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Neurotransmitter receptors and voltage-dependent Ca^{2+} channels encoded by mRNA from the adult corpus callosum

(glutamate receptors/acetylcholine receptors/*Xenopus* oocytes/glia)

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ABSTRACT **The presence of mRNAs encoding neurotransmitter receptors and voltage-gated channels in the adult human and bovine corpus callosum was investigated using *Xenopus* oocytes. Oocytes injected with mRNA extracted from the corpus callosum expressed functional receptors to glutamate, acetylcholine, and serotonin, and also voltage-operated Ca^{2+} channels, all with similar properties in the two species studied. Acetylcholine and serotonin elicited oscillatory Cl^- currents due to activation of the inositol phosphate–Ca^{2+} receptor–channel coupling system. Glutamate and its analogs *N*-methyl-D-aspartate (NMDA), kainate, quisqualate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) induced smooth currents. The non-NMDA responses showed a strong inward rectification at positive potentials and were potently blocked by 6,7-dinitroquinoxaline-2,3-dione, as observed for the AMPA/kainate glutamate receptors GLUR1 and GLUR3. Furthermore, *in situ* hybridization experiments showed that GLUR1 and GLUR3 mRNAs are present in corpus callosum cells that were labeled with antiserum to glial fibrillary acid protein and that, in primary cell cultures, had the morphology of type 2 astrocytes. These results indicate that glial cells in the adult corpus callosum possess mRNA encoding functional neurotransmitter receptors and Ca^{2+} channels. These molecules may provide a mechanism for glial–neuronal interactions.**

Glial cells are thought to participate in a large variety of functions in the nervous system, though most of these have not been clearly elucidated (1–3). In the last decade, many studies of glial physiology have concentrated on glial–neuronal interactions. Particularly interesting was the discovery of various neurotransmitter receptors and voltage-dependent channels in glial cells indicating that they might be sensitive to a large array of neuronal signals (4–6). However, most of that work was done in cultures of murine cells originating from the developing brain, or acutely dissociated cells, and it is known that the procedures used to isolate the cells, as well as the culture process itself, can alter the types and properties of the receptors and channels (7, 8). To avoid some of these problems we have used a different approach to gain additional information regarding the type and functional properties of receptors and channels expressed in mature glial cells, including those present in the adult human brain. Using electrophysiological techniques and *Xenopus* oocytes, which are a convenient system for the expression of heterologous functional receptors and channels (9), we recently observed that a human astrocytoma possessed mRNAs encoding receptors to glutamate and to acetylcholine (AcCho) (10). In the present study, we searched for mRNAs encoding neurotransmitter receptors and channels in the normal adult bovine and human corpus callosum (CC).

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MATERIALS AND METHODS

Immunohistochemistry. Standard immunoperoxidase techniques were applied to characterize the CC preparations used in this study. Blocks of bovine CC were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 days and then kept overnight in 20% sucrose, frozen in dry ice, and coronally cut in a cryostat. Sections (25 μm thick) were incubated with one of the following antibodies: rabbit anti-glial fibrillary acidic protein (GFAP; Dakopatts, Glostrup, Denmark), mouse pan-neuronal marker (SM1311; Sternberger–Meyer, Jarrettsville, MD), and mouse anti-microtubule-associated protein 2 (Boehringer Mannheim). The sections were then processed using the avidin–biotin–peroxidase method as described by the supplier (Vectastain; Vector Laboratories).

Isolation of mRNA, Injection into Oocytes, and Electrophysiology. Human CC tissue was obtained from 8 brains at autopsy, 5–24 hr after death. The patients were of both sexes, did not have detectable neurological disorders, and ranged from 36 to 77 years of age. Bovine CC tissue originated from 26 brains obtained at the slaughterhouse and dissected out at 4°C within an hour after death. In all instances, care was taken to remove the indusium griseum and septum pellucidum—i.e., the gray matter over and under the CC, respectively. All samples were snap-frozen immediately after dissection, in liquid nitrogen, and stored at −80°C until used. As controls, samples of bovine cerebral cortex and diencephalon were also taken. mRNA was extracted using the guanidinium/phenol/chloroform method (11), followed by oligo(dT)-cellulose chromatography. A total of 15 preparations was made: 8 from human CC, 3 from bovine CC, 2 from bovine cerebral cortex, and 2 from bovine diencephalon. Techniques for injection of mRNA and for electrophysiological recording from oocytes have been described (12–14). The presence of voltage-gated ion channels was measured by holding the membrane potential at −100 mV and giving pulses to −20 mV for Na^+ or 0 mV for Ca^{2+} channels. Aspartate (100 μM) was applied together with glycine (100 μM) to selectively activate *N*-methyl-D-aspartate (NMDA) receptors (9). Oocytes were injected with 50 nl (2 μg/μl) of mRNA and treated with collagenase 2 days later (15). Agonists and voltage-induced membrane currents were recorded 5–8 days after injection. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), quisqualate, and 6,7-dinitroquinoxaline-2,3-dione (CNQX) were purchased from Tocris Neuramin (Bristol, England). Other reagents were from Sigma. Current amplitudes are given as mean ± SEM.

Abbreviations: AcCho, acetylcholine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CC, corpus callosum; CNQX, 6,7-dinitroquinoxaline-2,3-dione; GFAP, glial fibrillary acidic protein; GLUR, glutamate receptor; 5-HT, 5-hydroxytryptamine; I–V, current–voltage; NMDA, *N*-methyl-D-aspartate.

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Northern and *in Situ* Hybridizations. mRNA was fractionated on a 1% agarose/formaldehyde gel, vacuum-blotted (Bio-Rad) onto a nylon membrane, and subsequently UV-crosslinked. Hybridization with specific probes was carried out at 68°C in 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 5% blocking reagent. Digoxigenin-labeled glutamate receptor GLUR1 and GLUR3 probes were synthesized from *Not* I and *Xho* I linearized plasmids (16, 17). Washings were in 2× SSC/0.1% SDS at 20°C and in 0.5× SSC/0.1% SDS at 65°C. Chemiluminescent immunological detection of label was as described by the supplier.

For *in situ* hybridization, tissue was fixed, frozen, and cut as described for immunohistochemistry. Sections were hybridized overnight at 42°C with specific digoxigenin-labeled probe diluted in a solution containing 50% formamide, 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate/1 mM EDTA), 0.5× Denhardt's solution, 5% dextran sulfate, 0.37% blocking reagent, 0.1% SDS, salmon sperm DNA (100 μg/ml), and yeast tRNA (100 μg/ml). Washings were in 2× SSC at 20°C and in 0.5× SSC at 42°C. Colorimetric immunological detection of label was as described by the supplier. Some sections were incubated with anti-GFAP antiserum and later treated with fluorescein isothiocynate-labeled anti-rabbit antiserum. Astrocyte cultures were prepared from samples of the human tissue used for mRNA extractions. Cells from CC were mechanically dissociated and seeded into culture flasks (Primaria; Falcon). After 6–8 weeks, cells were trypsinized and transferred to 96-well plates. Several days later, the cells were fixed with 4% formalin for an hour and processed as mentioned above. Unless otherwise stated, reagents were purchased from Boehringer Mannheim.

RESULTS

Cellular Characteristics of the CC Preparation. After the initial dissection the CC samples contained neurons from the indusium griseum and from the septum pellucidum (Fig. 1 *A* and *D*). However, most of these areas were later removed so that the samples used for mRNA extractions (Fig. 1 *B* and *E*) were practically devoid of neurons, as assessed by immunohistochemical techniques using antibodies to microtubule-associated protein 2 and the pan-neuronal marker SM1311 (Fig. 1 *B*, *C*, *E*, and lower half of *F*).

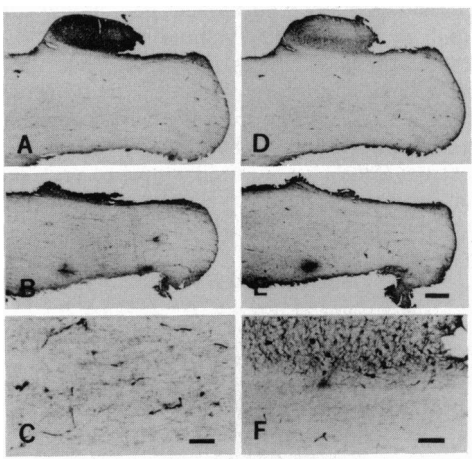

FIG. 1. Coronal sections of the bovine CC stained with antibodies to microtubule-associated protein 2 (*A–C*) and with pan-neuronal marker antibody SM1311 (*D–F*). Normal tissue (*A* and *D*) and tissue used for this study after removal of gray matter (*B* and *E*) are shown. No neurons are labeled within the CC with either antibody (*C* and lower half of *F*), whereas many neurons in the indusium griseum are heavily stained (*A*, *D*, and upper half of *F*). *C* and *F* correspond to areas shown in *B* and *D*, respectively, viewed at a higher magnification. (*A*, *B*, *D*, and *E*, bar = 500 μm; *C* and *F*, bars = 50 and 100 μm, respectively.)

Expression of Neurotransmitter Receptors and Voltage-Activated Channels. Membrane current responses were recorded from hundreds of oocytes taken from >10 frogs. Glycine and γ-aminobutyric acid (both at 1 mM) induced similar small currents (1–3 nA) in control, noninjected oocytes or oocytes injected with human or bovine CC mRNA. No membrane currents were elicited by substance P (200 nM) or by norepinephrine or dopamine (both at 100 μM) in injected oocytes, whereas, in the same oocytes, we observed responses to glutamate, AcCho, and serotonin (5-HT) (Fig. 2). The amplitudes of these responses were at least an order of magnitude smaller than those elicited in oocytes injected with bovine cerebral cortex mRNA extracted from the same brains (not shown). The currents elicited by AcCho and 5-HT were oscillatory in nature and were carried by Cl^- ions. These responses were due to stimulation of the endogenous inositol phosphate–Ca^{2+} receptor–channel coupling system. Therefore, the activated AcCho receptors are of the muscarinic type (9, 13), whereas the subtype of 5-HT receptor expressed still remains to be determined. The injected oocytes also acquired voltage-dependent Ca^{2+} channels (Fig. 2), similar to those encoded by mRNA extracted from rat brain (18), whereas tetrodotoxin-sensitive Na^+ channels were not expressed beyond the small number present in native oocytes (9).

Excitatory Amino Acid Receptors. Glutamate elicited small smooth inward currents in oocytes injected with mRNA from bovine or human CC (10.4 ± 1.9 nA and 6.4 ± 2 nA, respectively; Fig. 2). In contrast, glutamate-induced currents in oocytes injected with mRNA from bovine cerebral cortex were several hundred nA in amplitude and were comprised of smooth and oscillatory components again involving the inosi-

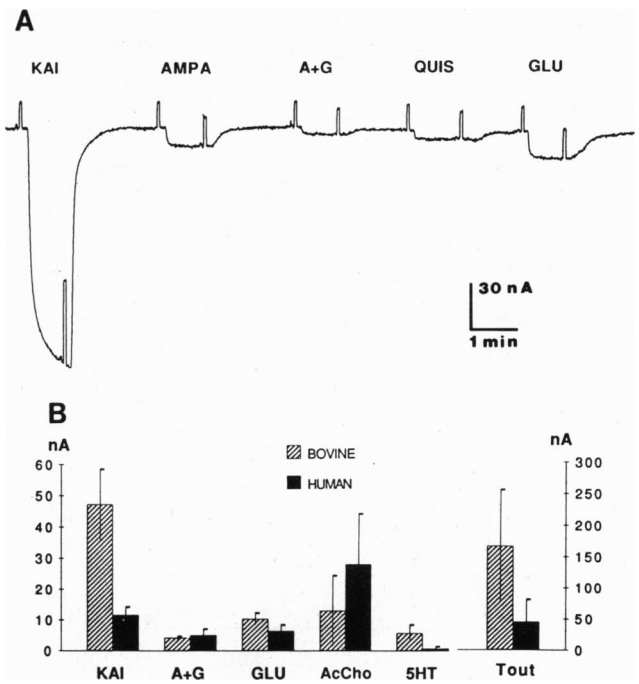

FIG. 2. (*A*) Sample currents elicited by kainate (KAI), AMPA, aspartate plus glycine (A+G), quisqualate (QUIS), all at 100 μM, and 1 mM glutamate (GLU) in an oocyte injected with mRNA from bovine CC. Membrane potential was held at −60 mV. Agonist was applied during the time elapsed between pulses. (*B*) Histogram showing the peak amplitudes of the currents elicited by the corresponding agonists and by stepping the membrane potential from −100 to 0 mV (Tout). Each bar represents the average ± SEM of 3–14 oocytes taken from two to four frogs.

tol polyphosphate/Ca^{2+} system, as observed previously (18–20). The glutamate responses elicited in oocytes injected with mRNAs from human and bovine CC were similar to those expressed in oocytes injected with mRNA extracted from a human astrocytoma (10).

Oocytes injected with CC mRNA also responded to various glutamate analogs: kainate, quisqualate, AMPA, and NMDA (Fig. 2). Hence, it appears that one or more types of excitatory amino acid receptors are encoded by CC mRNA. The kainate responses were blocked in a rapid and reversible manner by CNQX ($IC_{50} \approx 0.4$ μM; Fig. 3). We also observed small NMDA (i.e., aspartate plus glycine) responses in oocytes injected with bovine or human CC mRNA (4.2 ± 0.5 nA and 5 ± 1.9 nA, respectively; Fig. 2). Occasionally, the NMDA currents were up to about 20 nA, whereas aspartate and glycine applied separately did not elicit appreciable currents in the same oocytes.

Properties of the Kainate Receptors. Dose–response curves were constructed for the currents elicited by kainate (Fig. 4*A*). In oocytes injected with mRNA extracted from human or bovine CC, as well as bovine cerebral cortex and diencephalon, kainate responses were detectable at a concentration of 5 μM and saturated at, or slightly above, 1 mM. The concentration of kainate required to elicit half-maximal responses (EC_{50}) from the receptors encoded by CC mRNA was around 150 μM, close to that observed for receptors expressed by mRNA from a human astrocytoma (10), and by mRNAs from human fetal brain (21) and mouse cortical neurons developing in culture (22). In contrast, kainate receptors expressed by mRNA from bovine retina (23), bovine cerebral cortex, and diencephalon (not shown) showed EC_{50} values that were three to four times smaller.

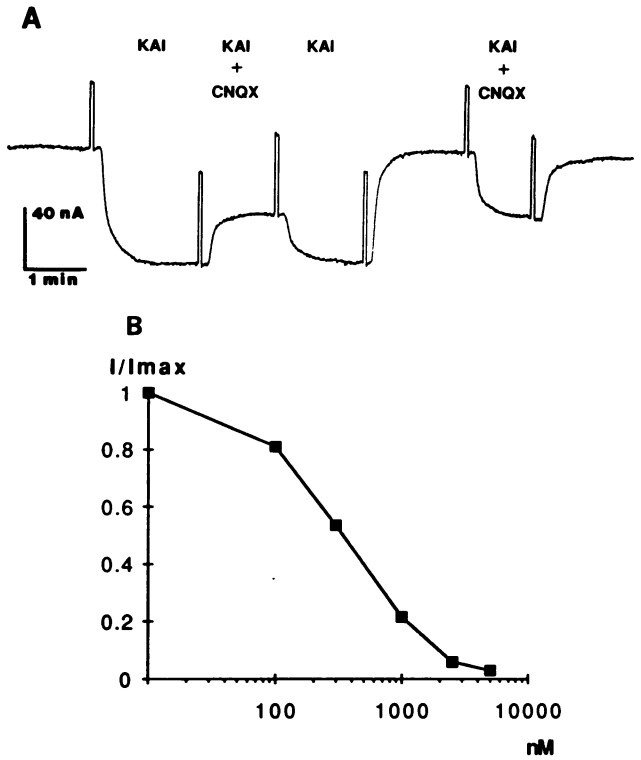

FIG. 3. (*A*) Inhibitory effect of 300 nM CNQX on current elicited by 100 μM kainate (KAI) in an oocyte injected with mRNA from bovine CC. Agonist and blocker were applied during the time elapsed between pulses. (*B*) Blocking effect of CNQX on kainate (100 μM) responses. Data points represent average membrane currents in eight oocytes taken from two frogs (SEM is smaller than the size of simbols). Values were normalized with respect to the current obtained with kainate alone.

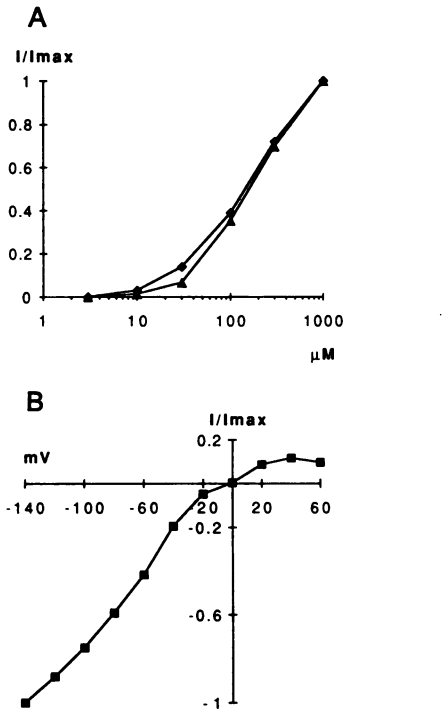

FIG. 4. (*A*) Normalized dose–response curves of membrane currents elicited by kainate in oocytes injected with bovine (▲, nine oocytes from two frogs) and human (◆, two oocytes from one frog) CC mRNA (SEM < 0.03). (*B*) Voltage dependence of the membrane currents elicited by kainate (1 mM) in oocytes injected with bovine CC (average from four oocytes from two frogs, SEM < 0.05). Responses were normalized with respect to the current obtained at −140 mV.

The Hill coefficients of the kainate responses in oocytes injected with mRNA from CC were around 1.4 in both species.

The kainate current–voltage (I–V) relationships had an equilibrium potential close to 0 mV (Fig. 4*B*), similar to that observed previously in oocytes injected with mRNA from bovine retina (23) or human brain (21). This finding indicates that the channels opened by kainate had similar ionic selectivities and were permeable mainly to Na^+ and K^+ (18). Furthermore, the I–V curves of the kainate receptors encoded by CC mRNA were strongly rectifying at positive potentials (Fig. 4*B*), a feature shared by kainate receptors expressed in oocytes injected with mRNA extracted from a human astrocytoma (10). A similar inward rectification has been seen recently for kainate receptors present in glial cells of the cerebellar cortex (24, 25) and in oocytes injected with

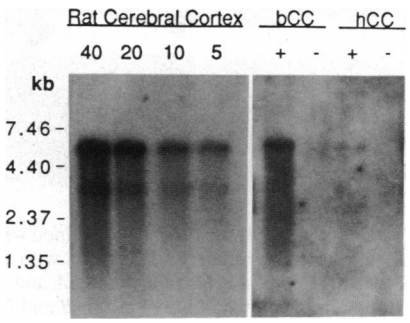

FIG. 5. Northern blot analysis of mRNA from cerebral cortex and from CC for the presence of GLUR1 message. Numbers at the top indicate the amount (in ng) of cerebral cortex mRNA loaded. Two micrograms of mRNA from CC per lane was used. b, Bovine; h, human; + and −, poly(A)$^+$ RNA and poly(A)$^-$ RNA, respectively.

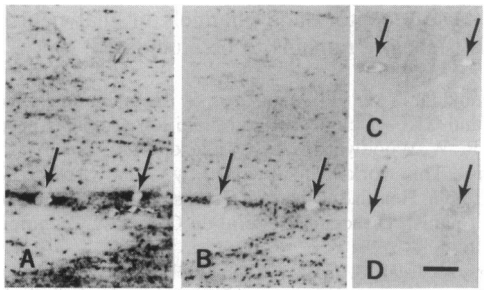

FIG. 6. *In situ* hybridization labeling in four neighboring coronal sections of the bovine CC using GLUR1 complementary RNA (*A*), GLUR3 complementary RNA (*B*), GLUR1 sense RNA (*C*), and GLUR3 sense RNA (*D*) probes labeled with digoxigenin. Arrows point to the same two blood vessels shown in the four photographs. (Bar = 200 μm.)

some combinations of the subunits of the cloned AMPA/kainate receptors (17, 26–28). Overall, the electrophysiological properties of the kainate receptors expressed by CC mRNA suggested that they might be molecularly related to the GLUR1 and GLUR3 receptor subtypes. Indeed, Northern blot analysis using RNA probes revealed that bovine and human CC contained GLUR1 mRNA in amounts that were much smaller than present in rat cerebral cortex (Fig. 5).

Localization of GLUR1 and GLUR3 Receptor mRNAs. Antisense GLUR1 and GLUR3 RNA probes hybridized with mRNA in cells of the bovine CC (Fig. 6 *A* and *B*), whereas sense probes gave no hybridization signal (Fig. 6 *C* and *D*). Most labeled cells were GFAP positive, suggesting that they are astrocytes (Fig. 7 *A* and *B*). To characterize further the type of astrocytes expressing the receptors, we cultured CC cells, from the same human brains used for mRNA extractions, and those cells were then used for *in situ* hibridization. The results show that GLUR1 and GLUR3 mRNA are present in a stellate, process-bearing population of cells with morphology similar to that of type-2 astrocytes (Fig. 7*C*). These findings are consistent with data indicating that GLURs occur *in vitro* in the precursor cell O-2A and type-2 astrocytes from the optic nerve and cerebellum (7, 8).

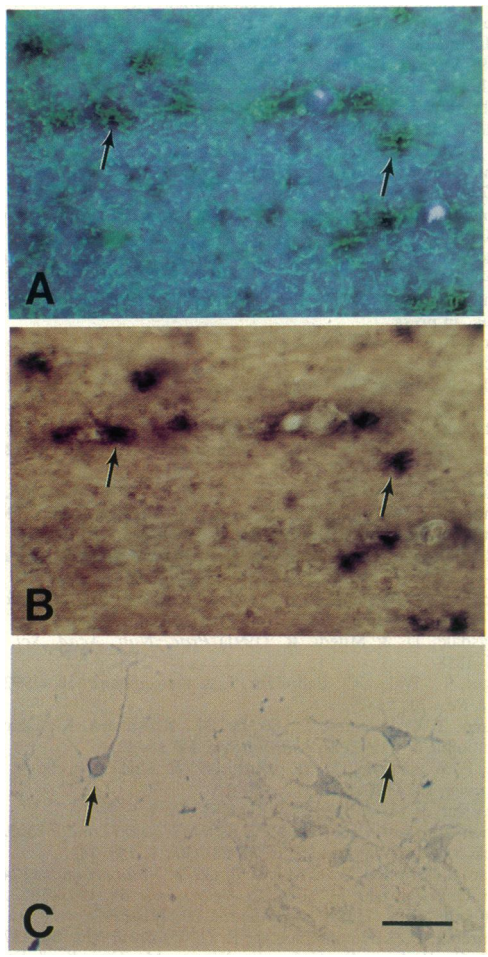

FIG. 7. Astrocytes (arrows) in the same coronal section of bovine CC labeled with anti-GFAP antibodies (*A*) and GLUR1 complementary RNA-digoxigenin (*B*). (*C*) Human process-bearing astrocytes *in vitro* (arrows) stained with GLUR1 complementary RNA probe. (Bar = 50 μm.)

DISCUSSION

Our results show that the bovine CC and the human CC possess mRNAs coding for glutamate, AcCho, and 5-HT receptors and Ca^{2+} channels. The amplitudes of the currents induced by activation of these receptors and channels were rather small when compared to those observed following injection of mRNAs extracted from cerebral cortex and diencephalon from the same brains. Since the amplitude of the current gives a relative measure of the amount of receptor and channel mRNAs in the sample (e.g., ref. 29), the results suggest that the quantity of the respective encoded proteins in callosal cells is much smaller than in gray matter areas. As with previous studies with human brain (21), the expressional potency of the human CC mRNA was particularly weak. It is, of course, possible that the mRNA was somewhat degraded during the postmortem period. However, at least in the rat (30), several postmortem hours may elapse without causing much loss of expressional potency of the mRNAs. Thus, it seems that the differences in the expressional potency of bovine and human CC might reflect some species peculiarity.

Further differences were observed between the channels and receptors encoded by mRNA from CC and other brain areas. For instance, in contrast to mRNA from bovine cerebral cortex and diencephalon taken from the same brains, mRNAs from CC did not induce γ-aminobutyric acid receptors or Na^+-tetrodotoxin-sensitive channels. Also, the dose–response curve of the currents elicited by kainate had a lower EC_{50} than that obtained with mRNA extracted from cerebral cortex and diencephalon.

It is very unlikely that the receptors and channels expressed are of neuronal origin, because this type of cell was virtually absent from the CC samples used in this study. Moreover, double-labeling experiments indicated that GLUR1 and GLUR3 cRNA probes hybridize predominantly with a subpopulation of GFAP-positive cells that probably represents type-2 astrocytes *in vitro*. A number of cells labeled with the GLUR1 and GLUR3 probes were GFAP negative, indicating that they were not astroglial cells, a finding that is consistent with observations suggesting that some oligodendrocytes may express GLURs (31). Thus, the kainate receptors expressed in CC are mostly restricted to astrocytes and appear to be molecularly related to some of the AMPA/kainate clones previously isolated (16, 17, 26, 28).

The kainate receptors encoded by CC glial cells showed a nonlinear I–V relationship, in contrast to more linear I–V curves obtained *in vitro* from rat astrocytes and O-2A progenitor cells (5, 7, 8). These differences may be a consequence of the different methods employed, or they may reflect regional differences in the properties of the receptors. Interestingly, kainate-activated currents with properties resembling those shown in this study, including I–V curves, have been observed recently in developing glial cells of the mouse CC and in cerebellar Bergmann cells using a slice preparation (24, 31). Also consistent with our findings is the fact that the receptors expressed in Bergmann glial cells

appear to be of the GLUR1 and GLUR3 types (25). Therefore, the observations reported here strongly suggest that the GLURs present in adult glial cells of white matter share some properties with those of developing glial cells and also with those occurring in glial cells located in the vicinity of synaptic specializations.

In contrast with previous studies of GLURs in glial cells, our results show definitely the presence of a small amount of mRNA encoding NMDA receptors in the adult CC as well as in a human astrocytoma (10), findings that may be important since NMDA receptor activation induces changes in the levels of nerve growth factor and its receptor mRNAs in C6 glioma cells (32). The discrepancy between these results and those reported by others might be due to the different experimental procedures used (normal adult CC vs. astrocyte cultures and slice preparations from newborn or young animals), to species differences, or to the sensitivity of the assaying techniques.

It is now well established that glial cells are endowed with a variety of voltage-gated channels (6). However, the degree to which these channels are expressed appears to depend on cell type, on species, and also on the procedures used for culturing the cells (33). For instance, Ca^{2+} channels require some experimental intervention before they can be activated by depolarization (34). Using *Xenopus* oocytes, we could overcome these limitations and gain information about the Ca^{2+} channels present in adult CC. The properties of these channels have yet to be fully investigated, but preliminary experiments suggest that they are similar to the neuronal Ca^{2+} channels. No Na^+ channels were observed in this study in accord with findings using acutely isolated hippocampal astrocytes from young adult rats (33) and the lack of such channels in oligodendrocytes (6). In contrast, Na^+ channels have been observed in astrocytes under certain conditions (6). These apparent discrepancies might be due to regional differences in the properties of glial cells. It could also be that glial cells require intimate interaction with neuronal somata in order to express Na^+ channels (7), a condition that might not be fulfilled in the CC because of the lack of neuronal cell bodies.

It has been proposed that neurotransmitter receptors and channels might be critical for glial–neuronal signaling, although their specific functions *in vivo* remain unclear. In Bergmann cells, activation of kainate receptors leads to a concomitant blockade of the resting K^+ conductance that might serve to propagate glutamate signals occurring in the neighboring Purkinje cells (24), although that putative role may not occur in white matter glial cells, which lack synaptic specializations. Nevertheless, the close spatial relation between processes of type-2 astrocytes and nodes of Ranvier (35, 36) points to important interactions. It is known that Schwann cells, associated with cholinergic motor axons, acquire the property of synthesizing and releasing AcCho after the axon has degenerated (37) and glutamate is released from glial cells in culture (38) and from electrically stimulated nerves (39, 40). Thus, the neurotransmitter receptors present in the astrocytes may be activated by neurotransmitters released from axons or from the glial cells themselves and this, in turn, may lead to the release of cytokines and growth factors (41). It may be that such interactions are not primarily concerned with the fast process of nerve impulse propagation but may be involved in slower processes, such as preserving axon–glial integrity or guiding axons during injury repair.

In summary, we provide evidence that mRNA from glial cells of the adult CC expresses various types of neurotransmitter receptors and Ca^{2+} channels in *Xenopus* oocytes. The role of these receptors and channels in glial–neuronal and glial–glial signaling remains to be elucidated.

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