Molecular Neurobiology

ISSN 0893-7648/04/29(1): 61–71/$25.00

Ion Channels and Amino Acid Transporters Support the Growth and Invasion of Primary Brain Tumors

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Abstract

The malignant growth of glial support cells causes gliomas, highly invasive, primary brain tumors that are largely resistant to therapy. Individual tumor cells spread by active cell migration, invading diffusely into the normal brain. This process is facilitated by Cl^- channels that endow glioma cells with an enhanced ability to quickly adjust their shape and cell volume to fit the narrow and tortuous extracellular brain spaces. Once satellite tumors enlarge, their growth is limited by the spatial constraints imposed by the bony cavity of the skull and spinal column. Glioma cells circumvent this limitation by active destruction of peritumoral neural tissue through the release of glutamate, inducing peritumoral seizures and ultimately excitotoxic neuronal cell death. Hence, primary brain tumors support their unusual biology by taking advantage of ion channels and transporters that are designed to support ion homeostatic functions in normal brain.

Index Entries: Glioma; glutamate; excitotoxicity; chloride; cell migration; invasion; metastasis; cell volume; ion channel.

Introduction

Malignancies only develop form growth competent cells, i.e. cells that continue to be replenished throughout life. Since most neurons are postmitotic and cannot be replaced, they do not give rise to tumors. However, support cells in the brain, most notably glial cells, retain the ability to divide throughout life. They do so very rarely and significant glial proliferation ("gliosis") is primarily evident as brain pathology following acute injury *(1,2)* or in association with neurodegeneration *(3).* A number of genetic mutations have been shown to characterize the malignant transformation of glial cells to become gliomas *(4).* These include loss of function of tumor suppressor genes *(5)* or gain of function mutations in oncogenes such as the epidermal growth factor receptor *(6).* While pathologists categorize gliomas as astocytomas, oligodendrogliomas,

Received 6/5/03; Accepted 7/24/03

and glioblastomas amongst others, the precise lineage relationship of either of these tumors to a defined nonmalignant glial cell of origin is tentative. It is possible that astrocytes or oligodendrocytes dedifferentiate and give rise to tumors. Alternatively, these tumors may all originate from undifferentiated glial progenitor cells *(7).* Genetic manipulation of glia progenitor cells has indeed been shown to yield gliomas in mice that present with some of the hallmarks of human gliomas *(8).* Gliomas constitute a fascinating class of malignancies characterized by unique features not found in other systemic tumors and two of these features are discussed in this article.

Firstly, gliomas can spread rapidly and invade diffusely into normal brain. They do not use hematogenous spread as is common amongst other tumors but instead migrate actively through the narrow extracellular spaces in brain. This process is akin to the directed migration of glial and neuronal progenitor cells during development. In this process, glioma cells digest the extracellular matrix using matrix metalloproteinases *(9)* and can lay down their own matrix molecules that are more conducive to cell migration *(10).* Guidance cues used by gliomas are poorly understood *(11).* However, cells appear to migrate along nerve fibers and blood vessels *(12)* and certain substrates *(13)* or blood-born molecules enhance their motility *(14).* Migration along the corpus callosum is frequent allowing interhemispheral spread (*see* Fig. 1A). In light of the physical constraints in the mature, myelinated brain, the rapid spread of these tumor cells is astonishing. The intact extracellular space is believed to be only 20 nm in width, just a tiny fraction of a cells' size *(15).* Recent evidence suggests that glioma cells are endowed with Cl^- channel that allow them to rapidly secrete their cytoplasm thereby adjusting cell size and molding their shape to fit through the tortuous spaces in the extracellular space *(16).*

A second, equally fascinating specializations of gliomas, relates to their growth within the confines of the brain and spinal cord. Unlike systemic tumors which insert themselves into soft tissue where their growth does not become physically limited, the growth of malignant gliomas is confined to the bony cavity of the skull and spinal cord. This creates a number of serious consequences. Tumor growth often pushes brain tissue into the ventricular space thereby obstructing the normal flow of cerebrospinal fluid (Fig. 1A). Furthermore, tumor expansion ultimately requires the destruction of normal brain tissue to produce more room for the growing tumor mass (Fig. 1A). Hence, unlike systemic tumors that expand without active destruction of normal organ tissues, primary brain tumors grow on the expense of the normal brain. This mode of expansion has recently been demonstrated to involve the release of glutamate from tumor cells invoking an excitotoxic neuronal death pathway *(17,18)* similar to that previously described for other brain pathologies *(19,20).*

Glioma Cell Invasion Requires Cl^- Channel-Mediated Cell Shrinkage

At the time of clinical presentation, gliomas have seeded tumor cells throughout the brain. These cells actively migrate and navigate the tortuous extracellular brain spaces. Electron-microscopic evidence suggests that glioma cells adjust their shape to better fit these narrow extracellular spaces, as invading glioma cells appear wedge-shaped and elongated (Fig. 1B). It was hypothesized that this change in cell shape resembles a change in overall cell volume *(16),* and that this volume change is accomplished by the secretion of Cl^- ions with obligated water following the outward directed gradient for Cl^- (Fig. 1C).

This hypothesis has gained significant experimental support. Firstly, glioma cells show prominent surface expression of known Cl^- channels (CIC) of the ClC superfamily as illustrated for three representative examples in Fig. 1D. Importantly, glioma cells have an unusually

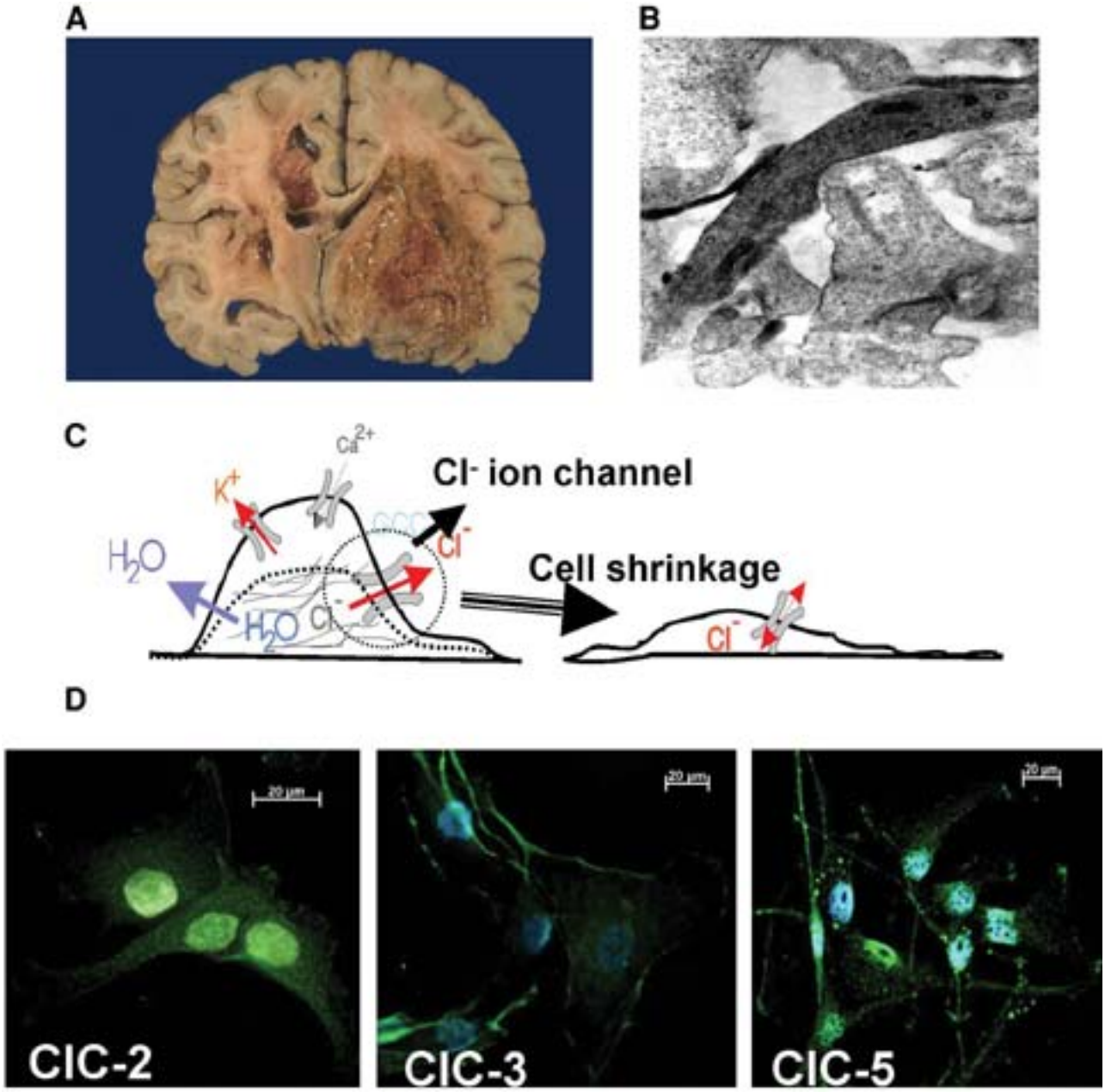
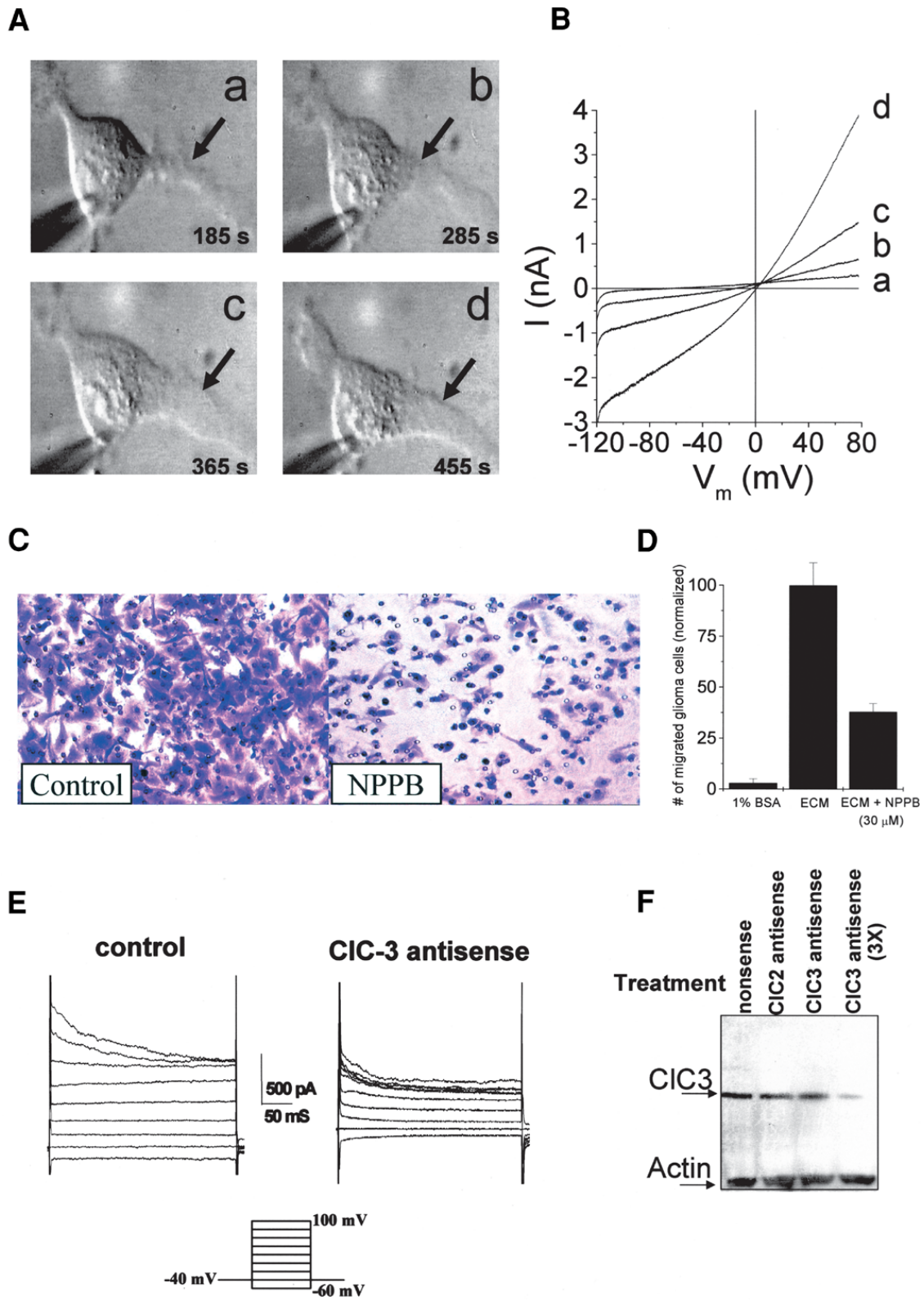


Fig. 1. Gliomas are characterized by relentless growth and invasion into normal brain. **(A)** Coronal section of a glioblastoma multiform by autopsy. The tumor shows massive tissue destruction as evidenced by prominent areas of necrosis and hemorrhage. Note also that this tumor has crossed the midline to the opposite hemisphere showing satellite tumors on the contralateral hemisphere. **(B)** Representative electronmicrograph (14,400 magnification) of glioma cells invading brain tissue demonstrates an elongated, wedge-like shape. This shape change is associated with overall cell shrinkage **(C),** and serves to facilitate invasion into the tortuous extracellular brain spaces. Cell shrinkage requires efflux of Cl^- through ion channels along with obligated water. **(D)** Representative immunostainings of human glioma cells with antibodies specific to ClC-2, ClC-3, and ClC-5 captured with a confocal microscope showing prominent surface expression of these channels. (From, WebPath, The University of Utah Internet Pathology Laboratory; also, *see* refs. *29,37.*)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-40 mV
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ClC2 antisense
ClC3 antisense
ClC3 antisense (3X)
ClC3
Actin

Fig. 2. Glioma cells express functional Cl^- channels that are essential for cell migration/invasion. **(A)** Cultured glioma cells often extend processes while being recorded with a patch-clamp pipet. **(B)** Process extension (arrows in A) correlates with the activation of Cl^- currents (time points indicated in small letters). **(C)** The dependence of cell migration on functional Cl^- channels was examined by a two chamber, Transwell migration assay. Under control conditions, the vast majority of glioma cells (trypan blue stained) transverse through 5 μm pores separating the two compartments (C, left) while in the presence of the Cl^- channel inhibitor NPPB (30μ*M*) cell migration is retarded (C, right) with numerous glioma cells stuck in the pores. **(D)** The relative cell migration for control (ECM = extracellular matrix) and drug-treated cells (ECM + NPPB) was compared to complete inhibition of cell migration in the absence of ECM (BSA = bovine serum albumin). **(E)** ClC-3 mediated outward currents and **(F)** ClC-3 protein content were markedly reduced by antisense treatment of glioma cells with specific antisense oligonucleotides. (*See* refs. *29,30.*)

high resting Cl^- permeability *(21)* that is enhanced as cells begin to extend process and migrate. This is evident in Figs. 2A and B, which show a glioma cell that is recorded with a patch-clamp electrode. As the cell extends a leading process (arrow), Cl^- currents become activated (Fig. 2B). The timecourse of process extension correlates well with the activation of the current. These currents are inhibited by the Cl^- channel inhibitor 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) which also inhibits process extension and cell migration/invasion. Indeed, when glioma cells are challenged to migrate across a Transwell barrier membrane, containing 5 μ*M* pores that separates two compartments (Fig. 2C), this migration/invasion was greatly retarded by NPPB (Fig. 2D).

To further substantiate the notion that known Cl^- channels contribute to glioma cell migration/invasion, a recent study used specific antisense oligonucelotides to demonstrated that whole-cell Cl^- currents were at least in parts mediated by ClC-2 *(22,23)* and ClC-3 (Fig. 2E, F). Treatment of glioma cells with antisense to ClC-3 selectively reduced the expression of ClC-3 channel protein (Fig. 2F) and reduced outward currents by approx 50% (Fig. 2E), while antisense to ClC-2 essentially eliminated Cd^{2+} sensitive inward Cl^- currents and decreased the resting Cl^- permeability threefold *(24).* The notion that glioma-cell invasion requires permeation of Cl^- through ion channels is further supported by ion replacement studies in which Cl^- was replaced by other anions. Cell migration across a barrier continued unabated if Cl^- was replaced with Br^- or NO_3^-, both anions known to permeate Cl^- channels, whereas cell migration was much reduced if Cl^- was replaced by gluconate or glutamate, which have much lower permeability *(16).* These studies collectively suggest that Cl^- channels, most likely of the ClC family, support cell-volume changes required for cell invasion. On another note, examination of biopsy tissues from patients demonstrated prominent expression of ClC Cl^- channels in human gliomas *(24)* but not surrounding normal brain.

Since glioma-cell invasion depends critically on the activity of Cl^- channels, one may speculate that pharmacological inhibition of these channels may be a suitable therapeutic intervention. Unfortunately, most Cl^- channel inhibitors, including the above mentioned drugs, have poor specificity and also inhibit Cl^- channels with important functions in other organs such as the kidney or stomach *(25).* A small putative Cl^- channel specific peptide, Chlorotoxin (Cltx), has been isolated from scorpion venom *(26)* and shown to inhibit Cl^- channels in glioma cells *(27).* A screen through patient biopsies revealed that biotin-labeled Cltx binds prominently and selectively to malignant gliomas and to a number of embryologically related tumors of neuroectodermal origin *(28).* When applied to various in vitro or *in situ* migration and invasion assays, Cltx showed marked inhibition on cell invasion *(16).*

Based on its anti-invasive properties, and specificity for glioma cells, Cltx has entered a Phase I/II clinical trial for patients with

advanced stage gliomas *(29).* The molecular interaction of Cltx with Cl^- channels is not fully understood. A recent study suggests that Cltx binds to and inhibits large conductance Cl^- channels in reactive astrocytes *(30),* however, as is the case in glioma cells *(27,31),* inhibition of channels required several minutes of exposure to the peptide to develop. This time-course of block is more suggestive of an indirect interaction of the peptide with the ion channel. Affinity purification of the principle receptor for Cltx on the surface of glioma cells isolated Matrix-metalloproteinase-II (MMP-2) as one major binding site *(32).* MMP-2 is a membrane-associated enzyme that is secreted by gliomas to digest the extracellular matrix *(9).* Binding of Cltx induced the endocytosis of MMP-2 into caveloi *(32)* thereby effectively removing the enzyme responsible for matrix degradation. Binding of Cltx to MMP-2 came as a surprise with regards to its presumed Cl^- channel inhibition properties and ongoing studies are reevaluating the interaction of MMP-2 with Cl^- channels. MMP-2 has been shown to regulate the CFTR Cl^- channels in human airways *(33)* and hence MMP-2 may have a more general regulatory role for Cl^- channel function.

The notion that Cl^- channels regulate cell volume in migrating cells may have much broader applicability and may not be restricted to invading brain tumor cells. In many respects, migrating glioma cells mirror the migration of progenitor cells during embryonic brain development *(34–36),* suggesting that glioma cells may recapitulate some features of gliogenesis or neurogenesis *(34,37)* and therefore could serve as a model system to study mechanisms involved in the migration of other central nervous system (CNS) cells. Of note, embryonic neurons also show intracellular Cl^- accumulation, which disappears upon cell differentiation *(38,39).* Hence it is conceivable that activation of Cl^- channels, including GABA-gated Cl^- channels on progenitor cells may similarly aid cell shape and cell volume changes of migratory progenitors cells. Indeed, it is possible that the depolarizing GABA response of immature neurons is primarily a consequence of a more general requirement for intracellular Cl^- accumulation in migratory cells.

The Growth of Primary Brain Tumors is Dependent on the Excitotoxic Release of Glutamate

In addition to their invasive behavior, gliomas have a tendency to expand very rapidly often putting pressure on surrounding brain tissue. A shift of the midline and obstruction of the ventricular space is therefore common (Fig. 1A). Ultimately, however, the space available for tumor growth becomes limiting, requiring the destruction of surrounding brain tissue. A recent study evaluated the significance of peritumoral cell death or necrosis and found that it resembled the strongest prognostic variable for these patients. Glioblastoma multiform (GBM) patients with little or no necrosis on preoperative magnetic resonance imaging (MRI) survive longer than patients with greater amounts of necrosis *(40).* The mechanism by which growing gliomas induce peritumoral necrosis has recently been elucidated. Surprisingly, glutamate neurotoxicity is at the heart of the problem *(17,18).* Unlike their nonmalignant counterparts that maintain low extracellular [Glu] in the face of significant neuronal release of Glu *(41),* glioma cells lack functional Na^+-dependent Glu transporters *(42)* and are thus not contributing to extracellular Glu homeostasis. To the contrary, glioma cells produce and release Glu into their environment. Indeed, the rate of glutamate release by glioma cells is astonishing, with a monolayer of cells elevating [Glu] in a 70 cc culture flask from 1 μ*M* to approx 500 μ*M* within just a few hours (Fig. 3A). This release increases with increasing extracellular cystine concentrations (Fig. 3B). When cultured together with neurons, glioma cells induce classic excitotoxicity as evidenced by uncontrolled and sustained elevations of neuronal $[Ca^{2+}]$ and NmDA-R dependent cell death (Fig. 3C–F). When glioma

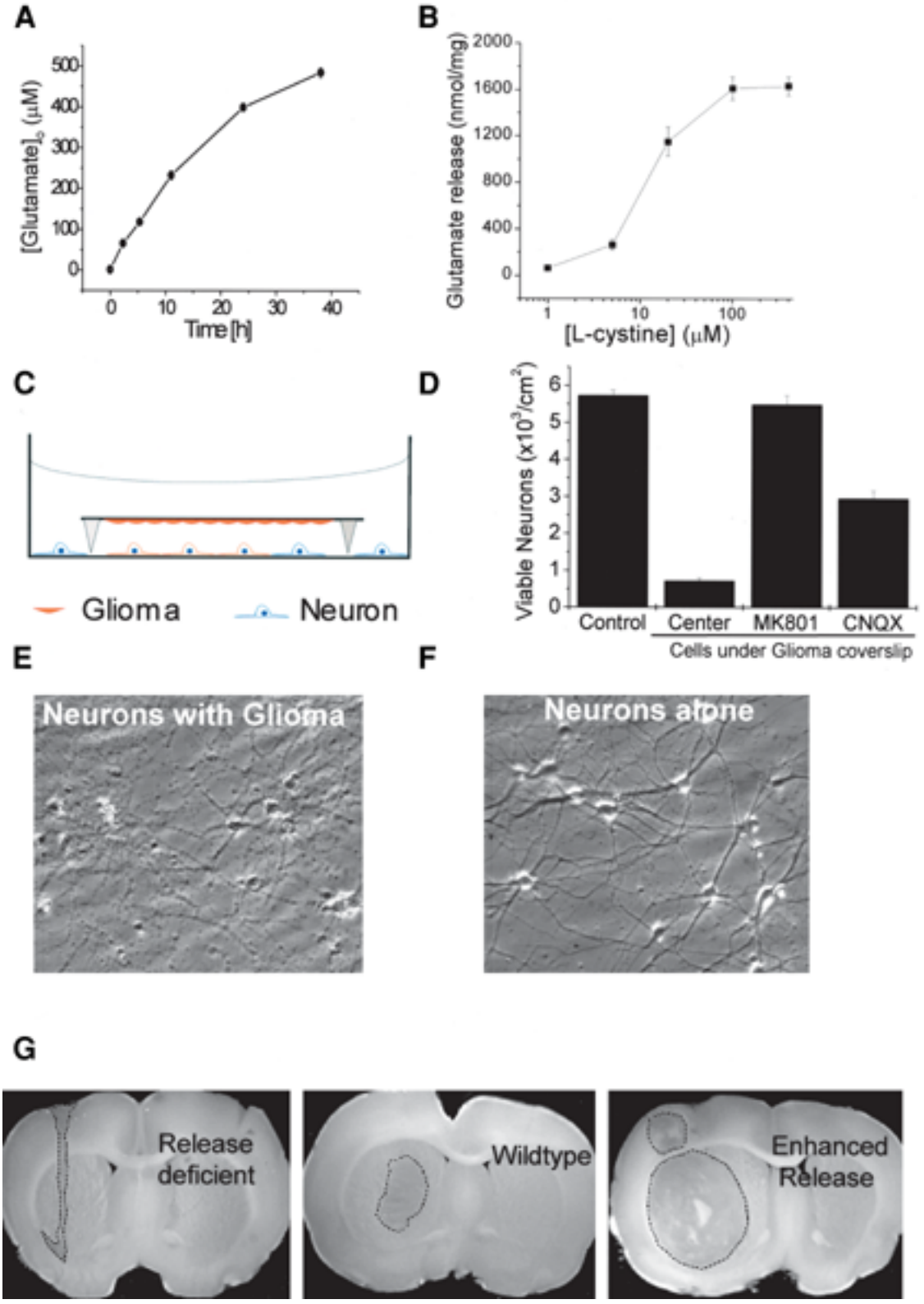

Fig. 3. Glioma cells release excitotoxic concentrations of glutamate. **(A)** Glioma cells release GLU into the medium, as shown by the progressive, time-dependent increase in GLU content. This release is dependent on extracellular cystine **(B).** Hippocampal neurons placed in a culture dish underneath a coverslip containing a monolayer of glioma cells **(C–F)** show pronounced cell death **(D,E)** that can be prevented by MK801 (D) or partially by CNQX (D), suggesting excitotoxic neuronal death. **(G)** Injection of glioma cells into rats yields significant tumor growth only in glioma cells capable of GLU release. (*See* refs. *38,47,48.*)

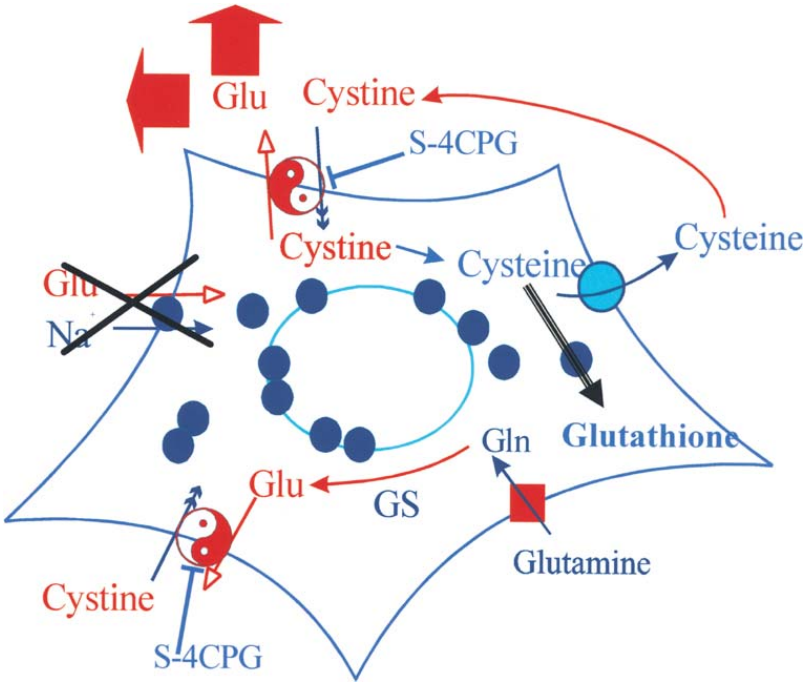

Fig. 4. Glutamate dynamics in gliomas. Pharmacological evidence suggests that the release of excitotoxic glutamate from glioma occurs via cystine-GLU exchange that can be inhibited by S-4CPG. GLU release is a byproduct of cystine uptake which serves to synthesize the antioxidant glutathione. The absence of Na^+-dependent Glu uptake in gliomas leads to an accumulation of Glu in the extracellular space.

cells are implanted into the rat brain, only those strains of gliomas that are able to release Glu are capable of growing solid tumors (Fig. 3G) and these will kill the animals within 7–10 wk *(17)*. On the other hand, cell lines deficient in Glu release fail to grow tumors and animals are uncompromised.

The pathway for glutamate release from glioma cells has also been identified *(42)*. A reversal of Na^+-dependent Glu transporters has been implicated in excitotoxicity in association with stroke *(43)*. However, these transporters are not functional in glioma cells. Instead, the pathway of release appears to be a neutral amino acid transporter that is responsible for the supply of cystine to the cell. Specifically, the recently cloned cystine-Glu exchanger *(44)*, also known as system X^c, is upregulated in glioma cells *(45)* and induced by oxidative stress. Its primary mode of operation is the supply of the essential amino acid cystine for the synthesis of glutathione (Fig. 4). The release of Glu and its accumulation in the extracellular space appears to be an advantageous byproduct of this transport. Enhanced levels of glutathione are believed to make these tumors more resistant to chemotherapeutic reagents *(46)*. Interestingly, a number of glutamate analogs, which are chemical derivatives of phenyl-glycine and belong to the family of metabotropic glutamate receptor agonists, are also competitive inhibitors of cys-Glu exchange in gliomas *(18,47)*. These effectively inhibit Glu release from gliomas and prevent the ensuing neuronal cell death *(18)*. Similarly, excitotoxicity can be prevented by blockade of neuronal NmDA receptors such as MK-801 or memantine *(17)*. Hence, these data propose at least two novel therapeutic strategies to contain the growth of primary brain tumors: (1)

The release from tumors may be inhibited by drugs that interfere with cys-Glu exchange; and (2) NMDA-R inhibitors may be employed to ameliorate peritumoral necrosis. Of note, approx 30% of glioma patients present with intractable seizures *(48)* which are most likely a direct consequence of Glu release from the tumor. The above noted strategies have the potential to control these peritumoral seizures.

As these two examples illustrate, primary brain tumors have adopted the function of two classes of proteins and misdirected them to advance their own biology. In both cases, a better understanding of the underlying tumor biology may now serve to provide novel and possibly tumor-type specific intervention methods. In addition, the intriguing similarity of some of the behavior of gliomas to immature progenitor cells in the brain suggests that these tumors may serve as a valuable model system to study certain aspects of developmental neurobiology.

Acknowledgments

The author is grateful for the continued supported by grants from the National Institutes of Health RO1 NS-31234, RO1 NS-36692, PO1-HD38760, and P50-CA97247.

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