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"Expression of GABA and Glycine Receptors by Messenger RNA's  
from the Developing Rat Cerebral Cortex"

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## Expression of GABA and glycine receptors by messenger RNAs from the developing rat cerebral cortex

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The ontogenesis of mRNAs coding for GABA and glycine receptors in the cerebral cortex of the rat was examined by extracting poly(A)<sup>+</sup> mRNA from the brains of embryonic, postnatal or adult rats and injecting it into *Xenopus* oocytes. The ability of a messenger to express functional receptors was then assayed by measuring the membrane currents elicited by the agonists. The size of the GABA-induced current increased progressively with age, being undetectable in oocytes injected with mRNA from embryonic day 15 and reaching a maximum in oocytes injected with mRNA from postnatal day 30. In contrast, the glycine-induced response was negligible in oocytes injected with mRNA from the cerebral hemispheres of embryos 15 days old; it increased sharply to a maximum with newborn animals and then decreased with age to become very small with mRNA from adult cortex. GABA and glycine receptors induced by mRNA from the cerebral cortex of all ages are associated with chloride channels.

### INTRODUCTION

The appearance of neurotransmitters and receptors in the cerebral cortex is believed to occur at different times in development (Johnson 1985; Telang & Enna 1985). A great deal of information is already available concerning the ontogenesis of various neurotransmitters in the rat brain (Lanier *et al.* 1976; Coyle 1977, 1982), mainly gained by using histochemical techniques for detecting the neurotransmitters or the enzymes that synthesize them. The ontogenesis of several neurotransmitter receptors has also been studied by using radioactive ligand binding. However, binding studies are somewhat complicated by a lack of specific probes and by uncertainties as to whether the measured binding sites correspond to functional receptors.

We describe here a novel way to study the developmental changes in the transcription of genes coding for neurotransmitter receptors in the brain. This method involves extracting poly(A)<sup>+</sup> messenger RNA (mRNA) from brains at various stages of development and injecting the mRNAs into *Xenopus* oocytes. As we have already shown, the oocytes translate the foreign mRNA and express a wide variety of functional brain receptors (Gundersen *et al.* 1983*a, b*, 1984; Sumikawa *et al.* 1986). By recording the membrane currents mediated by these receptor-channel complexes a relative measure of the amount of mRNA coding for each receptor can be obtained. This approach was used previously to study the

appearance of receptor mRNA in the developing chick brain (K. Sumikawa, I. Parker & R. Miledi, unpublished data). We now extend this technique to investigate developmental changes of the mRNAs coding for receptors to the inhibitory neurotransmitters GABA and glycine, in the cerebral cortex of the rat.

### METHODS

Sprague-Dawley rats were bred by placing males and females together overnight. The following day was called embryonic day 1 (E1) if the female was found to be sperm-positive. The gestation period was 21–23 days; the day of birth was called newborn or postnatal day 0 (P0), and the litter size ranged from 6 to 14 pups. Tissue samples were obtained from animals at embryonic days 15 and 18 (E15 and E18), newborn, postnatal days 5, 10, 15, and 30 (P5, P10, P15, and P30) and from adult animals (mass 200–280 g).

To obtain postnatal tissue, animals were deeply anaesthetized (except for newborns, P5 and P10, which were not anaesthetized before killing) and decapitated. The brain was then quickly removed and the cerebral cortex dissected away, placed immediately in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Embryonic tissue was obtained by deeply anaesthetizing a pregnant animal, removing the fetuses, and dissecting out their brains. Whole cerebral hemispheres were removed from E15 fetuses, only the cerebral cortices were removed from E18 fetuses (except for one E18 preparation, in which the whole cerebral hemispheres were used). The tissue was then frozen immediately. All dissections took less than 5 min.

Messenger RNA was isolated by using a modification of the procedure of Miledi & Sumikawa (1982). Briefly, total RNA was extracted from the frozen tissue by homogenization in pH 9 buffer (0.2 M Tris-Cl; 10 mM EDTA; 0.5% sodium dodecyl sulphate (SDS); 1 mg ml $^{-1}$  heparin), followed by deproteinization once with phenol, once with chloroform, twice with phenol-chloroform, and finally with chloroform again. Total nucleic acids (RNA and DNA) were precipitated from the aqueous phase overnight with 0.2 M NaCl and 2.5 volumes of ethanol at  $-20^{\circ}\text{C}$ . The precipitate was collected by centrifugation and dissolved in 20 mM HEPES (pH 7.5). From this, the RNA was selectively precipitated with 3 M NaCl, washed three times with 3 M sodium acetate (pH 6.0), washed with ethanol and dried. The RNA was dissolved in 20 mM HEPES, pH 7.5, and had an  $A_{260\text{nm}}/A_{280\text{nm}}$  ratio of about 2. Poly(A) $^{+}$  mRNA was then isolated from total RNA by oligo-dT chromatography, ethanol precipitated again, centrifuged and dried. For final injection into the oocytes, the mRNA was dissolved in sterile water at a final concentration of approximately 1 mg ml $^{-1}$ .

Each mRNA preparation was made from approximately 1 gram of starting tissue. As a rough guide, this required brains from 25–35 E18 animals or about two adult animals.

*Xenopus* oocytes were injected with 50 nl of 1  $\mu\text{g}$   $\mu\text{l}^{-1}$  mRNA and incubated in sterile Barth's solution (composition in mM: NaCl, 88; KCl, 1; NaHCO $_3$ , 2.4; MgSO $_4$ , 0.82; Ca(NO $_3$ ) $_2$ , 0.33; ClCl $_2$ , 0.41; Tris-HCl, 7.5, at pH 7.4) with nystatin (50 U ml $^{-1}$ ) and gentamycin (0.1 mg ml $^{-1}$ ) at 18  $^{\circ}\text{C}$  for 4–7 days before recording. In some cases, oocytes were treated with collagenase 1–3 days before recording to remove follicular and other enveloping cells (Kusano *et al.* 1982; Miledi & Parker

1984). Oocytes were voltage-clamped with a two-electrode voltage-clamp system (Kusano *et al.* 1982; Miledi 1982), usually at a potential of  $-60$  mV, and were continuously superfused with frog Ringer's solution (NaCl, 115 mM; KCl, 2 mM; CaCl $_2$ , 1.8 mM; HEPES, 5 mM, at pH 7.0) at room temperature. Drugs were bath-applied via this perfusate. To reduce the effects of variability by expression in different donors, oocytes from each donor were injected with several mRNA preparations. Moreover, we had already found that the expression of foreign mRNA coding for neurotransmitter receptors increases for several days after injection; and because recordings were usually made over a period of several days, beginning about 4 days after injection, oocytes injected with each type of mRNA were examined on each day. Thus the data would not be biased by an increase in expression of mRNAs with increasing time after injection.

### RESULTS

#### Isolation of mRNA

The mean recovery of total RNA from all preparations (E18 through adult) was  $1015 \pm 98$   $\mu\text{g}$  g $^{-1}$  wet mass of starting tissue (s.e.m., 17 preparations) and the mean yield of poly(A) $^{+}$  mRNA from total RNA was  $6.21 \pm 0.96\%$  (see table 1). There were no obvious developmental trends in the yield of poly(A) $^{+}$  mRNA, but this matter requires more careful examination.

TABLE 1. YIELD OF POLY(A) $^{+}$  mRNA EXTRACTED FROM RAT CEREBRAL CORTICES DISSECTED FROM BRAINS OF DIFFERENT AGES

(Results are means  $\pm$  s.e.m.; numbers in parentheses indicate the numbers of brains sampled.)

mRNA preparation	% total RNA from tissue	% mRNA from tissue	% mRNA from total RNA
E15 (1)	0.102	0.005	3.0
E18 (3)	$0.079 \pm 0.020$	$0.007 \pm 0.002$	$10.3 \pm 4.5$
new (3)	$0.111 \pm 0.027$	$0.005 \pm 0.001$	$4.5 \pm 0.9$
P5 (1)	0.089	0.008	9.5
P10 (2)	$0.130 \pm 0.055$	$0.008 \pm 0.002$	$6.3 \pm 0.9$
P15 (3)	$0.103 \pm 0.021$	$0.006 \pm 0.002$	$5.8 \pm 1.6$
P30 (1)	0.079	0.004	5.4
adult (3)	$0.085 \pm 0.017$	$0.003 \pm 0.001$	$4.4 \pm 1.7$

Each oocyte was injected with a roughly constant amount of mRNA (50 nl of a solution with an average concentration of  $1.15 \pm 0.31$  mg ml $^{-1}$ , 17 preparations (mean  $\pm$  s.d.). However, it was possible that the proportion of translationally active mRNA varied with age, or between different mRNA preparations. To examine this, most ( $n = 13$ ) of the mRNA preparations were tested for translational activity in a reticulocyte lysate cell-free system (Pelham & Jackson 1976). All samples tested showed translational activity within 24% of the overall mean value, and there was no obvious trend with developmental stage (see table 2). Thus, each oocyte was injected with a similar amount of translationally active mRNA.

TABLE 2. *IN VITRO* TRANSLATION EFFICIENCY OF DIFFERENT RAT CORTEX mRNAs

(Values were obtained by translating the mRNA preparations in reticulocyte lysate cell-free system. Counts represent the incorporation of [<sup>3</sup>H]leucine. Each number is the mean number of counts for duplicate samples, minus the control blank.)

mRNA preparation	counts - control	mRNA preparation	counts - control
E18 B	19840	P15 A	19312
new A	22929	P15 B	17138
new B	29467	P15 C	15856
new C	12686	P30 A	21542
P10 A	23826	adult A	12047
P10 B	21716	adult B	17275

#### Ontogenesis of GABA and glycine mRNAs

Bath application of GABA and glycine (both  $10^{-3}$  M) to oocytes injected with cerebral cortex mRNA isolated from P10 onwards (for GABA) or from newborns (for glycine) elicited large inward currents at a clamp potential of  $-60$  mV (figure 1). In contrast, control (non-injected) oocytes from the same donors showed only small responses: mean response to GABA,  $0.71 \pm 0.40$  nA, 14 oocytes from 4 donors; mean response to glycine,  $3.00 \pm 0.71$  nA, 16 oocytes, from 4 donors. The currents elicited by 1 mM GABA and glycine declined during prolonged application of the agonists (cf. figure 1), probably because of receptor desensitization.

Oocytes injected with mRNA from E15 cerebral hemispheres (the earliest age examined so far) showed no detectable response to GABA, and very little or no response to glycine. Messenger RNA from newborn cerebral cortex induced larger currents to both GABA and glycine, but at this stage the response to glycine had reached a maximum while the response to GABA was still quite small (figures 1 and 2). During postnatal development, the size of the current elicited by GABA continued to increase and reached a maximum by about P30. In marked contrast, the response to glycine declined after birth, to become very small with mRNA from adult animals (figures 1 and 2).

Quantitative comparison of the ability of mRNA from brains of different ages to induce sensitivity to GABA and glycine was made by measuring the peak currents elicited by 1 mM GABA and glycine at a clamp potential of  $-60$  mV. These agonist concentrations were sufficient to give nearly maximal responses so possible developmental changes in receptor affinity would not alter the peak response size. Figure 2 shows results obtained in this way, from 7 donors, and include between one and three different preparations of mRNA for each developmental age. The average membrane current elicited by glycine was very small in oocytes injected with E15 mRNA ( $2.0 \pm 0.7$  nA), increased to a maximum of about 170 nA in oocytes injected with newborn mRNA, and declined to about 16 nA in oocytes injected with P30 and adult mRNA (figure 2a). In contrast, the average membrane current elicited by GABA was very small in E15, E18 and newborn oocytes (less than 25 nA), increased in postnatal oocytes and reached a maximum of about 200 nA in P30 and adult oocytes (figure 2b). Thus, during

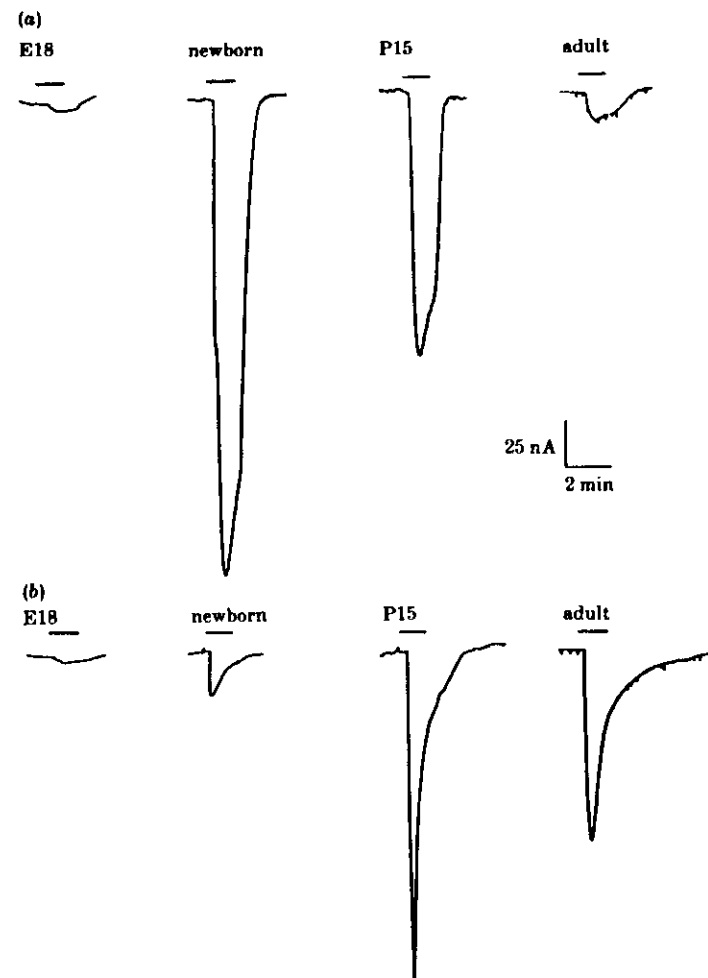


FIGURE 1. Membrane currents elicited by glycine (a) and GABA (b) in oocytes injected with poly(A)<sup>+</sup> mRNA obtained from rat cerebral cortex at different developmental stages. Each pair of responses was recorded from a single oocyte, and E18, newborn and P15 oocytes were from the same donor. In this and other figures, downward deflections correspond to inward membrane currents and, except where indicated, recordings were made at a clamp potential of  $-60$  mV. GABA and glycine (both 1 mM) were bath-applied for the times indicated by the bars.

ontogenetic development of the brain, the mRNA's ability to express GABA, and glycine sensitivities, were markedly different. This is shown even more strikingly by considering the ratio of the GABA response size relative to the glycine response obtained in the same oocyte (figure 3). The ratio increased progressively during development and showed a 522-fold change between E18 and adult.

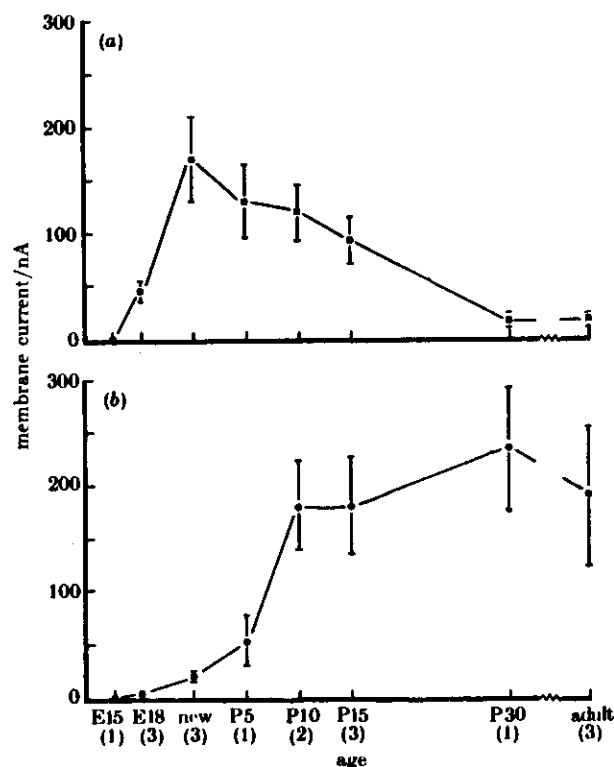


FIGURE 2. Peak sizes of membrane currents recorded from oocytes injected with cerebral cortex mRNA (except for the E15 and one E18 mRNA preparation in which the entire cerebral hemispheres were used) from different stages of rat development, measured from records like those in figure 1. Each point represents the mean  $\pm$  standard error of measurements from 8–38 oocytes from 7 donors. The numbers of replicate mRNA preparations examined are indicated below each age. Oocytes from each donor were injected with several mRNAs, except for E15 mRNA, which was used in three donors which were not injected with the other mRNAs. (a) Glycine,  $10^{-3}$  M; (b) GABA,  $10^{-3}$  M.

#### Properties of GABA and glycine currents

Glycine- and GABA-activated membrane currents were examined in oocytes injected with mRNA from different ages (glycine: E18, newborn, P5, P10; GABA: P5, P10, P30). In all cases, the current decreased as the membrane was depolarized and inverted direction at about  $-20$  mV. This corresponds to the chloride equilibrium potential in *Xenopus* oocytes (Kusano *et al.* 1982). GABA and glycine currents had similar reversal potentials (figure 4): the average reversal potential for glycine was  $-19.4 \pm 1.9$  mV (15 oocytes), and that for GABA  $-21.9 \pm 2.2$  mV (7 oocytes). Furthermore, there were no obvious differences in reversal potential with mRNA from brains of different ages. Figure 5 shows current-voltage relations for GABA and glycine responses in one oocyte injected with P5 mRNA. Both

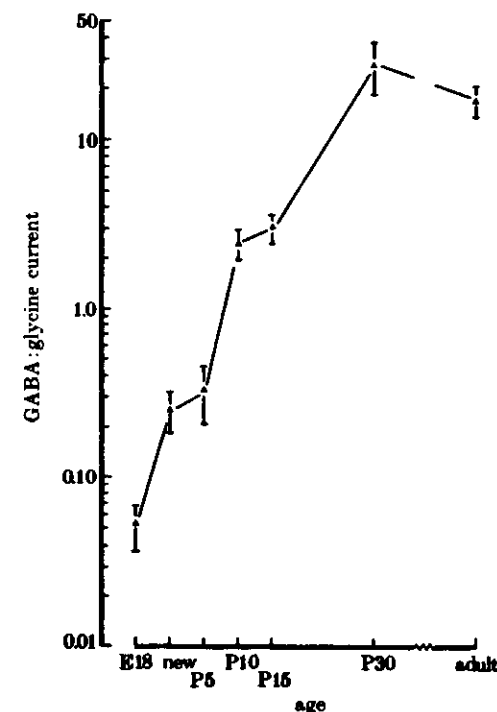


FIGURE 3. Ratios of GABA to glycine response sizes in individual oocytes. Each point represents the average of 8–35 oocytes taken from 2–4 donors; bars indicate standard error of mean. For each developmental age data from 1–3 mRNA preparations were used. Glycine responses in oocytes injected with mRNAs from older ages (P30 and adult) were often undetectable (less than 1 nA) and those data were not included in the mean.

responses rectified at negative potentials, with the current failing to increase with increased hyperpolarization beyond about  $-100$  mV, despite the increased driving force for chloride efflux. A similar rectification was also seen for glycine responses in oocytes injected with newborn mRNA and for the GABA response in an oocyte injected with P30 mRNA.

Dose-response curves were obtained by application of various concentrations of agonists and measuring the peak membrane current. In both E18 and P10 oocytes glycine currents were detectable at a concentration of  $5 \times 10^{-5}$  M and reached a maximum at a concentration of about  $10^{-3}$  M (figure 6). The dosage required to obtain a half-maximal response was  $3.85 \times 10^{-4}$  M. GABA responses, in P10 and P30 oocytes were first detectable at a concentration of  $5 \times 10^{-6}$  M, a concentration lower than that required for glycine response detection, and the GABA response reached a maximum at  $10^{-3}$  M. The dosage required to obtain half-maximal GABA responses at both ages was  $10^{-4}$  M. Dose-response curves were not obtained for glycine at later ages or for GABA at earlier ages, because responses were too small to obtain accurate data at the low concentrations.

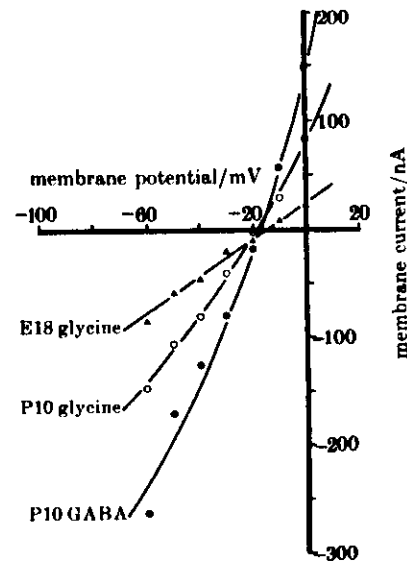


FIGURE 4. Current-voltage relations of glycine responses from oocytes injected with E18 or P10 mRNA, and for GABA responses in oocytes injected with P10 mRNA. Each point represents the average of two to five oocytes from the same donor. Measurements were obtained by briefly stepping the oocyte to different membrane potentials during steady application of the agonists. Points indicate the drug-induced current at each potential after subtraction of passive membrane currents evoked by the same voltage steps in the absence of agonists.

#### Co-injection of newborn and adult mRNAs

As described above, the expression of glycine receptors is maximal with mRNA from the newborn cerebral cortex and decreases postnatally. Presumably this is because the amount of mRNA coding for glycine receptors decreases with age. However, an alternative possibility is that a substantial amount of glycine receptor mRNA exists in the adult cerebral cortex, but that its translation in the oocyte, or the expression of functional glycine receptors are inhibited by some other messenger present in the adult cortex mRNA. These possibilities were examined by injecting oocytes with a fixed amount (25 ng) of mRNA from newborn cortex, together with an equal amount of mRNA from adult cortex. Oocytes injected with both messengers gave a mean response to glycine of  $143 \pm 71$  nA (12 oocytes from a single donor). By comparison, oocytes from the same donor injected with newborn mRNA alone (25 ng) gave a mean response of  $111 \pm 32$  nA (10 oocytes), and oocytes injected with adult mRNA alone gave a response of  $1.4 \pm 0.5$  nA (11 oocytes) (figure 7). Thus, the expression of glycine receptors by mRNA from newborn cortex was not significantly reduced by the co-injection of adult mRNA. Similar results were seen for the GABA induced response. Oocytes injected with both messengers showed a mean GABA response of  $86 \pm 24$  nA (12 oocytes), whereas oocytes injected with only newborn mRNA gave a mean response of  $17 \pm 6$  nA (9 oocytes) and oocytes injected with only adult mRNA gave

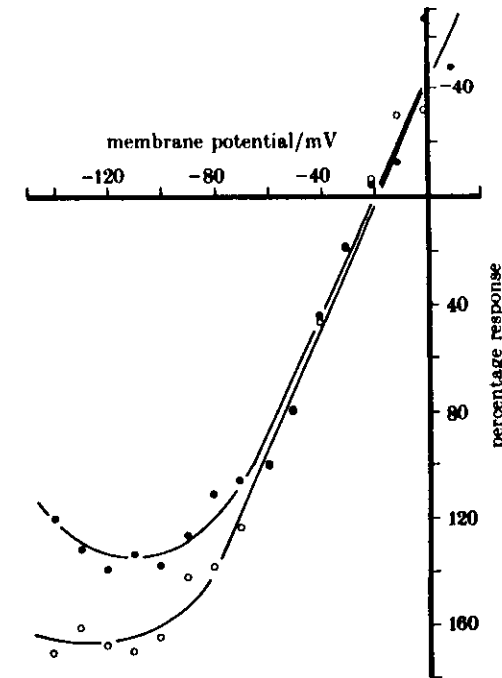


FIGURE 5. Rectification of GABA and glycine currents. Current-voltage relation of glycine (filled circles) and GABA (open circles) responses from an oocyte injected with P5 mRNA. Measurements were obtained by briefly stepping the oocyte to different membrane potentials during steady application of the agonists (GABA,  $10^{-6}$  M; glycine,  $4 \times 10^{-4}$  M). Points indicate the drug-induced current at each potential after the subtraction of passive membrane currents evoked by the same voltage steps in the absence of agonists and are expressed as a percentage of the drug-induced current at a holding potential of  $-80$  mV.

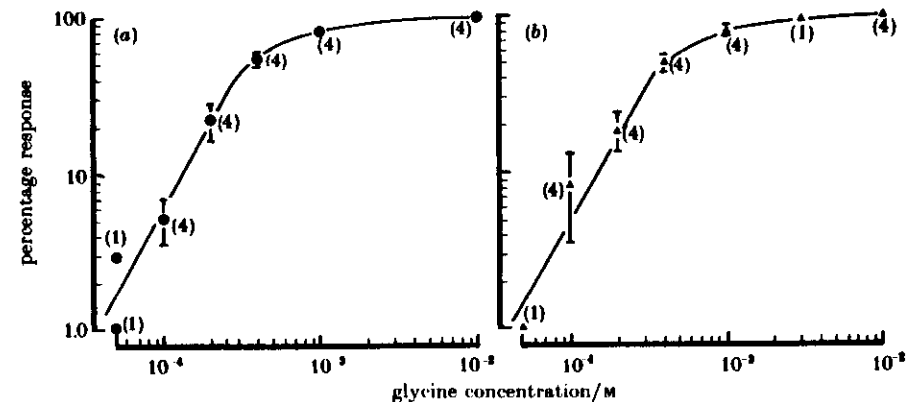


FIGURE 6. Glycine dose-response curves obtained in oocytes injected with E18 (a) or P10 (b) cerebral cortex mRNA. Each point represents one value or the mean of four values (actual numbers are in parenthesis next to each point). Error bars indicate standard error. Two E18 mRNA preparations and one P10 mRNA preparation were used. All oocytes came

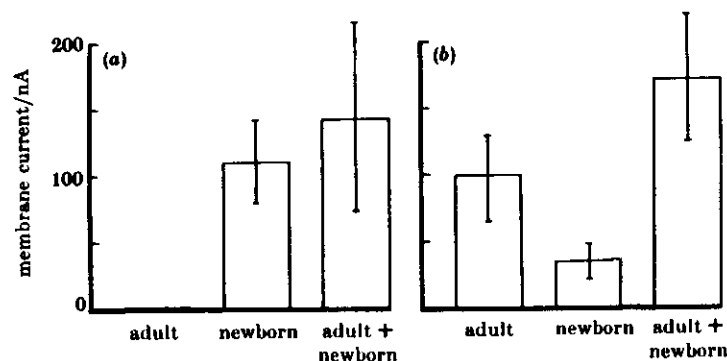


FIGURE 7. Peak sizes of membrane currents induced by glycine ( $10^{-3}$  M) (a) and GABA ( $10^{-3}$  M) (b), recorded from oocytes injected with rat cortex mRNAs from adult brain alone (25 ng); newborn brain alone (25 ng), or a mixture of adult mRNA (25 ng) plus newborn (25 ng). Bars indicate mean  $\pm$  standard error from 8–12 oocytes from the same donor.

#### DISCUSSION

GABA and glycine are the two major neurotransmitters underlying fast inhibition in the central nervous system. In the adult rat, GABA is found in highest concentrations in the grey matter of the brain, whereas glycine is most concentrated in the grey matter of the ventral spinal cord and the brain stem, but is present at only low concentrations in the cerebral cortex and cerebellum (Alger 1985). In agreement with this, we had previously found that poly(A)<sup>+</sup> mRNA from adult rat cerebral cortex induced the appearance of GABA and glycine receptors in the oocyte but the sensitivity to GABA was greater than that to glycine (Sumikawa *et al.* 1984, 1986). In contrast, the main finding in the present study is that in younger animals (i.e. E18, newborn, P5) the opposite case exists, i.e. the cerebral cortex mRNA induces greater sensitivity to glycine than to GABA.

The oocytes were injected with roughly constant amounts of mRNAs, which were also actively translated in the cell-free system. Hence the simplest interpretation is that changes in the size of a particular response elicited in oocytes injected with mRNAs isolated from different ages reflects changes in the proportion of mRNA coding for that receptor. Our results, therefore, suggest that in the rat cerebral cortex the quantity of mRNA coding for GABA receptors increases during embryonic and postnatal development; whereas the quantity of mRNA coding for glycine receptors reaches a maximum at birth and then declines with further development.

In addition to the amount of mRNA, there may be other factors affecting the size of the responses induced in the oocytes. For example, the efficiency of translation of different messengers may vary with age; or proteins that regulate the expression of the receptors may also be translated in the oocyte. Therefore, it could be that mRNA extracted from adult cerebral cortex contains the same proportion of mRNA coding for glycine receptors as the newborn mRNA, but may

also include mRNA coding for a protein that inhibits the production of functional glycine receptors. However, this possibility is unlikely because the glycine responses induced by injection of newborn mRNA were not significantly reduced by the co-injection of mRNA from adult cortex.

It is also possible that the changes we observed are partly due to changes in the lifetime and conductance of the GABA- and glycine-activated membrane channels expressed by the different mRNAs. All this notwithstanding, the most likely explanation of our results is that, during development, the amounts of mRNAs coding for GABA and glycine receptors change, essentially as the size of the currents they express in the oocytes. Although the amount of messenger RNA coding for a particular receptor may vary with age this might not necessarily correspond directly to the number of receptors in the surface membrane of brain cells, because there may be other mechanisms in the mammalian neuron which control translational activity, the rate of assembly, membrane insertion, or degradation of receptors. It is therefore interesting to compare our results with what is known about the number of GABA and glycine receptors in the developing brain.

Studies of the appearance of GABA-benzodiazepine receptors in the rat cerebral cortex have been made measuring the binding of [<sup>3</sup>H]GABA (Coyle & Enna 1976; Aldinio *et al.* 1980). Their results are qualitatively similar to ours in that the number of GABA receptors was found to increase from low levels before birth to reach adult levels by about P21.

On the other hand, little is known about the developmental appearance of glycine receptors in the rat cortex. Benavides *et al.* (1981) measured [<sup>3</sup>H]-strychnine binding in the whole rat brain, and found low levels in the newborn, rising to adult levels by about P15. This differs from our conclusion that mRNA coding for glycine receptors is maximal at birth and falls thereafter; but the results may not be comparable because the strychnine binding was measured in the whole brain, rather than the cortex, and there is uncertainty as to whether strychnine binds to all glycine receptor sites (Bristow *et al.* 1986).

For all ages examined the equilibrium potential for GABA and glycine action corresponded to the chloride equilibrium potential in *Xenopus* oocytes and was similar to that for GABA-activated currents induced by chick optic lobe mRNA (Miledi *et al.* 1982), and glycine-activated currents induced by fetal human brain mRNA (Gundersen *et al.* 1984). Moreover, the channel rectification seen in oocytes injected with mRNA from the developing brain was similar to that reported previously (Gundersen *et al.* 1984; Parker *et al.* 1986). Thus, it seems that from the moment they first appear during development the GABA and glycine mRNAs express receptors that are permeable mainly to chloride ions and behave essentially like those coded by mRNA from the adult brain.

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