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Uncultured Lobster Muscle, Cultured Neurons and Brain Slices: the Neurophysiology of Zinc*

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The vertebrate central nervous system (CNS) is a repository of information continually received by neurons and effectively coded in the 'language of the nervous system', before being transmitted between individual cells in the form of electrical signals or action potentials. To understand precisely how individual neurons function in this network of cells, and how they are able to conduct and process action potentials, remains as one of the great challenges in neuroscience.

A close look at neuronal cell membranes will show that they are bristling with membrane-bound proteins which can have a considerable influence on the electrical excitability in the nervous system. These proteins can determine, for example, whether individual nerve cells respond to a depolarizing stimulus by 'firing' just a single action potential, or whether they react by producing bursts of action potentials, sometimes in a synchronized pattern. One major class of membrane-bound protein, which directly influences the electrical response characteristics of a cell to a direct stimulus, includes the neurotransmitter receptor and ion channel proteins. These proteins are capable of allowing ionic current to flow across the cell membrane, not only initiating, but also modulating action potentials and forming the very basis of neuronal excitability (Hille 1984; Bloom 1988; Llinas 1988; Nicoll 1988; Krueger 1989). Neurotransmitter receptor proteins can form, or be associated with, individual ion channels, which can then be modulated in many different ways by other ligands, proteins, or by 'messenger' molecules within or outside the cell, and also by the electric field or membrane potential across the cell membrane (Catterall 1988; Levitan 1988) (Fig. 1).

After much research, ion channels are now being classified, partly according to their functional operation. Some channels are operated by voltage alone, a depolarization or hyperpolarization can simply open or close an ion channel or operate a more subtle form of modulation by controlling the amount of time a channel can remain open in a conducting state (Catterall 1988; Jan & Jan 1989). Other channels require the presence of a ligand (or neurotransmitter) for activation or even deactivation (Hille 1984), whilst yet further examples of ion channels can be modulated by different types or classes of membrane proteins such as guanine nucleotide binding proteins (G proteins) which can be considered as 'signal transducers' following receptor activation (Gilman 1987). G proteins can be activated by the binding of guanosine triphosphate (GTP) and influence ion channel function either directly (Brown & Birnbaumer 1988), or more usually, participate in a complex series of reactions resulting in the formation and release of second messengers, or activation of intracellular enzymes leading to phosphorylation of more proteins (Gilman 1987; Neer & Clapham 1988). Typical second messengers and enzymes which have been implicated in modulating ion channel function include calcium ions, inositol phospholipids formed following the induction of phosphoinositide turnover (Oron et al 1985; Higashida & Brown 1986; Fisher & Agranoff 1987; Sawada et al 1987), metabolites of the arachidonic acid cascade system (Piomelli et al 1987), protein kinases (DeRiemer et al 1985; Higashida & Brown 1986; Doerner et al 1988; Nicoll 1988) and many others (e.g. Dudai 1987; Gold & Nakamura 1987; Ashcroft 1988; Hemmings et al 1989).

It is now possible to appreciate that this broad array of receptor and ion channel proteins can provide a wide degree of functional diversification for a neuron to respond to excitatory or inhibitory stimuli. Note that these few examples of membrane-bound proteins are by no means totally exclusive.

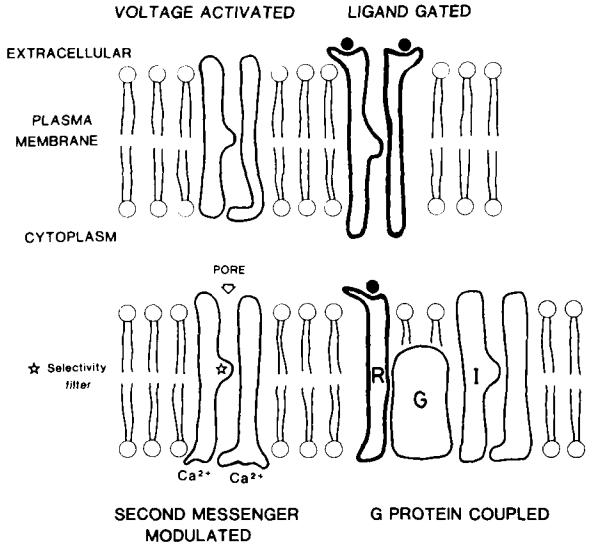

FIG. 1. Schematic representation of various types of membrane ion channel. Ion channels (I) are usually defined by the formation of a hydrophilic pore which 'runs' through a membrane protein that spans the thickness of the membrane. This enables the passage of ions across the lipophilic membrane at quite high transport rates. The type of ions which flow through the channel can be specified by the selectivity filter (☆) residing within the ion channel. The passage of ionic current by ion channels can be modulated in various ways, including controlling the opening or closing of the conducting pore with membrane voltage changes, activation of the channel by the presence of a specific ligand (●), activation/inactivation by intracellular messengers (e.g., calcium) and also direct, or indirect modulation, controlled by G proteins (G) following ligand activation of a receptor (R). This cartoon represents only a limited view of the number of possible ways of modulating ion channels and some ion channels are subject to more than one form of modulation.

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All these ion channel proteins can play an integral role in the passage and modulation of information transmitted in the form of digitized electrical signals (action potentials) around the nervous system. Because of their importance, these proteins have become prime evolutionary targets for natural toxins and venoms, and consequently, crucial sites for therapeutic drug action and the design of future agents.

One of the preferred methods for studying electrical events in the nervous system, is to directly record from single cells in in-vitro preparations. What I shall illustrate is an indication of some electrophysiological techniques and different types of neuronal preparation, currently used to study the properties and function of receptors and ion channels. These techniques will be used as a foundation to describe how an endogenous substance in the brain, the heavy metal zinc, may interact with post-synaptic membrane proteins, in particular a receptor protein which is sensitive to the major inhibitory neurotransmitter in the mammalian central nervous system, γ-aminobutyric acid (GABA) (Schwartz 1988), and perhaps have a profound influence on neuronal excitability in both health and disease states.

Distribution of zinc in the mammalian brain

Zinc is not ubiquitously distributed throughout the CNS; it is concentrated into specific neuronal structures, usually nerve terminals, found principally within the hippocampus, cortex and pineal body (Crawford & Connor 1972; Donaldson et al 1973; Friedman & Price 1984). Within these nerve terminals, zinc can be actively uptaken (Wolf et al 1984; Wensink et al 1988) into synaptic vesicle-type structures throughout the entire telencephalon (cerebral hemispheres; Ibata & Otsuka 1969; Perez-Clausell & Danscher 1985; Holm et al 1988). Later experiments by three different groups utilized the hippocampal brain slice, which is a convenient neuronal preparation possessing discrete yet compact neuronal cell layers and some functional innervation (Seifert 1983). These experiments discovered that zinc could be released in a calcium-dependent manner from nerve terminals following stimulation of the mossy fibre pathway in the hippocampus using either electrical, high potassium or kainate-induced stimulation (Assaf & Chung 1984; Howell et al 1984; Charlton et al 1985). Assaf & Chung (1984) have estimated the likely zinc concentrations that may be attained in the extracellular space following stimulation, to be quite high, approximating 300 μM!

If zinc can be released into the synaptic cleft by nerve fibre stimulation in-vivo, then I believed it plausible that zinc might subsequently interact with pre- or postsynaptic membrane proteins. Zinc is a small cation (hydrated radius 0·44 nm) with a double positive charge ensuring a high field strength around the ion; these are excellent credentials for any ion postulated to interact with proteins. This interaction could occur by affecting ligand binding to neurotransmitter receptors, or perhaps by binding within and blocking the lumen of membrane ion channels. This type of modulation of receptors and ion channels could ultimately exert considerable control over neuronal excitability.

Why look at the interaction of zinc with a GABA receptor?

Current and voltage-clamp recording methods

To understand why I chose to study the effect of zinc on a GABA receptor, it is necessary to go down the evolutionary tree to the invertebrate species and in particular, the invertebrate muscle GABA receptor. At that time, I was interested in the pharmacological and physiological properties of the lobster muscle GABA receptor (Smart 1983). Lobster muscle possessed large muscle fibres (approximate dimensions: 4 mm length by 500 μm width) which facilitated the easy insertion of three microelectrodes for monitoring membrane potential and passing current across the cell membrane. This intracellular method of recording drug-induced responses is open to detailed quantitation. By passing a current pulse across the membrane and monitoring the deflection of the membrane potential in the presence and absence of GABA, it is possible to calculate the conductance induced by GABA due to the opening of GABA-operated chloride ion channels. A more desirable, but technically more difficult alternative to the current-clamp technique, is to use voltage clamp. This method does not allow the membrane potential to change during application of the agonist but monitors the current which is passed by the amplifier to clamp the membrane potential at the desired value. This injection of current is then directly related to the drug-induced response. This latter technique has an advantage that voltage-sensitive channels are not activated by drugs which would normally cause a depolarization or hyperpolarization of the cell; this clarifies the analysis when it is required to concentrate solely on the ligand-gated conductances. The number of electrodes employed in these techniques depends on the size of the cell and its electrical properties. Muscle fibres behave much like electrical cables and often require measurements of membrane potential at two points along the muscle fibre, whilst spherical neuronal cells are invariably small (5–50 μm) and can accommodate usually only one electrode. This electrode is coupled to a different type of amplifier, which rapidly switches or alternates the function of the electrode between measuring voltage and also passing current. Exceptionally, sometimes two electrodes can be inserted into a large neuron ($\geqslant 50$ μm), one to record voltage and one to pass current into the cell.

pH and zinc sensitivity of the invertebrate muscle GABA receptor

In 1980, I observed that GABA-evoked responses monitored using intracellular recording from lobster muscle (*Homarus vulgaris*) were sensitive to the external pH of the Ringer solution. On increasing the alkalinity of the superfusing medium, the GABA conductance change (measured using a three-electrode current-clamp method) decreased in a sigmoid fashion. This curve was well described by the Henderson-Hasselbach equation for a monovalent site with a pK_a value of 6·1 (Fig. 2).

pH minimally affects the GABA molecule over a wide pH range, existing mostly in the zwitterionic form (99% at pH 7·6; 97% at pH 5·6). This research was performed before the discovery of the structure for the mammalian $GABA_A$ receptor (Schofield et al 1987; Pritchett et al 1989) or any other ligand-gated neurotransmitter receptor (e.g. nicotinic acetylcholine receptor; Noda et al 1983). We believed the receptor was most likely to be a protein and, as such, there are not many chemical moieties within a protein possessing a pK_a of 6·1; indeed only one candidate seemed likely, an

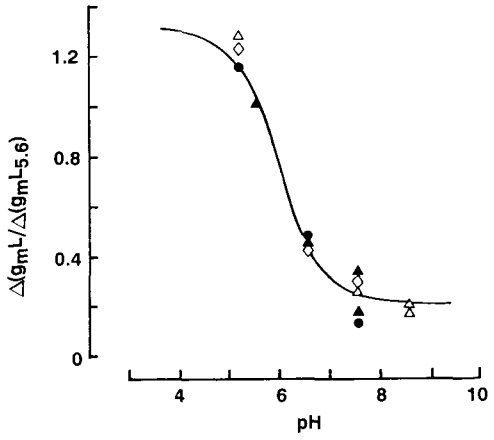

FIG. 2. pH titration of the GABA-activated chloride conductance recorded intracellularly from lobster muscle. All points are single determinations of conductance (Δg_mL), where g_m is the membrane conductance and L is the muscle fibre half length) evoked by 40 μM GABA and normalized with respect to the conductance obtained at pH 5·6. The different symbols indicate data obtained on different muscle fibre preparations (n = 4). The solid line was generated from the Henderson-Hasselbach pH equation representing a monovalent, titratable group with a pK_a of 6·1. After each pH perturbation the preparation was always returned to pH 7·6. Reproduced with permission, Smart & Constanti (1982).

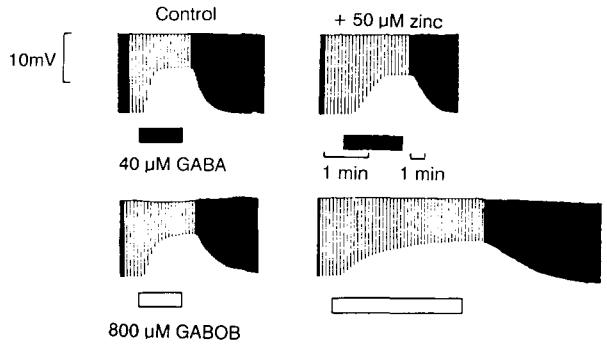

FIG. 3. Zinc antagonises GABA and GABA-agonist responses recorded from lobster muscle. These chart records illustrate conductance changes evoked by GABA and γ-amino-β-hydroxybutyric acid (GABOB) in control Ringer (lefthand column) and in the presence of 50 μM zinc (righthand column). Both the onset and offset rates of the responses are slowed by zinc. The downward deflections represent hyperpolarizing electrotonic potentials (0·2 Hz, −300 nA) recorded at the centre of a single muscle fibre. Drug exposure times are indicated by the bars. Reproduced with permission, Smart & Constanti (1982).

imidazole group in a histidine residue (pK_a 6·0). If this site exerted some influence over the GABA conductance, then it might be modified by agents which could bind tightly to this group. Divalent cations from the top row of the transition series seemed an ideal initial choice. Two ions were of particular interest for their ability to bind to imidazole groups, namely copper and zinc. In keeping with the above predictions, both zinc and copper proved to be effective GABA antagonists against different GABA agonists on lobster muscle over a wide concentration range (Smart & Constanti 1982; Fig. 3). Furthermore, we could extend the prediction, since if the site at which zinc binds was also sensitive to pH, then we might expect pH to interfere with the zinc antagonism of GABA responses. Once again, as expected, by acidifying the bathing medium and increasing $[H^+]$, the blockade by zinc of GABA responses could be considerably alleviated (Fig. 4).

Comparison with vertebrate $GABA_A$ receptors

Whilst the invertebrate observations were interesting, it was clear that if zinc had any important physiological role, it was likely to be in the mammalian CNS. It was therefore of interest to extend these preliminary observations with zinc to a vertebrate neuronal preparation. The vertebrate $GABA_A$ receptor population is quite likely to differ fundamentally in structure from invertebrate GABA receptor isoforms, since both benzodiazepines and barbiturates bind and modulate the vertebrate receptor, but not the invertebrate muscle GABA receptor, although both types of GABA receptor

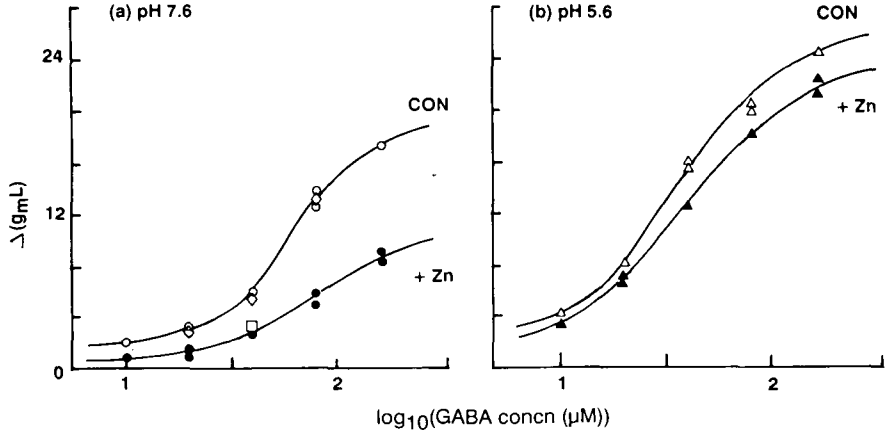

FIG. 4. Low pH can reverse the inhibitory effect of zinc on GABA responses in lobster muscle. Log GABA concentration-conductance curves were constructed in the absence (○) and presence (●) of 50 μM zinc at pH 7·6 followed by a recovery after washout of zinc (◇) (a). In the same muscle fibre, the Ringer pH was altered to 5·6 and this caused a small enhancement in the GABA dose-conductance curve (△) (b); however, subsequent application of zinc at pH 5·6 produced only a slight inhibition of the GABA response (▲). Whilst still in the presence of 50 μM zinc at pH 5·6, the pH of the external Ringer was rapidly altered back to 7·6 to produce a more effective inhibition (□) (a). Reproduced with permission, Smart & Constanti (1982).

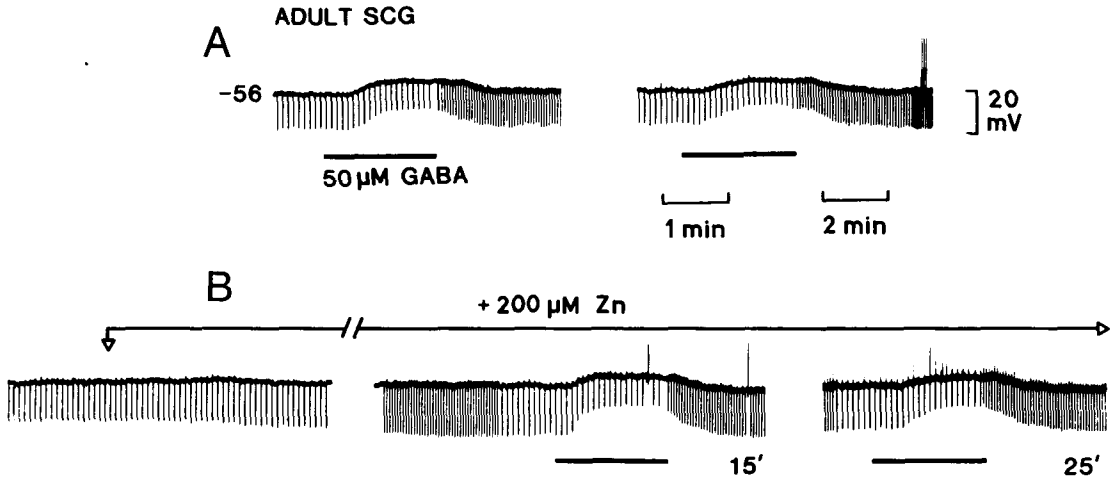

FIG. 5. GABA responses recorded from adult sympathetic ganglia are insensitive to zinc. (A) GABA application produced a small depolarization (upward deflection) and conductance increase (reduced hyperpolarizing electrotonic potential amplitude evoked by −0·16 nA, 300 ms, 0·2 Hz current pulses), measured using single-electrode intracellular recording from rat superior cervical ganglia (SCG). (B) Bath application of zinc (200 μM), produced a small hyperpolarization and reduction in the input conductance but no effect on the GABA response after 25 min. Modified and reproduced with permission, Smart & Constanti (1990).

mediate an increase in chloride conductance following activation with GABA agonists.

Two different preparations were chosen for the investigation, offering previously well studied GABA receptors at different locations in the nervous system: (i) a peripheral $GABA_A$ receptor located in rat superior cervical ganglia, and (ii), a CNS $GABA_A$ receptor situated in the rat and guinea-pig cortex and hippocampus.

Superior cervical ganglion studies

Ganglion cells were impaled with a single intracellular microelectrode and GABA was applied via bath superfusion. GABA responses were always depolarizing on sympathetic ganglia (Adams & Brown 1975); however, subsequent application of zinc (100–300 μM) was unable to antagonize the GABA response at concentrations which would have severely depressed the invertebrate GABA response (Fig. 5).

A lack of tissue penetration by zinc was not considered a problem, since the ganglia were stripped of their connective tissue sheath and on the addition of zinc, the input conductance of the ganglion cells consistently decreased, coupled with a small hyperpolarization and the appearance of spontaneous action potential firing. GABA responses were apparently resistant to zinc concentrations up to 500 μM. It appeared from these results that zinc was able to distinguish between vertebrate and invertebrate GABA receptors.

Studies using pyriform cortex brain slices

We then turned our attention to pyriform cortical slices, where much to our surprise, zinc produced an entirely different effect on the GABA response. As observed with ganglionic preparations, bath-applied GABA routinely evoked a depolarization in these CNS cells (Brown & Scholfield 1979). The application of zinc induced a slow *depolarization* and decrease in the input conductance without an initial, overt effect on the GABA evoked depolarization and conductance increase (Smart & Constanti 1983; cf. Hori et al 1987); however, after a 10–20 min incubation with zinc, the GABA response was clearly *enhanced*, concurrent with the production of spontaneous action potentials. After a prolonged washout of zinc (> 1 h), the GABA response recovered (Fig. 6).

We could try to conclude many things from these observations. In 1983, I was in favour of suggesting that zinc could distinguish between three putative 'types' of GABA receptor: producing an effective blockade of invertebrate GABA receptors, whilst having little or no effect on peripheral ganglionic $GABA_A$ receptors, yet surprisingly enhancing the response of CNS $GABA_A$ synaptic and/or extrasynaptic receptors.

The interaction of zinc with GABA receptors progressed very little for some years thereafter, until two other labora-

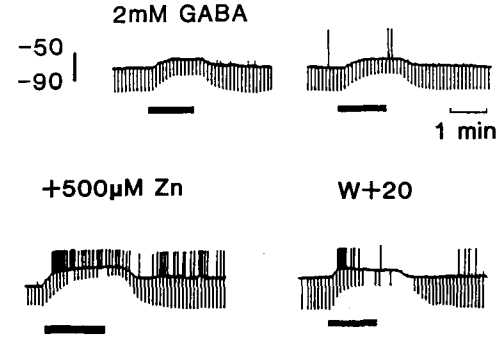

FIG. 6. GABA responses recorded from CNS neurons are enhanced by zinc. GABA produced small depolarizations and conductance increases in cortical neurons measured using intracellular current-clamp recording. Hyperpolarizing electrotonic potentials were generated from injected current pulses (−0·7 nA, 150 ms, 0·2 Hz). Larger doses of GABA were required to evoke a response in brain slices, due to the presence of avid GABA uptake mechanisms. Bath application of zinc induced a gradual depolarization, but no block of the GABA response. After approximately 10–15 min in zinc the GABA response became noticeably enhanced and the appearance of spontaneous action potential firing was evident. This effect was partly reversible on washing. Modified and reproduced with permission, Smart & Constanti (1983)

tories (Westbrook & Mayer 1987; Akaike et al 1987; Yakushiji et al 1987) observed that GABA responses recorded from either embryonic mouse hippocampal neurons in tissue culture, or acutely dissociated frog sympathetic ganglionic neurons, were *inhibited* by zinc. These studies implied that zinc may after all, modulate the $GABA_A$ receptor in the CNS. This prompted a reinvestigation of the actions of zinc on GABA receptors which we had previously studied. We utilized similar preparations as before, but now we employed tissue culture methods and the whole-cell recording technique for the ganglionic neurons, and an ionophoretic analysis of the GABA response on cortical and hippocampal brain slices.

Patch clamp recording from cultured neurons

The use of tissue culture preparations for electrophysiological studies was greatly enhanced by the promulgation of a novel recording method, the patch clamp recording technique (Sakmann & Neher 1983). This technique, unlike those discussed previously, requires a blunt and heat polished electrode tip to be placed on a clean cell surface thus electrically isolating a small patch of membrane. The electrical seal between the membrane and glass microelectrode is greatly improved by the application of suction to the inside of the electrode. When the seal resistance exceeds at least 1 GΩ, a satisfactory 'patch' has been obtained. This method requires clean cell surfaces to be able to achieve these high seal resistances; there are many ways of cleaning cell membranes in-vitro, including using dissociated tissue cultures (Sakmann & Neher 1983; Figs 7, 8), acute enzymatic isolation of cells (Kay & Wong 1986) and also cleaning cells in-situ in brain slices, using a stream of solution from an adjacent blunt pipette and avoiding the use of proteolytic enzymes (Edwards et al 1989). Once the 'cell-attached' patch has been obtained, the resolution of our recordings is markedly improved such that currents flowing through *single* ion channel proteins (order of picoamps) can now be resolved adding to our information concerning the functioning of receptors and ion channel proteins. Furthermore, we can manipulate this patch of membrane to either gain access to the cell's cytoplasmic milieu and modify this if desired by changing the patch pipette solution (whole-cell recording; Fig. 8), or withdraw the electrode and remove various configurations of isolated patches of membrane which can often retain functional and pharmacologically responsive receptor and ion channel proteins (inside-out and outside-out patches).

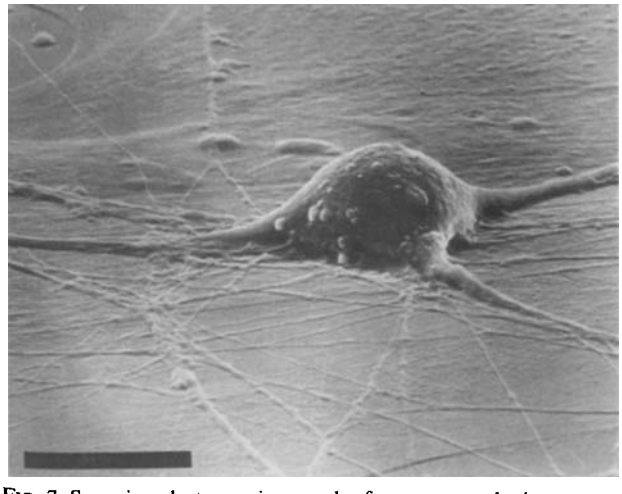

FIG. 7. Scanning electron micrograph of a rat sympathetic neuron maintained on a laminin substratum and grown in tissue culture for three weeks. After only a few days these neurons form an extensive network of fibres which pervade every area of the culture dish. Many of the fibres observed with cells grown in the presence of laminin may be axonal in origin (Lein & Higgins 1989). Note the bulbous appendages on the cell soma. Scale: 20 μm.

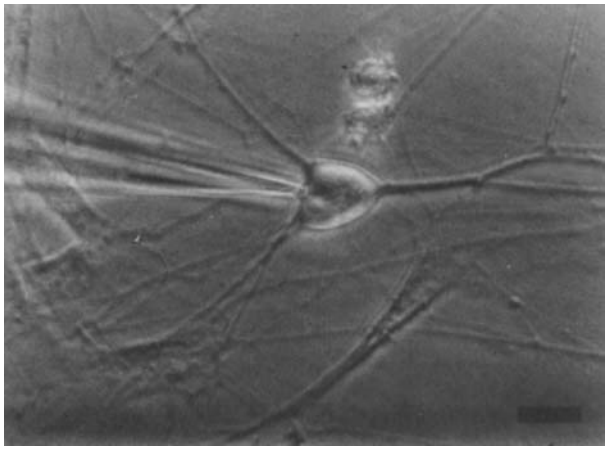

FIG. 8. This phase-contrast photomicrograph shows a single sympathetic neurone being used for whole-cell recording. The patch electrode is placed against the side of the cell and sealed to the membrane by using suction inside the pipette. A further application of suction ruptures the membrane beneath the pipette tip, allowing access to the cell interior to record whole-cell membrane currents. Sympathetic neurons can occasionally allow recordings to continue for up to 3 h. Scale: 10 μm.

Cultured rat sympathetic neurons: zinc and peripheral $GABA_A$ receptors

The bath application of GABA to sympathetic neurons, always produced an inward current and conductance increase, measured using the whole-cell voltage clamp. An increase in membrane current 'noise' was also apparent and due to the stochastic opening and closing of many individual GABA-operated chloride ion channels. There was a slight fade during the response which was probably due to desensitization. Application of zinc to these cultured cells now produced a reversible *inhibition* of the GABA response, similar to that reported previously on cultured mouse hippocampal neurons (Westbrook & Mayer 1987) (Fig. 9).

This inhibition was not restricted to bath-applied GABA responses, being easily reproduced by brief 'pressure-application' of GABA from a GABA-containing pipette positioned adjacent to the cell. In addition, the inhibitory action of zinc was shared by another divalent cation from the periodic table group IIB, cadmium (100–300 μM) (Fig. 10), but not by group IIA cations such as barium (100 μM–2 mM).

The action of zinc on embryonic cultured neurons was not confined to sympathetic ganglia; the GABA response recorded from embryonic cultured rat sensory neurons (dorsal root ganglia) or cerebellar neurons, was also reduced by similar concentrations of zinc. The inhibition of GABA responses was due to a genuine reduction in the GABA

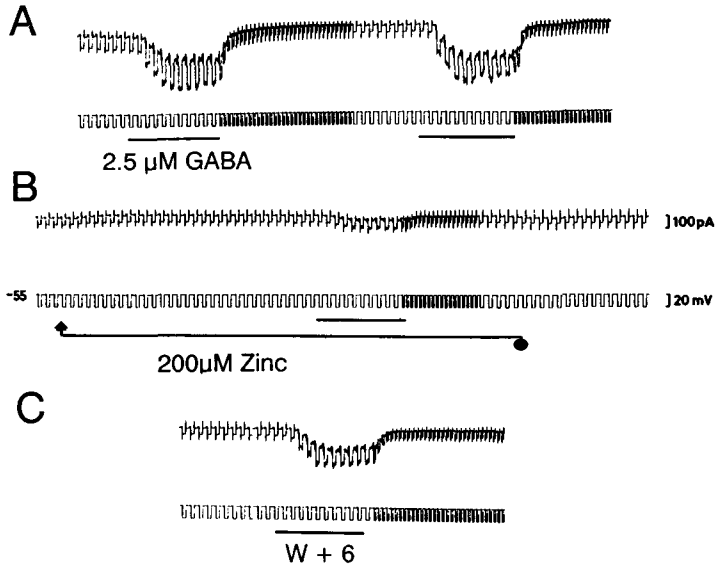

FIG. 9. Zinc inhibits GABA responses on embryonic cultured sympathetic ganglia. Bath-applied GABA responses were recorded under voltage clamp using the whole-cell recording technique. (A) GABA induced an inward (depolarizing) current and conductance increase (upper traces) observed following voltage commands from −55 mV (holding potential) to −75 mV (lower traces; 300 ms, 0·1 Hz). (B) Zinc (200 μM) was applied for 10 min. which induced a small outward (hyperpolarizing) current and also clearly inhibited the GABA response. (C) This effect was reversible on washing. Drugs were applied for the duration of the solid lines. The chart recorder speed was increased every 10 s for the duration of the voltage pulse and slowed during the washout of GABA. Reproduced with permission, Smart & Constanti (1990).

conductance and not due to a change in the GABA reversal potential determined from current-voltage relationships.

How can we account for the disparity between these results with tissue cultured neurons where zinc inhibits the GABA response, and those I have illustrated from previous studies utilizing intact ganglionic tissues, where zinc was quite ineffective?

It was conceivable that the inhibited GABA response observed with zinc in tissue culture was due to some artifact of cell culture, or perhaps caused by proteolytic modification of the receptor protein following the necessary enzymatic pretreatment for tissue dissociation (Inoue & Akaike 1987). However, we can discount these assertions by recording from similar age, young *post-natal intact* ganglia, which retain their cytoarchitecture and most important, have not been exposed to proteolytic enzymes. Intracellular recordings from young ganglia (1–4 days postnatal (P+1−4)) proved difficult with cells quickly becoming electrically leaky and

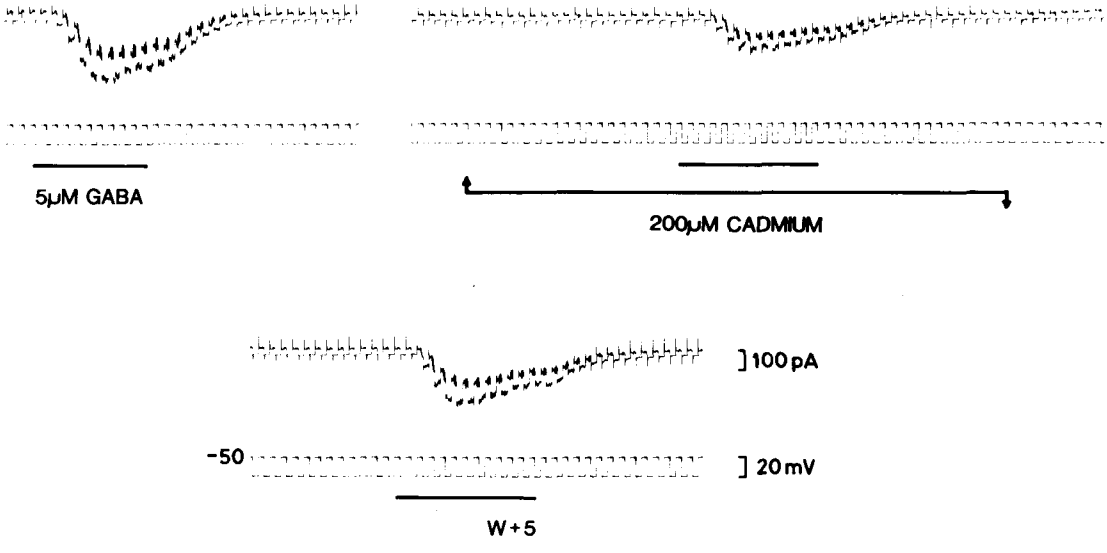

FIG. 10. Cadmium can also reduce the GABA response observed on cultured sympathetic neurons. Using similar whole-cell recording methods as in Fig. 9 at a holding potential of −50 mV and applying 20 mV hyperpolarizing voltage steps (lower traces; 300 ms, 0·1 Hz), bath application of 5 μM GABA produced a desensitizing inward current response and conductance increase (upper trace). Cadmium (200 μM) produced a reduction in the GABA response which was easily reversed following washout of cadmium (5 min). Unpublished results, T. G. Smart.

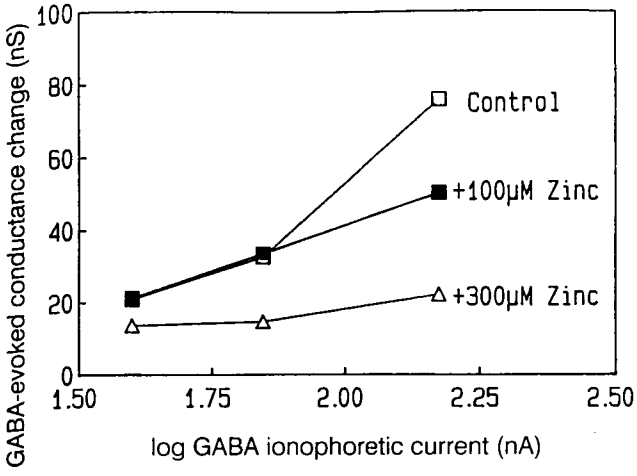

FIG. 11. GABA responses recorded from young post-natal intact sympathetic ganglia are inhibited by zinc. Responses to ionophoretically applied GABA were recorded under current clamp using intracellular recording. The GABA-evoked conductance change was measured from hyperpolarizing electrotonic potentials and plotted against the ionophoretic current used to eject GABA onto the cell. The dose-response plot revealed zinc (100 and 300 μM) as an effective non-competitive GABA antagonist in young (P6) post-natal ganglia. Unpublished results, A. Constanti & T. G. Smart.

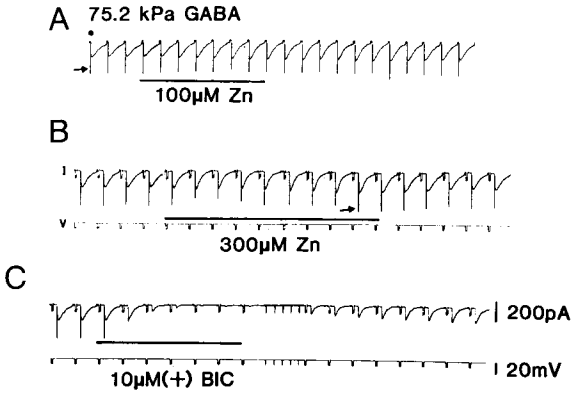

FIG. 12. GABA responses recorded from adult cultured sympathetic neurons are occasionally resistant to zinc. Whole-cell voltage clamp recordings were performed on adult cells at −55 mV holding potential. GABA was pressure-applied (75·2 kPa, 50 ms, 1 pulse every 30 s) to the neuron via a pressure ejection pipette, positioned adjacent to the patch pipette, producing transient inward currents (A). Membrane conductance was monitored by applying hyperpolarizing voltage commands (−10 mV, 300 ms, 0·03 Hz). Bath application of 100–300 μM zinc did not inhibit the GABA response, but caused the appearance of uncontrolled action potentials initiated at some distal site in the cell (arrow) (B). Neurons displaying GABA responses resistant to zinc were all blocked by application of bicuculline, a $GABA_A$ receptor antagonist (C). Modified and reproduced with permission, Smart & Constanti (1990).

unstable. The youngest ganglia which could be used successfully were P + 5 − 6. GABA, applied by ionophoresis, caused the expected depolarization with a conductance increase, and interestingly, superfusion of zinc produced little inhibition at low GABA doses but depressed the GABA responses at high doses. At 300 μM, zinc reduced the dose-conductance curve in a non-competitive manner (Fig. 11) (Smart & Constanti 1988, 1989).

The ability of zinc to inhibit GABA responses in young post-natal or foetal neurons, might conceivably be due to a developmental change in the $GABA_A$ receptor with neuronal development. To address this aspect, we turned our attention to tissue cultures of adult neurons and intact adult SCG preparations. The age of these animals was at least P > 90, and, from our previous experience with intact ganglia, I reasoned that any putative developmental influences affecting the $GABA_A$ receptor might now be complete.

Zinc was consistent in still producing no effect on the adult GABA response recorded from intact SCG neurons as we observed previously (Smart & Constanti 1983); however, whole-cell recording from adult cultured neurons of a similar age yielded a variety of responses. The GABA responses were still inhibited by zinc, but now by much less than we observed in the foetal cultures; also, there were a considerable number of cells where zinc was totally ineffective as a GABA antagonist. These latter cells would display a normal $GABA_A$ receptor pharmacology to barbiturates and the $GABA_A$ antagonist, bicuculline (Fig. 12).

This postulated developmental change in the sensitivity of GABA responses to zinc was further highlighted by a full dose-response curve analysis on cultured adult and foetal sympathetic neurons. In both cases, 100 μM zinc caused a non-competitive reduction in the GABA response; however, the GABA responses observed with the foetal cells were far more affected by zinc than those recorded on the adult cells. There was a small increase in the degree of zinc blockade with the agonist dose, but overall the agonist dependence was very slight (Fig. 13).

An additional and equally important question, was whether zinc inhibited the GABA response by merely complexing with GABA molecules and thereby preventing the agonist gaining access to the receptor. GABA, in its zwitterionic form, could conceivably complex with zinc ions, but the shape of the dose-response curves does not support such a theory as the sole explanation for the observed antagonism of GABA responses. A complexation mechanism predicts that the slope of the dose-response curve should be increased in the presence of zinc and that the maximum of the curve should be unaffected. Both points are incompatible with the experimental evidence. Furthermore, complexation would not explain why GABA receptors on foetal neurons are more susceptible to zinc block than those receptors resident on adult neurons.

Pyriform cortex studies: zinc and CNS GABA receptors

Our postulation of a developmental modification for the $GABA_A$ receptor on sympathetic neural tissue could explain much of the data so far, but how does this help to explain the effect of zinc on intact CNS slices? The $GABA_A$ receptor in these slices should be 'mature' and adult, and therefore unaffected by zinc (unless zinc affects only a particular subpopulation of $GABA_A$ receptors!). Clearly zinc, from our previous brain slice work, enhances the GABA response. This conclusion was again supported by more recent experiments on olfactory cortical neurons which respond to bath-applied GABA with a slow depolarization and conductance increase. Preincubation with zinc (100–300 μM) enhanced the GABA response along with the following features which are the typical signature of zinc application to a brain slice preparation (Fig. 14A): An increase in membrane noise due to the release of spontaneous synaptic potentials, a 5–10 mV

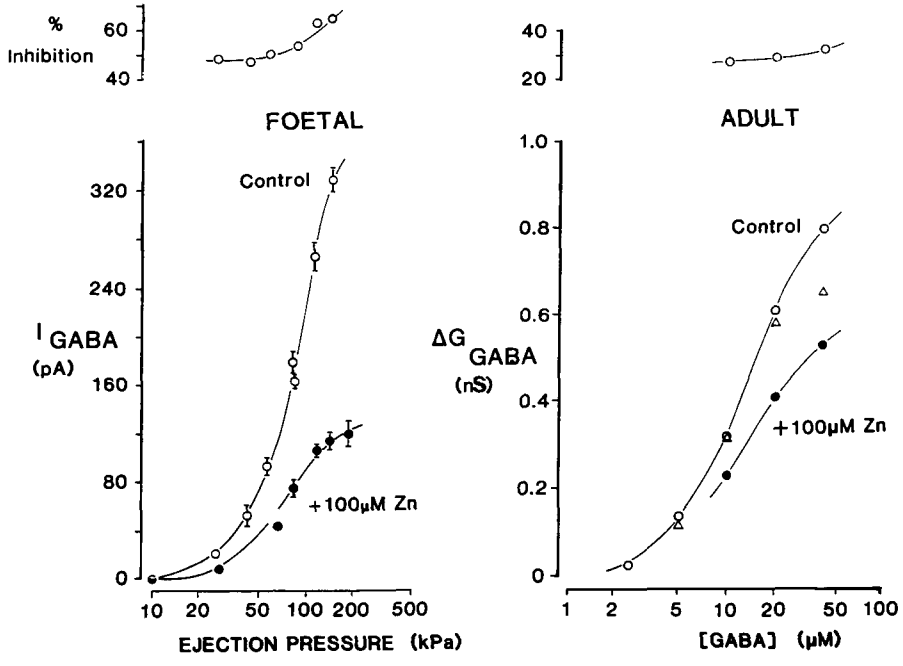

FIG. 13. Dose-response curve analysis revealed zinc as a more potent inhibitor of GABA responses on foetal compared to adult sympathetic neurons. Dose-response curves were constructed from data recorded from foetal (A) and adult (B) neurons using whole-cell recording at a holding potential of −55 mV. GABA was applied either by pressure ejection and the resulting GABA current measured (I_{GABA}) (A), or by bath application and the GABA-evoked conductance change (ΔG_{GABA}) calculated (B). In control Krebs, the GABA log dose-response curve was sigmoidal with an EC50 of 12 μM (B) (n = 3). Bath application of 100 μM zinc resulted in a non-competitive depression in both curves, but clearly, zinc was more effective on the foetal cells (A). The insets show the percentage depression in the control GABA response by zinc. The points in (A) are the mean values ± standard error of the mean determined from 5 successive exposures to the same pressure application of GABA. The open triangles in (B) represent recoveries following 10 min washout of zinc. (A) reproduced with permission, Smart & Constanti (1990); (B) unpublished results, T. G. Smart.

depolarization coupled with a decreased input conductance and increased spontaneous neuronal action potential firing.

The enhanced GABA response was not due to a blockade of the GABA uptake carrier (Brown & Scholfield 1984) which would increase the reactivation of GABA receptors by GABA, since the enhancement also occurred with equal magnitude in the presence of nipecotic acid (1 mM), an established GABA uptake inhibitor (Johnston et al 1976). Furthermore, zinc could also enhance the GABA response measured in other brain slices, including hippocampal slices recording intracellularly from either CA1 or CA3 neurons (X. Xie & T. G. Smart, unpublished observations).

On close examination, it appeared that the decrease in the cell's input conductance was occurring at the same time as zinc was enhancing the GABA response. To determine the time course of the zinc-induced enhancement and to be able to apply more doses of GABA over a shorter time period, GABA was focally applied to single neurons using ionophoresis from a separate GABA-containing micropipette. This also avoided the slow washout of bath-applied drugs which occurs with a multilayer preparation such as a brain slice.

A biphasic GABA response was now apparent following ionophoresis (Alger & Nicoll 1982; Scharfman & Sarvey 1987; Thompson 1988; Connors et al 1988), consisting of a small initial hyperpolarization (somatic) followed by a much larger depolarization (dendritic) (Fig. 14A). These responses to GABA are believed to be generated by spatially discrete populations of GABA receptors. The direction of the membrane potential change is believed to be a function of the different transmembrane chloride ion gradients maintained in the dendrites compared with the neuronal soma (Misgeld et al 1986; Thompson et al 1988). The hyperpolarization is confined to the cell soma, whereas the depolarizing response most likely results from activation of dendritic $GABA_A$ receptors. These GABA responses were also enhanced by zinc and were commensurate with a reduction in the cell's resting input conductance (Fig. 14B). In fact, these two parameters were clearly temporally correlated, suggesting a possible causal relationship (Fig. 14D) (Smart & Constanti 1990).

Whether some or all of this enhancement in the GABA response could be accounted for by a change in the GABA reversal potential was studied by measuring the reversal potentials for the biphasic response. Such a shift did not appear to be the reason, since the GABA-evoked conductance change was also increased by zinc.

As for the sympathetic ganglia study, it was also important to try alternative divalent cations to observe how specific the enhancement of CNS GABA responses was to zinc. Group IIA and IIB divalent cations were again utilized, 300 μM barium depolarized the cell by 25 mV, decreased the input conductance dramatically and also enhanced the GABA response (cf. Newberry & Nicoll 1985). Some of these features were also apparent when using cadmium (200 μM). This suggested that the ability of zinc and barium (and also cadmium) to enhance the GABA response might reside

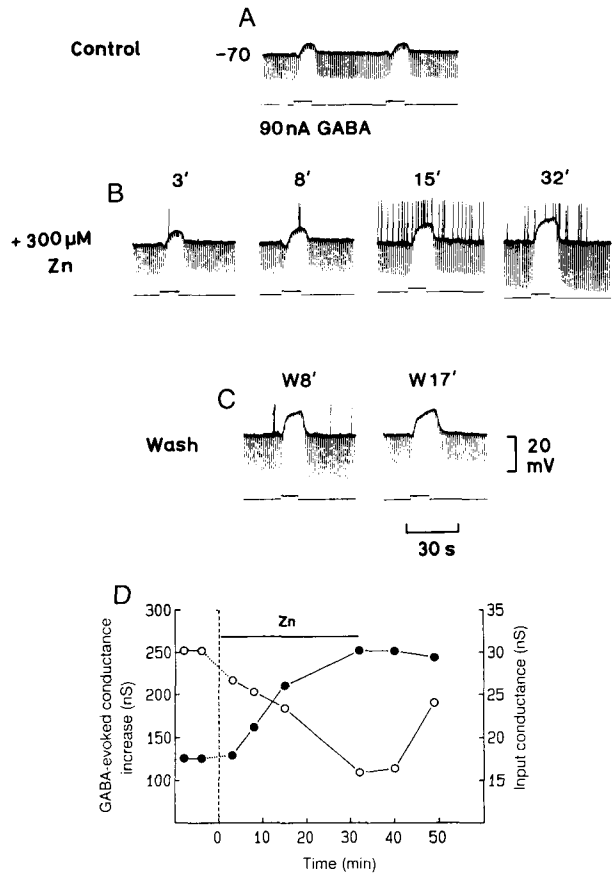

FIG. 14. Biphasic GABA responses produced by ionophoretically applied GABA in cortical neurons are enhanced by zinc. GABA was applied to the neuron maintained at −70 mV, using 90 nA current ejection for 10 s. Hyperpolarizing electrotonic potentials were applied (80 ms, −0·47 nA, 1 Hz) to monitor the membrane conductance. The reproducible control GABA response (A) was gradually enhanced following exposure to 300 μM zinc for 3, 8, 15 and 32 min (B). The enhancement occurred concurrently with the zinc-induced decrease in the resting input conductance and production of spontaneous action potential firing. Some recovery was obtained from the effect of zinc on washout (C). (D) illustrates a plot of the GABA-evoked conductance increase (●; left ordinate) and the cell input conductance (○; right ordinate) plotted against time. Zinc was applied for the period indicated by the bar. Reproduced with permission, Smart & Constanti (1990).

mostly in their ability to alter the electrical properties of the cell, rather than by some direct action on the GABA receptor protein complex.

Xenopus oocytes: mRNA expression studies

If the GABA receptor does become insensitive to zinc, at what stage does this occur? Is it a postranslational modification of the receptor by the neuron, or can the developmental modification occur at the level of the genes carrying the code for the receptor protein? The GABA receptor is an oligomeric protein consisting of at least three types of subunits designated as, α, β and γ (Schofield et al 1987; Pritchett et al 1989); more subunits are continually being postulated suggesting the possibility of a considerable amount of $GABA_A$ receptor heterogeneity (Levitan et al 1988; Shivers et al 1989) with important consequences for pharmacological studies.

The *Xenopus laevis* oocyte has an impeccable record for being able to translate and correctly post-translationally process foreign mRNA which can be easily injected into this large cell (1 mm diameter). For our purposes, this facilitates the study of neurotransmitter receptors and ion channel proteins in a much more experimentally convenient cell without the complications presented by the intact CNS, which includes uptake carriers for neurotransmitters, constant 'background' synaptic transmitter or modulator release influencing receptor function and tortuous diffusion pathways in intact preparations (particularly in brain slices). It is possible to take some mRNA from a cell and express, relatively exclusively, specific proteins of interest. We and others (Smart et al 1987; Sumikawa et al 1986; Snutch 1988; Dascal 1987) have previously demonstrated that this translation system can faithfully reproduce many receptor proteins with a recognizable pharmacology from comparative studies of similar proteins in their native cells (e.g. neurons, muscle cells, epithelia). It is also apparent that the efficiency of mRNA translation and functional expression of the proteins, depends to some extent on the source and species of mRNA. We have observed in preliminary studies, that $GABA_A$ receptors expressed in *Xenopus* oocytes exhibit a differential sensitivity to zinc inhibition, which can be correlated with the age of the animals used for mRNA extraction (D. Bowie & T. G. Smart unpublished observations). Our favoured hypothesis for this result is that the 'modification' to the GABA receptor which renders the receptor less sensitive to zinc, probably occurs at the gene level. This might occur perhaps by modification of one or more DNAs, but this does not discount the possibility that the same 'injected mRNA', extracted from certain ages of animals, can also direct post-translational processing of the receptor once it is inserted in the membrane. To provide categorical evidence about the developmental hypothesis, we need to address this question more clearly, using cloned $GABA_A$ receptors where the subunit type and composition of the receptor are known and under full experimental control.

Zinc and $GABA_A$ receptors: a conclusion?

I originally planned to try and answer the question of whether zinc has any neurophysiological role when starting this study. Unfortunately, as is often the case in science generally, it is easy to fail in the search for a satisfactory answer to the original question, but during the study, you can easily gain plenty of answers to questions you did not originally pose, nor even formulate.

The data I have presented has enabled two conclusions or 'working hypotheses' to be proposed: Firstly, the GABA response of young embryonic neurons is more sensitive to zinc than the adult response. This may be due to a developmental change in the $GABA_A$ receptor, or due to heterogeneity of $GABA_A$ receptor populations or individual subunits. I would suggest that zinc-sensitive receptors or subunits predominate in the younger embryonic cells. Secondly, CNS neurons present in brain slices will naturally possess an extensive dendritic arborization. The insertion of a recording microelectrode into the cell soma might be expected to faithfully monitor electrical events which occur in and around the soma; however, it is extremely doubtful

whether synaptic events or ion channel activation occurring more distally out on the 'dendritic tree' will also be recorded accurately due to the dissipation of the signal as it travels towards the soma.

How can this explain the enhanced GABA response recorded from intact brain slices after zinc treatment?

It may be due to zinc (or any other divalent cation or agent) blocking *critical leak conductance(s)*, enabling previously unresolved components of the dendritic GABA conductance to now be observed back at the somatic recording site. Thus any agent blocking this leak conductance should, in theory, be capable of enhancing the recorded GABA response.

GABA receptors, ion channels and their modulators

Finally, I have attempted to demonstrate various ways of modulating ion channel function in the nervous system. The particular example which we have dwelt upon, has demonstrated that GABA channels are not stationary in a developmental sense, but clearly show functional and perhaps structural development with time. These features have apparently been resolved using the endogenous transition metal, zinc.

I can envisage, that with the advances being made by molecular biologists in elucidating the structure of genes responsible for receptor and ion channel protein synthesis, for so long an unapproachable area, that an exciting new era is dawning, when we may be able to understand the behaviour of single neurotransmitter receptor and ion channel proteins in neuronal membranes. Then will come the major task of considering how individual neurons, neural networks and whole areas of the brain function at a molecular level.

On reflection, the data presented do tend to make our original early notion regarding neurotransmitter receptors as proteins merely controlling the opening or closing of an ion channel rather simplistic. They seem far more functionally and structurally 'plastic' than even the most optimistic molecular biologist realised.

As a general truism, it seems the more incisive our investigating techniques become, the deeper we study, the more we find, and then, the more complex the whole story becomes and so our methods are now once more honed to the new challenge!

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