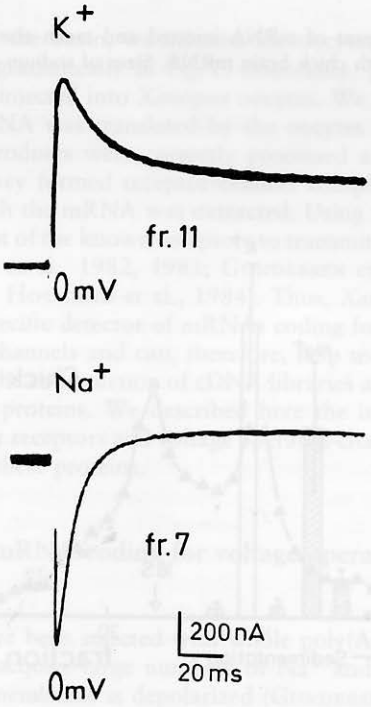

Fig. 4: Voltage-operated sodium and potassium currents recorded from two oocytes injected with fraction 11 (top) and fraction 7 (bottom) mRNA from chick brain. In this, and all other figures, downward deflexions correspond to inward membrane currents.

channels expressed are coded by a single mRNA species, or by several mRNAs of similar size.

It is interesting to note that the mRNAs coding for GABA, glycine and β-alanine receptors, all of which mediate synaptic transmission by opening Cl^- channels, sedimented in separate fractions. Fraction 13 gave responses to glycine and β-alanine but not GABA, while fraction 11 gave larger responses to GABA than to glycine, and a similar response to β-alanine. Thus, it seems very likely that GABA, β-alanine and glycine binding sites are on different proteins coded by separate mRNAs, but we still do not know whether their associated Cl^- channels are formed by the same proteins that carry the receptor sites, or by proteins coded by a separate mRNA.

It is known that barbiturates and benzodiazepines potentiate GABA mediated synaptic transmission in the central nervous system (Haefely et al., 1979; Barker et al., 1978). Since the responses to GABA in oocytes injected with rat brain mRNA fraction 11 were potentiated by barbiturates and benzodiazepines, it follows that the GABA receptor complex induced by this mRNA fraction must consist of a functional assembly, containing binding sites for GABA, modulating drugs and their associated Cl^- channels. This suggests that the proteins constituting the GABA receptor assembly are coded by one mRNA or by separate mRNAs of very similar sizes.

The Na^+ channel purified from brain has been shown to be an oligomeric protein with the largest subunit having a molecular weight of 260,000 (Hartshorne and Catterall, 1984). This agrees with our finding that the mRNA coding for Na^+ channel, which migrated near the bottom of the gradient, is the largest among the mRNAs coding for receptors and channels. In addition to the 260,000 subunit, the Na^+ channel contains two other subunits with molecular weights of 39,000 and 37,000 (Hartshorne and Catterall, 1984). It is thought that these subunits are coded by smaller mRNAs than that coding for the 260,000 subunit. Thus, it is likely the chick brain mRNA fraction 7 and rat brain mRNA fraction 5, both of which express functional Na^+ channels (Sumikawa et al. 1984), contain only large mRNAs, including that coding for the 260,000 subunit, but not mRNAs for the smaller subunits. This would suggest that only the largest subunit is required to form a functional Na^+ channel, or if one or both of the two small subunits are required, then the mRNAs coding for these subunits are usually large relative to the sizes of these proteins and are very similar in size to that for the heavy subunit.

It is interesting that most transmitters used here, except for kainate, open Cl^- channels. For instance, Cl^- channels are involved in the smooth responses to GABA, glycine and β-alanine, as well as in the slow oscillatory responses produced by ACh, serotonin, glutamate, dopamine and norepinephrine. The characteristics of the oscillatory membrane currents elicited by these different transmitters appear to be indistinguishable. However, the receptor molecules are entirely distinct, as shown by their very different pharmacological properties (Gundersen et al., 1984a), and because it was possible to partly separate the mRNAs coding for different receptors by size-sedimentation (Sumikawa et al., 1984a). Several characteristics of the oscillatory responses suggest that they are mediated by intracellular second messengers, which link receptor activation to channel opening. Furthermore, all these oscillatory responses are abolished when oocytes are injected intracellularly with the calcium chelating agent EGTA (Parker et al., 1985), suggesting that calcium may act as an intracellular second messenger in these receptor systems.

It is known that the native oocytes (not injected with mRNA) already contain a system that elicits oscillatory Cl^- currents, either spontaneously or during exposure to ACh (Kusano et al., 1982). Therefore, it could be that the mRNA injected into the oocytes merely causes the synthesis of transmitter binding proteins that link in to an already existing cascade that consists of an internal messenger system and Cl^- channels. Alterna-

the oocytes injected with fraction 11 showed no inward current, suggesting that there was no detectable amount of Na^+ channel mRNA in this fraction. Different to this, oocytes injected with whole mRNA showed both the inward Na^+ and outward K^+ currents.

Partial purification of mRNA coding for the neurotransmitter receptors that are directly coupled to channels

In addition to the voltage operated channels, brain mRNA induces the oocyte membrane to acquire also functional neurotransmitter receptors. Of the several animal species tested so far, rat brain has yielded the mRNA most efficient in expressing receptors to neurotransmitters and related substances. Binding of these substances with the receptors induced by the mRNA causes membrane channels to open, and the consequent ionic current gives a measure of the number of channels opened. Furthermore, the amplitude of the current is directly proportional to the amount of mRNA injected, as illustrated for the receptor to kainate in Fig. 5.

We fractionated the rat brain mRNA by sedimentation in a sucrose gradient (Fig. 6). In order to assay the gradient fractions for the presence of neurotransmitter receptor mRNAs, these fractions were translated in the oocytes and the oocytes were examined electrophysiologically to determine the amount of functional receptors expressed. As shown in Fig. 7 and 10, the fractionated mRNAs were more potent than whole mRNA in expressing the receptors. Figures 7 and 8 show that the mRNA species coding for kainate,

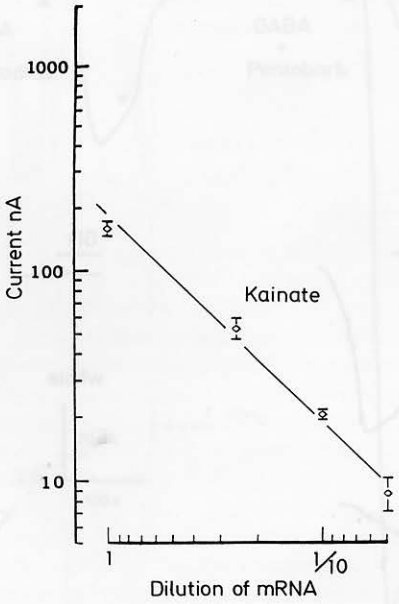

Fig. 5: Relation between amount of mRNA injected and mean size of kainate-activated currents in oocytes injected with rat brain mRNA. Responses were recorded to bath application of 10^{-4} M kainate, at a membrane potential of −60 mV.

The mRNA fractions which gave maximum responses to glutamate, serotonin or ACh (muscarinic) peaked in fractions 11, 9 or 13, respectively. The mRNA coding for the glutamate receptor was sharply sedimented, while the mRNAs coding for the other receptors were widely spread among different fractions (SUMIKAWA et al., 1984a). Subsequently, we found that dopamine and norepinephrine also elicited oscillatory Cl^- currents in oocytes injected with fractions 10 or 11 (Fig. 11). Thus, the mRNA species sedimenting in fractions 9 to 13 encode receptors that function by activating a system that generates oscillatory chloride currents.

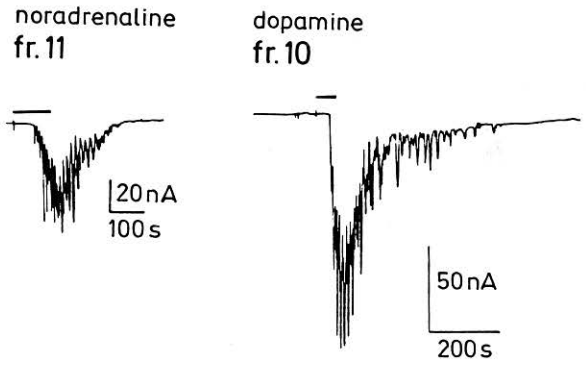

Fig. 11: Oscillatory chloride currents elicited by dopamine and noradrenaline in oocytes injected with the rat brain mRNA fractions indicated. Both agonists were bath applied at a concentration of 10^{-3} M. Clamp potential −60 mV.

Discussion

An important first step in receptor gene cloning is the partial purification of the mRNA coding for a receptor. This requires a sensitive and specific method to identify a particular mRNA fraction carrying the required mRNA. Our assay system, which involves the translation of mRNA in *Xenopus* oocytes followed by electrophysiological recording from the oocytes, requires the expression of functional receptors and channels, and this could pose considerable problems. For instance, a functional receptor could demand the expression of various protein subunits, or of a protein necessary to assemble the subunits into a functional receptor-channel complex. If these proteins were coded by messengers of very different length, then size fractionation of the mRNA could yield fractions incapable of expressing functional receptors. This was not the case, and the results obtained clearly demonstrate that fractionated mRNA is still effective in expressing many functional receptors and channels. In fact, we found that the fractionated mRNA was even more potent than total mRNA in expressing functional receptors and channels. For example, fractions 10 and 11 were capable of expressing norepinephrine and dopamine receptors, while whole mRNA hardly expressed these receptors (SUMIKAWA et al. 1984b). Moreover, expression of particular receptors or channels tended to be restricted to fractions containing messengers larger than 18S. Thus, our results suggest that the functional receptors or

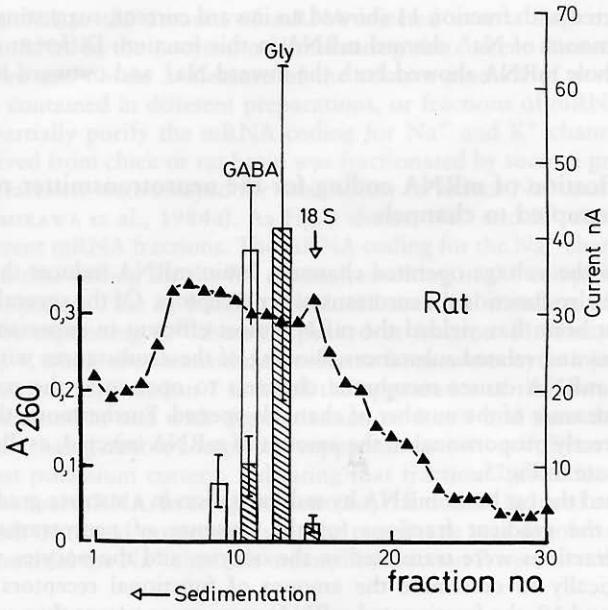

Fig. 6: Sedimentation profile at poly(A)$^+$mRNA and profiles of drug-activated responses induced by mRNA fractions derived from rat brain. Open columns represent size of GABA responses (1 mM) and shaded columns glycine responses.

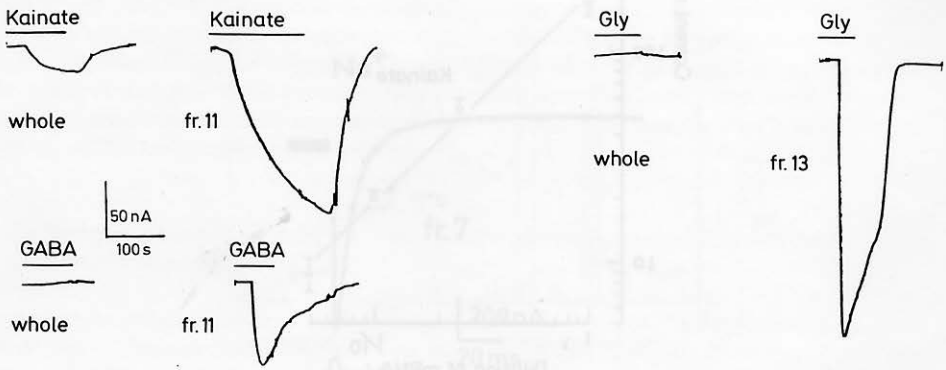

Fig. 7: Drug-activated currents recorded from oocytes injected with whole poly(A)$^+$ rat brain mRNA and fractions derived from the whole mRNA. Oocytes were voltage clamped at –60 mV, and drugs were bath applied at a concentration of 10^{-4} M kainate, and 1 mM GABA and glycine.

glycine, β-alanine or GABA, all of which elicit smooth inward membrane currents, were sedimented mainly in fractions 11 and 13, corresponding to mRNAs slightly larger than 18S rRNA. Oocytes injected with fraction 13 gave large responses to glycine and none to GABA, while oocytes injected with fraction 11 induced larger response to GABA than to glycine. The barbiturate pentobarbital and benzodiazepines potentiated the responses to GABA (Fig. 9), but not those to glycine.

Partial purification of mRNA coding for the receptors coupled to second messenger systems

The oocytes injected with total poly(A)$^+$mRNA expressed receptors to serotonin, muscarinic ACh and glutamate, all of which appear to be coupled to a second messenger system. The currents induced by these transmitters usually started after a long delay, showed oscillations and were carried largely by chloride ions. Screening of the different fractions for mRNA coding for these receptors indicated that the mRNAs coding for them are larger than 18S rRNA and here again the fractionated mRNA induced more receptors for these transmitters than the whole mRNA (Fig. 10).

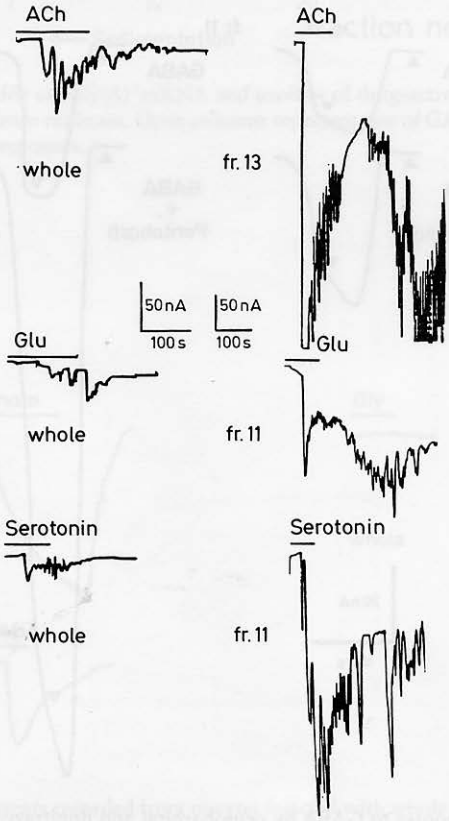

Fig. 10: Oscillatory chloride currents elicited in oocytes injected with the mRNA fractions indicated. ACh concentration 10^{-4}M, glutamate 10^{-5} M, and serotonin 10^{-7} M. Clamp potential −60 mV.

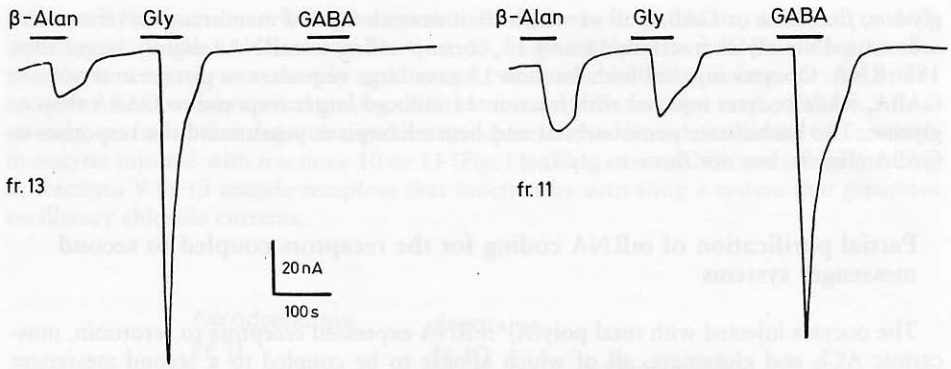

Fig. 8: Smooth chloride currents elicited by ‹inhibitory› amino acids in oocytes injected with rat brain mRNA fractions. Clamp potential was −60 mV. All drugs applied at a concentration of 1 mM.

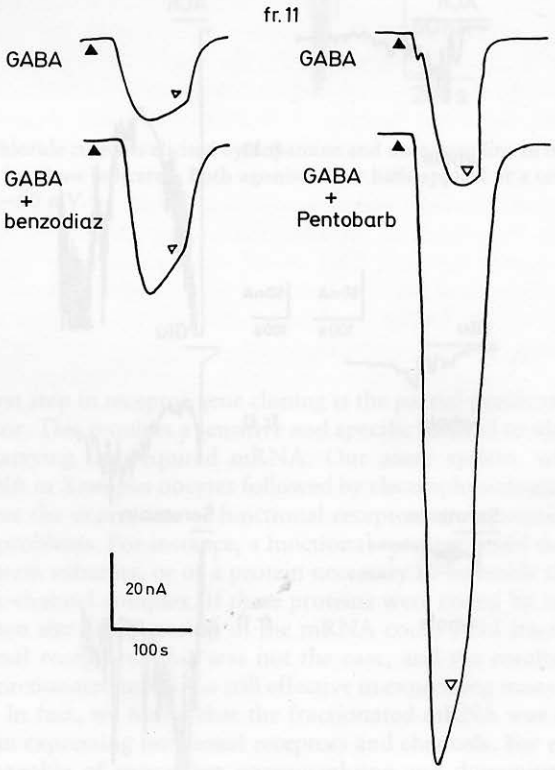

Fig. 9: Potentiation of responses to GABA by pentobarbital and flunitrazepam in an oocyte injected with rat brain mRNA fraction 11. Clamp potential was −60 mV. Drug concentrations were 10^{-5} M GABA, 10^{-4} M pentobarbital and 10^{-5} M flunitrazepam.