The Journal of Neuroscience, September 1, 2002, 22(17):7373–7379

Selective Electrical Silencing of Mammalian Neurons *In Vitro* by the Use of Invertebrate Ligand-Gated Chloride Channels

Eric M. Slimko,[1] Sheri McKinney,[2] David J. Anderson,[2] Norman Davidson,[2] and Henry A. Lester[2]

[1]*Computation and Neural Systems Program and* [2]*Division of Biology, California Institute of Technology, Pasadena, California 91125*

Selectively reducing the excitability of specific neurons will (1) allow for the creation of animal models of human neurological disorders and (2) provide insight into the global function of specific sets of neurons. We focus on a combined genetic and pharmacological approach to silence neurons electrically. We express invertebrate ivermectin (IVM)-sensitive chloride channels (*Caenorhabditis elegans* GluCl α and β) with a Sindbis virus and then activate these channels with IVM to produce inhibition via a Cl^- conductance. We constructed a three-cistron Sindbis virus that expresses the α and β subunits of a glutamate-gated chloride channel (GluCl) along with the green fluorescent protein (EGFP) marker. Expression of the *C. elegans* channel does not affect the normal spike activity or GABA/glutamate postsynaptic currents of cultured embryonic day 18 hippocampal neurons. At concentrations as low as 5 nM, IVM activates a Cl^- current large enough to silence infected neurons effectively. This conductance reverses in 8 hr. These low concentrations of IVM do not potentiate GABA responses. Comparable results are observed with plasmid transfection of yellow fluorescent protein-tagged (EYFP) GluCl α and cyan fluorescent protein-tagged (ECFP) GluCl β. The present study provides an *in vitro* model mimicking conditions that can be obtained in transgenic mice and in viral-mediated gene therapy. These experiments demonstrate the feasibility of using invertebrate ligand-activated Cl^- channels as an approach to modulate excitability.

Key words: silencing; excitability; hippocampal neurons; chloride channel; Sindbis virus; transfection of neurons; EGFP; EYFP; ECFP

This paper focuses on an approach to understanding the role of individual neuronal cell types in development, information processing, and behavior. Several investigators have suggested that such information can be obtained by using gene transfer *in vitro*, or transgenic animals, to produce spatially and temporally controlled inactivation of specific neurons (White et al., 2001a). Recent studies report the use of transcriptional induction of various K^+ channels (Johns et al., 1999; Falk et al., 2001), and this method silences *Drosophila* neurons or muscle *in vivo* (Paradis et al., 2001; White et al., 2001b; Nitabach et al., 2002), providing useful insights into the role of defined neuronal populations. We previously studied the possibilities for neuronal silencing by using expressed G-protein-gated inwardly rectifying K^+ (GIRK) channels (Ehrengruber et al., 1997), weakly inwardly rectifying K^+ channels (Nadeau et al., 2000), and neuron-restrictive silencing factor (Nadeau and Lester, 2002). However, there are indications that K^+ channels may cause unwanted side effects, such as apoptosis caused by sustained K^+ efflux, in the cultured mammalian CNS neurons that were used to test this strategy (Nadeau et al., 2000).

The emerging genetic techniques seem to present some advantages over previous exclusively pharmacological strategies. Target areas and nuclei indeed have been inactivated *in vivo* via the focal application of a GABA agonist, muscimol (Jasnow and Huhman, 2001; Maren et al., 2001). This procedure has been valuable in determining the role of the amygdala and hippocampus in fear conditioning (Helmstetter and Bellgowan, 1994; Muller et al., 1997; Holt and Maren, 1999; Wilensky et al., 1999, 2000). However, the muscimol strategy suffers from limitations such as the inability to know exactly which neurons were silenced, the inability to silence specific cell types in a given brain region, the inability to silence distributed but functionally related neuronal populations, and the irreproducible localization of focal injections. Also, specific neuronal populations have been ablated irreversibly (Kobayashi et al., 1995; Sawada et al., 1998; Watanabe et al., 1998; Isles et al., 2001), but further insights could arise from a reversible technique.

We suggest a strategy that combines the genetic and acute pharmacological manipulations. Because GABA and glycine are the major inhibitory neurotransmitters in the brain and because $GABA_A$ and glycine receptors inhibit activity by activating a Cl^- conductance, we have explored the possibility of using ligand-activated Cl^- channels as silencing genes. The glutamate-gated chloride channel (GluCl) family of ligand-gated Cl^- channels, found in several invertebrate phyla, is the target for various pesticides, anthelminthic, and antiparasitic drugs, including ivermectin (IVM) (Cully et al., 1994). GluCl channels are part of the nicotinic receptor superfamily, characterized by four transmembrane segments and a large N terminus, and are most similar to $GABA_A$ and glycine receptors. Glutamate is the putative *in vivo* ligand for these channels. No GluCl channels have been detected yet in mammals (Martin et al., 1998; Xue, 1998).

The GluCl channels may represent a promising set of silencing genes. We have used a recombinant Sindbis virus to express these channels in cultured neurons and then tested whether the ex-

Received March 25, 2002; revised June 5, 2002; accepted June 13, 2002.

This work was supported by National Institutes of Health Grants NS 11756 and MH 49176, by the Sidney Stern and Plum Foundations, and by the William T. Gimbal Discovery fund in Neuroscience. We thank Doris Cully for generously supplying the *C. elegans* GluCl channel cDNAs and for discussion; Birgit Hirschberg, Charles Cohen, and Christof Koch for discussion; and Dong Ju for technical assistance.

Correspondence should be addressed to Henry A. Lester, M/C 156-29, Division of Biology, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125. E-mail: lester@caltech.edu.

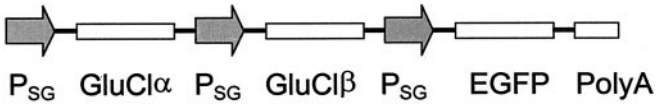

Figure 1. Modified pSinRep5 construct, pSinRep5tsgGluClαβEGFP, designed to express three genes with three subgenomic promoters: the GluCl α subunit, the GluCl β subunit, and the reporter EGFP. The resulting Sindbis virus is vSinGluClαβEGFP.

pressed channels can be activated with high sensitivity and high selectivity by IVM. Indeed, we do find that IVM at low nanomolar concentrations induces a large Cl^- conductance in some infected neurons, effectively clamping the membrane at a potential near the original resting potential. As a result, neither current injection nor focal application of excitatory transmitter elicits action potentials. We also studied possible unwanted additional effects of either GluCl channel expression or IVM application and found these potential side effects to be minimal or nonexistent. The inducible and reversible nature of this inactivation, which we term the GluCl/IVM method, represents a potential powerful new technique for use in probing the contributions of specific regions of the nervous system to the global function.

MATERIALS AND METHODS

Viral vectors and preparation. Wild-type *Caenorhabditis elegans* GluCl α and GluCl β genes (Cully et al., 1994) cloned into pBluescript II SK^+ were a kind gift from Merck Research Laboratories (Whitehead, NJ); the Sindbis vector pSinRep5dsgEGFP was a kind gift from Drs. H. Nawa and M. Kawamura of Niigata University (Niigata, Japan). The oligos pSG1 (5′-6CG ACG TCA TCT CTA CGG TGG TCC TAA ATA GTT TAA ACG CAT G –3′) and pSG2 (5′-6CG TTT AAA CTA TTT AGG ACC ACC GTA GAG ATG ACG TCG CAT G-3′) were annealed and ligated into the *Sph*I site of pSinRep5dsgEGFP, resulting in pSinRep5tsgEGFP. GluCl α was amplified by PCR with the primers 5′-ATA GAT ACG CGT TCA ATA CTG CAT AAA T-3′ and 5′-TAG ATT CAC GTG AAA GCA TTC TCG ATC A-3′ and cloned into the *Mlu*I and *Aat*II sites of pSinRep5tsgEGFP, resulting in pSinRep5tsgGluClαEGFP. GluCl β was amplified by PCR with the primers 5′-AAT GCA GCA TGC ACT ACA CCT AGT TCA T-3′ and 5′-ACC GGT GCA TGC TAT GAT GTT TGC AAA T-3′ and cloned into the *Sph*I site of pSinRep5tsgGluClαEGFP, resulting in pSinRep5tsgGluClαβEGFP (Fig. 1). Then this plasmid was used to generate recombinant Sindbis virus according to the manufacturer's instructions. Briefly, RNA was transcribed from this plasmid and the DB-26S helper plasmid by using the Invitrogen InvitroScript kit (Carlsbad, CA). These two RNA species were transfected by electroporation into baby hamster kidney cells and incubated for 48 hr. The supernatant was collected and filtered at 0.2 μm, and the viral particles (termed vSinGluClαβEGFP) were concentrated by centrifugation at 35,000 rpm for 1 hr.

Neuronal culture. Rat embryonic day 18 (E18) hippocampal neurons were prepared as described previously (Li et al., 1998). Hippocampal cultures then were infected with 1–5 μl of the above virus stocks per 35 mm culture dish containing ~2 ml of medium. Neurons were infected after 12–16 d in culture, and recordings were made 24–48 hr later. For neuron transfection experiments, Lipofectamine 2000 from Invitrogen was used in conjunction with Nupherin-neuron from Biomol Research Laboratories (Plymouth Meeting, PA) per the manufacturer's instructions. Briefly, 5 μg of DNA of each tagged subunit was incubated with 120 μg of Nupherin-neuron in 400 μl of Neurobasal medium without phenol red while 10 μl of Lipofectamine 2000 was mixed in 400 μl of Neurobasal. After 15 min the two solutions were combined and incubated for 45 min. Neuronal cultures in 35 mm culture dishes were incubated in the resulting 800 μl mixture for 5 min, spun in a swinging bucket centrifuge at 1000 rpm for 5 min, and incubated for 4 hr; then the mixture was removed and replaced with the original 2 ml medium. Recordings were made 24–48 hr later.

Electrophysiology. The bath solution contained (in mM): 110 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, and 10 D-glucose, pH 7.4, with an osmolarity of 230 mOsm. Patch pipettes were filled with a solution containing (in mM): 100 K-gluconate, 0.1 $CaCl_2$, 1.1 EGTA, 5 $MgCl_2$, 10 HEPES, 3 ATP, 3 phosphocreatine, and 0.3 GTP, pH 7.2, at 215 mOsm. Whole-cell voltage clamp was maintained by using an Axopatch-1D amplifier controlled by a personal computer running pClamp 8 software via a Digidata 1200 interface (Axon Instruments, Foster City, CA). Data were filtered at 2 kHz and digitized at 5 kHz. Drugs were applied focally to single voltage- and current-clamped neurons by using a Picospritzer with a U-tube system providing the wash (Khakh et al., 1995).

Statistics. Pooled data are shown as means ± SEM.

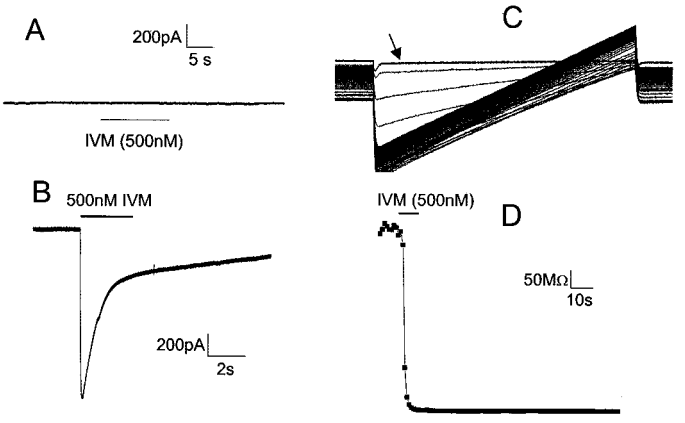

Figure 2. Both GluCl α and β are required for functional channels in HEK 293 cells. *A*, Voltage-clamp record of a HEK 293 cell transfected with GluCl α and EGFP shows that this subunit alone does not respond to applications of 500 nM IVM. Five of five cells showed no response to this subunit alone. *B*, Voltage-clamp record of a HEK 293 cell transfected with GluCl α and β subunits shows a transient IVM-induced current. Five of five cells transfected in this manner showed a robust response to IVM. *C*, Voltage ramps from –90 to –40 mV before (*arrow*), during, and after the application of 500 nM IVM. The increasing slopes show the IVM-induced conductance. The waveforms also show a clear change in reversal potential during the development of the conductance. Ramps 100 msec in duration were delivered at 1 sec intervals. *D*, Input resistance of the cell in *C*. A conductance develops and then remains relatively constant after the application of IVM.

RESULTS

Initial experiments were performed on transfection of the GluCl α and β subunits into human embryonic kidney (HEK) cells. Robust responses to IVM (500 nM) were detected only in cells that expressed both the α and β subunits (Fig. 2*A,B*). Unlike recordings from *Xenopus* oocytes (Cully et al., 1994), the current was transient; however, when we used successive voltage ramps to measure the conductance and reversal potential of the current, we found that the conductance remained relatively constant and the reversal potential changed after the application of IVM (Fig. 2*C,D*). In experiments that are not shown, we found that the IVM-induced conductance was stable for at least 30 min. These results encouraged us to design and construct the Sindbis virus, vSinGluClαβEGFP, which expressed three genes: GluClα, GluClβ, and green fluorescent protein (GFP) as a reporter for infected cells.

The GluCl/IVM method suppresses neuronal excitability

We infected 10- to 14-d-in-culture rat E18 hippocampal cultures with vSinGluClαβEGFP or the control virus vSinEGFP. As expected from previous studies (Khakh et al., 2001), the cultures were infected readily by either Sindbis virus, showing robust GFP expression in 20–50% of the neurons as early as 6 hr after infection. Previous studies also show that Sindbis-infected neurons die after ~48 hr, presumably because the virus recruits all of the ribosomes of the cell and thereby effectively shuts off host

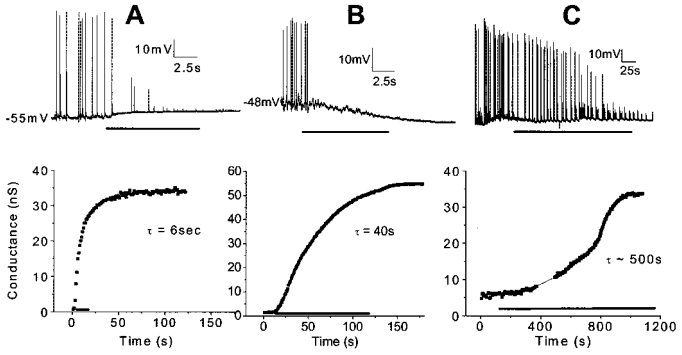

Figure 3. Activation of GluCl conductance by IVM over a 100-fold concentration range (*A*, 500 nM; *B*, 50 nM; *C*, 5 nM). *Top panels*, Current-clamp data from cells that displayed spontaneous activity. *Bottom panels*, Input conductance measured with voltage-clamp ramps from −80 to −50 mV for 200 msec duration at intervals of 1 sec. Each *panel* represents data from a different cell. Uninfected and GFP-infected neurons had no response to IVM applied similarly.

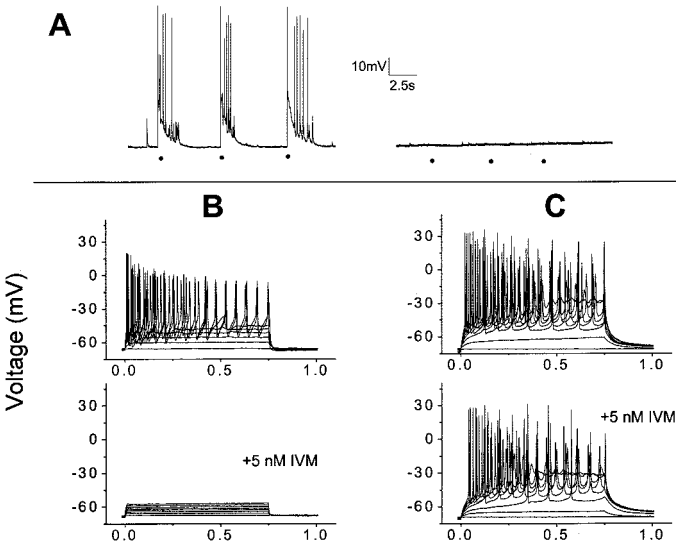

Figure 4. IVM silences GluCl-expressing neurons. *A*, Silenced cells no longer respond to glutamate. Shown are current-clamp traces from a GluCl-infected cell. *Dots* indicate 10 msec applications of 100 μM glutamate. *A, Left*, Before activation with IVM the cell responds to glutamate with action potentials. *A, Right*, After activation with 5 nM IVM the cell no longer responds to the same glutamate application. The 1 sec applications of glutamate could produce a slight depolarization, but no action potentials (data not shown). *B*, GluCl-infected cells in current clamp, showing responses to depolarizing current pulses (0–30 pA, 5 pA increments). In control solutions the cells responded with action potentials, but the IVM-induced (5 nM) Cl^- conductance prevented action potentials (*bottom panel*). Cells remained silenced even in responses to current pulses as high as 200 pA (data not shown). *C*, An uninfected cell showing no reduction in excitability by 5 nM IVM.

protein synthesis (Frolov and Schlesinger, 1994; Perri et al., 2000); therefore, we performed our experiments at 24 hr after infection. Neurons that were infected with vSinGluCl$\alpha\beta$EGFP and vSinEGFP had morphology indistinguishable from uninfected controls at this stage and appeared to be healthy (data not shown).

In ~20% of GFP-positive cells that were infected with vSinGluCl$\alpha\beta$EGFP, we detected a robust conductance increase with the application of 5–500 nM IVM (Fig. 3*A–C*, *bottom panels*). The time to activate this current was dose-dependent; the time constant decreased from 500 sec at 5 nM to 6 sec at 500 nM. Surprisingly, the maximal conductance did not seem to vary in this concentration range. Also shown in the top panels of Figure 3 is the robust silencing effect of this conductance increase. Spontaneously firing cells subject to the application of IVM stopped firing action potentials, and the membrane potential moved to values between −50 and −65 mV, which is near the value of E_{Cl} for the solutions that we used. Importantly, vSinEGFP-infected neurons and uninfected neurons showed no responses to IVM at these concentrations (data not shown).

Glutamate is the major excitatory transmitter in the brain, and brief applications of glutamate strongly elicit action potentials in hippocampal neurons (Storm-Mathisen, 1981; Crunelli et al., 1983). The major goal of the GluCl/IVM method is to insure that, once a GluCl$\alpha\beta$-positive cell is activated by IVM, it no longer fires action potentials in response to excitatory input. Figure 4*A* demonstrates this effect. The left panel shows that an infected neuron fires volleys of action potentials in response to 10 msec pulses of 100 μM glutamate; the right panel shows that, in the presence of 5 nM IVM, the same neuron no longer responds to identical glutamate pulses. Below (see Fig. 8), we show that neurons expressing this invertebrate glutamate receptor display no changes in glutamate responses.

Additionally, neurons that are infected with vSinGluCl$\alpha\beta$EGFP and activated with IVM show extreme reductions in impulse firing elicited by direct current injection. The top panel of Figure 4*B* shows current-clamp records from a vSinGluCl$\alpha\beta$EGFP-infected neuron. Increasing current pulses (up to 30 pA) elicit current-dependent firing. The bottom panel shows records from a similar neuron, incubated with 5 nM IVM. This neuron does not respond at all to the same current injection protocol. Moreover, injection of up to 200 pA (data not shown) failed to elicit a spike.

Figure 4*C* shows the same experiments for an uninfected control cell; 5 nM IVM did not affect the excitability. This lack of IVM effect was observed in all of 103 uninfected or 55 vSinEGFP-infected neurons, respectively.

Sindbis expression produces highly variable results

Previous reports show large cell-to-cell variability in expression levels, based on measurements that used dual promoters to express an ion channel subunit with the Sindbis virus (Okada et al., 2001). We expected similar cell-to-cell variability in our experiments. Unexpectedly, we found that the major variability in IVM responses occurred between culture dishes rather than within neurons in a given culture (Fig. 5). Each point in Figure 5 represents the average of five infected neurons in a single infected culture. Some cultures respond well, whereas others do not. We could observe no clear morphological differences or GFP fluorescence intensity differences between cultures that expressed well and those that expressed poorly. We attempted, with no success, to minimize this variability by varying the age of the cultures, the time after infection, temperature and position in the incubator, preparation of the virus, sonication of the virus, and glutamate concentration in the culture medium. In control dishes that were infected with vSinEGFP, there was no significant difference between the IVM^+ and IVM^- input conductance in any culture that was tested (data not shown). Because of this culture-to-culture variation, we selected only cultures that had neurons responding well to IVM for the measurement studies presented above. We found similar dish-to-dish variability in HEK 293 cultures that were infected with vSinGluCl$\alpha\beta$EGFP, but not in HEK cultures transfected with both plasmids encoding the GluCl

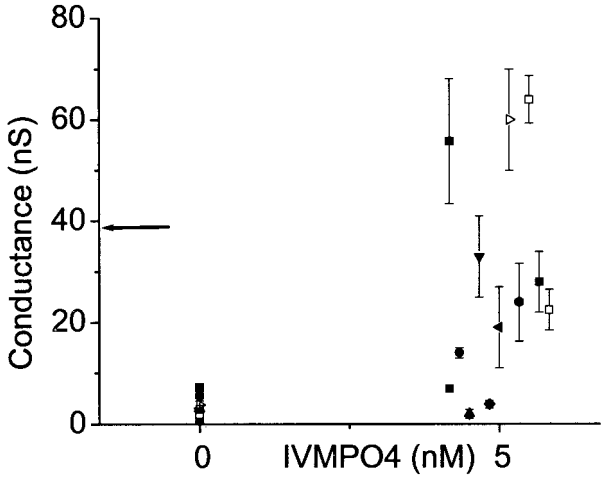

Figure 5. There is a large variation in expression levels that seems to be culture dependent. Each *point* represents the average of five cells in a culture dish. Approximately one-half of the cultures that were surveyed have no response to 500 nM IVM, and these cultures were excluded from the plot. The *points* plotted here represent the cultures that do respond to 500 nM IVM. Note that this graph represents data taken at 0 and 5 nM IVM; the *points* have been spread out to enhance visualization. The *arrow* represents average cell conductance in 0 nM IVM, 100 μM muscimol for comparison.

subunits; therefore, we believe that this variability is a characteristic of the Sindbis expression system rather than an inherent limitation of the GluCl/IVM method.

In Nupherin-mediated transfection, all fluorescent cells respond to IVM

We sought an expression method that could overcome the variability of the Sindbis system. We used the newly developed Nupherin-neuron transfection system to transfect GluCl channels into hippocampal cultures. In these experiments we modified the GluCl coding sequence to include fluorescent proteins (yellow fluorescent protein, YFP; cyan fluorescent protein, CFP) in the M3–M4 loop of each construct (GluClα and β subunits, respectively, as shown in Fig. 6*A*). Figure 6*C–E* shows images from an exemplar hippocampal culture. Each of 30 fluorescent cells in three cultures responded to 5 nM IVM with a conductance >30 nS, similar to the results with neurons in responsive Sindbis-infected cultures (Fig. 6*B*). This figure shows that neurons transfected with the GluCl α subunit alone do not develop a conductance in the presence of IVM. The variability between cultures is substantially less with Nupherin-mediated expression than with Sindbis-mediated expression.

IVM-induced Cl^- conductance is reversible

In previous reports IVM has been described as acting irreversibly on GluCl channels expressed in *Xenopus* oocytes (Cully et al., 1994) on a time scale of several minutes. Ligand-binding experiments show that IVM does dissociate from its receptor (presumably the GluCl channels), with a rate constant of 0.005–0.006/min (Cully and Paress, 1991). Therefore, we sought to measure the reversibility of IVM action on a time scale of several hundred minutes. We infected cultures of hippocampal neurons, incubated in 5 nM IVM, and we measured the input conductance of the neurons to verify that the channels were activated. We then washed the cultures and recorded the input conductance after 1 or 8 hr. Figure 7*A* summarizes these results. At 1 hr the conductance has not changed significantly from the activated conductance, whereas by 8 hr the conductance has returned nearly to baseline. The final bar shows that IVM subsequently can reactivate the channels. Formally, it is not possible to distinguish between the possibility that the reversibility is caused by IVM washing off the GluCl receptors or the possibility that the reversibility is attributable to receptor turnover, but the important feature here is that the technique appears to be reversible in terms of electrical activity.

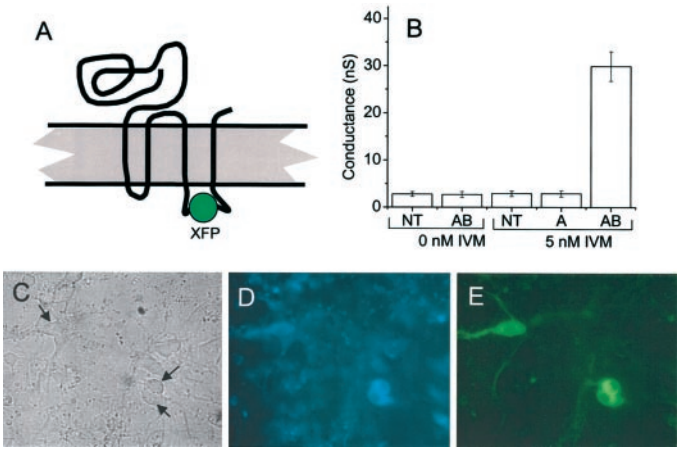

Figure 6. Neurons cotransfected with separate plasmids encoding EYFP-tagged GluCl α and ECFP-tagged GluCl β show fluorescence. *A*, Diagram indicating that the fluorescent protein was inserted in the M3–M4 loop of each subunit. *B*, Measured input conductance of neurons transfected with the GluCl α–EYFP fusion and GluCl β–ECFP fusion. *NT*, Neurons that have not been transfected; *AB*, neurons transfected with both subunits; *A*, neurons transfected with only the GluCl α–EYFP subunit. All *bars* represent data from three independent cultures, 10 cells per culture. *C*, A bright-field image at 40× showing several neurons, with *arrows* indicating three neurons that have been transfected. *D*, The field of view in *C* imaged with an ECFP filter set. *E*, The field of view in *C* imaged with an EYFP filter set.

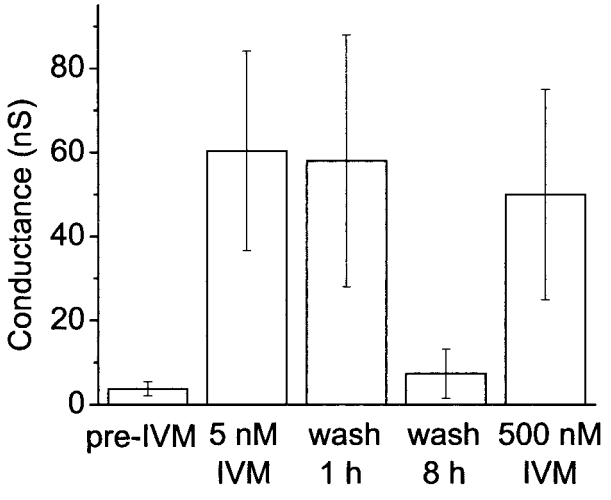

Figure 7. IVM-induced chloride conductance deactivates several hours after IVM washout. The input conductance of cells that were infected with vSin$\alpha\beta$EGFP was measured first without IVM and then in the presence of 5 nM IVM. After 1 hr of washing there is little recovery in cell conductance, whereas after 8 hr the conductance returns to uninfected levels. The *last bar* shows that IVM-induced conductance can be reactivated. Each *bar* represents 10 cells in two cultures.

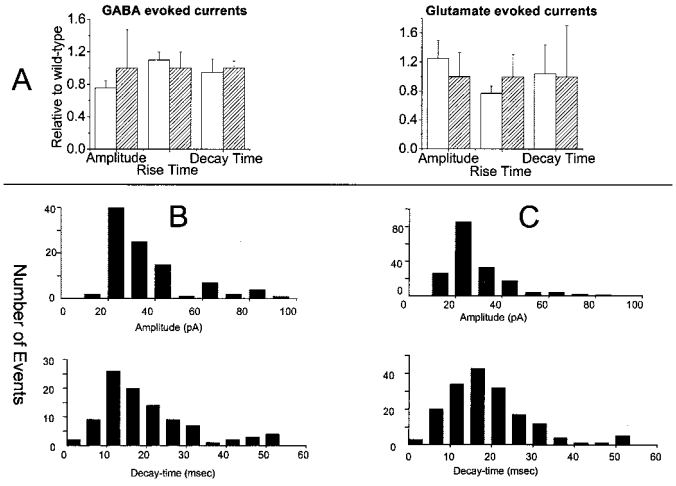

Figure 8. Expression of GluCl does not alter glutamate- or GABA-evoked currents. *A*, Evoked currents were measured in GluCl-infected and uninfected neurons with 10 msec agonist puffs (100 μM GABA, 1 mM glutamate). The amplitude, rise time, and decay time were measured. The plot shows these three parameters relative to the measurements in uninfected cells. GABA responses are shown on the *left* and glutamate responses on the *right*. For each of the two ligands the *bars* represent data pooled from 10 cells from two different cultures. *B, C*, Analysis of GABA mIPSCs in the absence (*B*) and presence (*C*) of 5 nM IVM. The peak amplitude histogram (*top*) and decay time histogram (*bottom*) are shown. The amplitude data are shown in 10 pA bins, and the decay time data are shown in 10 msec bins. Note that there is no apparent difference in histogram shape between the 0 and 5 nM IVM case for either measurement. In *B*, the data are pooled from 20 events each from five neurons in one culture; in *C*, the data are pooled from 30 events each from five neurons in one culture.

Neither GluCl expression nor IVM application affects other synaptic properties

In experiments to examine possible unwanted additional effects of IVM, we used 5 nM IVM, because this concentration produces adequate silencing in most neurons. Because of the homology between GluCl channels and $GABA_A$ receptors, we were concerned that one or both of these subunits might heteromultimerize with native $GABA_A$ receptor subunits. We therefore compared the amplitude, rise time, and decay time of GABA-evoked responses. The left panel of Figure 8*A* shows that these three parameters do not differ significantly between infected and uninfected cells, providing confidence that heteromultimerization with GABA subunits did not take place or at least had no functional consequences. After measuring the evoked responses, we applied 5 nM IVM to these cultures to ensure that these cultures were expressing the GluCl receptor at high levels.

The GluCl α and β heteromultimer is a glutamate receptor (Cully et al., 1994). We were concerned that this response might add to or distort the endogenous glutamate response of the neuron. The right panel of Figure 8*A* addresses these concerns in an experiment that used Sindbis-mediated expression: the glutamate response did not change significantly between uninfected and GluCl$\alpha\beta$-expressing neurons. As above, we used only cultures that responded well to IVM for these experiments. These experiments were performed with CsCl in the internal recording solution and were voltage clamped at −60 mV so that any additional conductance caused by glutamate activation of GluCl would result in an increase in amplitude or perhaps an increase in decay time. In infected cultures that were treated with CNQX and APV to block endogenous glutamate receptors, we found that steady application of 10 μM glutamate (the estimated steady concentration of glutamate in CSF) produced no detectable conductance increases (<0.1 nS). Steady application of 2 mM glutamate, however, produced slow, large (~30 nS) conductances.

IVM has various effects on other ion channels, including GABA (Krusek and Zemkova, 1994), $P2X_2$ (Khakh et al., 1999), and neuronal AChR (Krause et al., 1998). These effects occur with concentrations of IVM higher than 5 nM. However, we chose to study what we regard as the most likely side effect, GABA potentiation, with our desired IVM concentration of 5 nM. Figure 8*B* presents histograms of the amplitude and decay time of the GABAergic synaptic miniature IPSCs (mIPSCs) recorded from uninfected neurons in the presence of the glutamate receptor blockers CNQX and APV and the Na^+ channel blocker TTX. Figure 8*C* presents the same type of data, with the addition of 5 nM IVM in the recording medium. There is no apparent potentiation of GABA receptors at this concentration of IVM.

DISCUSSION

We describe a procedure that combines advantages of genetically based and pharmacologically based silencing by inhibitory ion channels. Our results demonstrate that the GluCl/IVM method modifies neuronal excitability in an inducible and reversible manner. Expression of GluCl alone seems to leave the excitability of the neuron unaltered, and IVM application to uninfected neurons within the effective range for silencing GluCl-expressing neurons never produces a detectable conductance. However, many GluCl-expressing neurons display a reduction in excitability when exposed to low concentrations of IVM. The reduction in excitability silences many neurons, and this silencing can be reversed by ~8 hr of washing. These are the desired results of the GluCl/IVM silencing method.

With the Sindbis virus construct there was an inherently high level of variability of expression among infected cells. Despite numerous attempts to understand and control this variability, we found no parameters to explain it. Both HEK cells transfected with the GluCl α and β subunits and *Xenopus* oocytes injected with RNA for this channel nearly always show expression, reinforcing our belief that this variability results from an aspect of Sindbis viral biology. Nupherin-mediated expression yields more consistent data, supporting this interpretation. Because our long-term goal involves transgenic methods to express the GluCl channels, the variability does not appear to be a source of significant concern for the GluCl/IVM silencing method.

Neurons expressing the channel at a high level are silenced strongly by 5 nM IVM. This low concentration is important, for previous reports show that IVM at considerably higher concentrations in the mammalian brain is toxic. Studies done with radiolabeled IVM in blood–brain barrier-impaired mice indicate that the toxic concentration of IVM in the brain is ~500 nM (Schinkel et al., 1994), which is two orders of magnitude above the concentration we are using. This gives us some confidence that *in vivo* applications of the GluCl/IVM method may succeed. A related complexity is the delivery of IVM to the brain. The blood–brain barrier contains mechanisms, primarily involving *mdr1*, that appear to pump IVM out of the brain, and this might prevent systemically applied IVM from reaching GluCl channels in the brain. However, a clinically relevant intraperitoneal dose, 0.2 mg/kg, results in 1.5 ng/gm IVM in brain tissue (Schinkel et al., 1994), or ~2 nM. This is very close to the concentration we believe to be necessary for silencing neurons expressing GluCl. Perhaps *in vivo* applications of the GluCl/IVM method will

require relatively simple methods of IVM delivery to the animal. Of course, more sophisticated options exist for easing drug delivery, such as using blood–brain disrupting drugs (Jette et al., 1995) or using mice with disrupted drug pumping at the blood–brain barrier, as the foundation for transgenic mice expressing this channel.

Previous reports suggest that IVM "irreversibly" activates GluCl channels, an unusual situation for ligand-gated channels. The impression of irreversibility may arise from the limited time scale of previous experiments on *Xenopus* oocytes or from drug retention by the large yolk volume of the cells. Data from our washout experiments (Fig. 7), suggesting reversibility after 8 hr, are approximately consistent with the disassociation constant determined in biochemical binding experiments (Cully and Paress, 1991). Both the onset and recovery from silencing will depend on the pharmacokinetics of IVM in the animal; for instance, IVM can be stored in the fat.

In addition to the channel-based silencing strategies noted in the introductory remarks, other investigators have used genetic manipulations of synaptic transmission, for instance by using tetanus toxin (Baines et al., 2001). It is unclear whether tetanus toxin-based procedures are reversible. Additionally, exogenous expression of K^+ channels has been used to modulate biochemical signaling pathways in HEK 293 cells (Holmes et al., 1997).

There are several potential applications for the GluCl/IVM method, or for improved versions, in research and in therapeutics. One application would involve cell-specific expression of GluCl channels under the control of appropriate promoter(s). This strategy may enable genetic manipulation of excitability at selected times in development and in specific regions in the CNS. The requirement for both the GluCl α subunit and the GluCl β subunit at first may seem like a complication; however, this gives the experimenter the flexibility to use different promoters to drive the two genes so that only neurons in which both promoters are active will be silenced with the administration of IVM. Additionally, it may be possible to construct viruses with these two genes that can be used *in vivo*. Stereotaxic injection of such viruses then may circumvent the need for promoters that drive expression in specific brain nuclei. From a clinical standpoint, a targeted and controlled "excitability modulator" may form the basis for new treatments of epilepsy, chronic pain, or other diseases arising from excess neuronal activity. It may be possible to titrate silencing by varying the dose of IVM.

REFERENCES

Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M (2001) Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J Neurosci 21:1523–1531.

Crunelli V, Forda S, Kelly JS (1983) Blockade of amino acid-induced depolarizations and inhibition of excitatory postsynaptic potentials in rat dentate gyrus. J Physiol (Lond) 341:627–640.

Cully DF, Paress PS (1991) Solubilization and characterization of a high-affinity ivermectin binding site from *Caenorhabditis elegans*. Mol Pharmacol 40:326–332.

Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LH, Schaeffer JM, Arena JP (1994) Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 371:707–711.

Ehrengruber MU, Doupnik CA, Xu Y, Garvey J, Jasek MC, Lester HA, Davidson N (1997) Activation of heteromeric G-protein-gated inward rectifier K^+ channels overexpressed by adenovirus gene transfer inhibits the excitability of hippocampal neurons. Proc Natl Acad Sci USA 94:7070–7075.

Falk T, Kilani RK, Yool AJ, Sherman SJ (2001) Viral vector-mediated expression of K^+ channels regulates electrical excitability in skeletal muscle. Gene Ther 8:1372–1379.

Frolov I, Schlesinger S (1994) Comparison of the effects of Sindbis virus and Sindbis virus replicons on host cell protein synthesis and cytopathogenicity in BHK cells. J Virol 68:1721–1727.

Helmstetter FJ, Bellgowan PS (1994) Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. Behav Neurosci 108:1005–1009.

Holmes TC, Berman K, Swartz JE, Dagan D, Levitan IB (1997) Expression of voltage-gated potassium channels decreases cellular protein tyrosine phosphorylation. J Neurosci 17:8964–8974.

Holt W, Maren S (1999) Muscimol inactivation of the dorsal hippocampus impairs contextual retrieval of fear memory. J Neurosci 19:9054–9062.

Isles AR, Ma D, Milsom C, Skynner MJ, Cui W, Clark J, Keverne EB, Allen ND (2001) Conditional ablation of neurones in transgenic mice. J Neurobiol 47:183–193.

Jasnow AM, Huhman KL (2001) Activation of $GABA_A$ receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. Brain Res 920:142–150.

Jette L, Murphy GF, Leclerc JM, Beliveau R (1995) Interaction of drugs with P-glycoprotein in brain capillaries. Biochem Pharmacol 50: 1701–1709.

Johns DC, Marx R, Mains RE, O'Rourke B, Marban E (1999) Inducible genetic suppression of neuronal excitability. J Neurosci 19:1691–1697.

Khakh BS, Humphrey PP, Surprenant A (1995) Electrophysiological properties of P2X-purinoceptors in rat superior cervical, nodose, and guinea-pig coeliac neurones. J Physiol (Lond) 484:385–395.

Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA (1999) Allosteric control of gating and kinetics at $P2X_4$ receptor channels. J Neurosci 19:7289–7299.

Khakh BS, Smith WB, Chiu CS, Ju D, Davidson N, Lester HA (2001) Activation-dependent changes in receptor distribution and dendritic morphology in hippocampal neurons expressing $P2X_2$-green fluorescent protein receptors. Proc Natl Acad Sci USA 98:5288–5293.

Kobayashi K, Morita S, Sawada H, Mizuguchi T, Yamada K, Nagatsu I, Fujita K, Kreitman RJ, Pastan I, Nagatsu T (1995) Immunotoxin-mediated conditional disruption of specific neurons in transgenic mice. Proc Natl Acad Sci USA 92:1132–1136.

Krause RM, Buisson B, Bertrand S, Corringer PJ, Galzi JL, Changeux JP, Bertrand D (1998) Ivermectin: a positive allosteric effector of the α7 neuronal nicotinic acetylcholine receptor. Mol Pharmacol 53: 283–294.

Krusek J, Zemkova H (1994) Effect of ivermectin on γ-aminobutyric acid-induced chloride currents in mouse hippocampal embryonic neurones. Eur J Pharmacol 259:121–128.

Li YX, Zhang Y, Lester HA, Schuman EM, Davidson N (1998) Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. J Neurosci 18:10231–10240.

Maren S, Yap SA, Goosens KA (2001) The amygdala is essential for the development of neuronal plasticity in the medial geniculate nucleus during auditory fear conditioning in rats. J Neurosci 21:RC135.

Martin RJ, Murray I, Robertson AP, Bjorn H, Sangster N (1998) Anthelmintics and ion channels: after a puncture, use a patch. Int J Parasitol 28:849–862.

Muller J, Corodimas KP, Fridel Z, LeDoux JE (1997) Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. Behav Neurosci 111:683–691.

Nadeau H, Lester HA (2002) NRSF causes cAMP-sensitive suppression of sodium currents in cultured hippocampal neurons. J Neurophysiol 88:409–421.

Nadeau H, McKinney S, Anderson DJ, Lester HA (2000) ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. J Neurophysiol 84:1062–1075.

Nitabach MN, Blau J, Holmes T (2002) Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. Cell 109:485–495.

Okada T, Yamada N, Kakegawa W, Tsuzuki K, Kawamura M, Nawa H, Iino M, Ozawa S (2001) Sindbis viral-mediated expression of Ca^{2+}-permeable AMPA receptors at hippocampal CA1 synapses and induction of NMDA receptor-independent long-term potentiation. Eur J Neurosci 13:1635–1643.

Paradis S, Sweeney ST, Davis GW (2001) Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. Neuron 30:737–749.

Perri S, Driver DA, Gardner JP, Sherrill S, Belli BA, Dubensky Jr TW, Polo JM (2000) Replicon vectors derived from Sindbis virus and Semliki Forest virus that establish persistent replication in host cells. J Virol 74:9802–9807.

Sawada H, Nishii K, Suzuki T, Hasegawa K, Hata T, Nagatsu I, Kreitman RJ, Pastan I, Nagatsu T, Kobayashi K (1998) Autonomic neuropathy in transgenic mice caused by immunotoxin targeting of the peripheral nervous system. J Neurosci Res 51:162–173.

Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP (1994) Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. Cell 77:491–502.

Storm-Mathisen J (1981) Glutamate in hippocampal pathways. Adv Biochem Psychopharmacol 27:43–55.

Watanabe D, Inokawa H, Hashimoto K, Suzuki N, Kano M, Shigemoto R, Hirano T, Toyama K, Kaneko S, Yokoi M, Moriyoshi K, Suzuki M, Kobayashi K, Nagatsu T, Kreitman RJ, Pastan I, Nakanishi S (1998) Ablation of cerebellar Golgi cells disrupts synaptic integration involving GABA inhibition and NMDA receptor activation in motor coordination. Cell 95:17–27.

White BH, Osterwalder T, Keshishian H (2001a) Molecular genetic approaches to the targeted suppression of neuronal activity. Curr Biol 11:R1041–R1053.

White BH, Osterwalder TP, Yoon KS, Joiner WJ, Whim MD, Kaczmarek LK, Keshishian H (2001b) Targeted attenuation of electrical activity in *Drosophila* using a genetically modified K^+ channel. Neuron 31:699–711.

Wilensky AE, Schafe GE, LeDoux JE (1999) Functional inactivation of the amygdala before, but not after, auditory fear conditioning prevents memory formation. J Neurosci 19:RC48.

Wilensky AE, Schafe GE, LeDoux JE (2000) The amygdala modulates memory consolidation of fear-motivated inhibitory avoidance learning, but not classical fear conditioning. J Neurosci 20:7059–7066.

Xue H (1998) Identification of major phylogenetic branches of inhibitory ligand-gated channel receptors. J Mol Evol 47:323–333.