

Codon optimization of *Caenorhabditis elegans* GluCl ion channel genes for mammalian cells dramatically improves expression levels

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Received 18 October 2002; received in revised form 11 December 2002; accepted 11 December 2002

Abstract

Organisms use synonymous codons in a highly non-random fashion. These codon usage biases sometimes frustrate attempts to express high levels of exogenous genes in hosts of widely divergent species. The *Caenorhabditis elegans* GluCl α 1 and GluCl β genes form a functional glutamate and ivermectin-gated chloride channel when expressed in *Xenopus* oocytes, but expression is weak in mammalian cells. We have constructed synthetic genes that retain the amino acid sequence of the wild-type GluCl channel proteins, but use codons that are optimal for mammalian cell expression. We have tagged the native and codon-optimized GluCl cDNAs with enhanced yellow fluorescent protein (EYFP, GluCl α 1 subunit) and enhanced cyan fluorescent protein (ECFP, GluCl β subunit), expressed the channels in E18 rat hippocampal neurons and measured the relative expression levels of the two genes with fluorescence microscopy as well as with electrophysiology. Codon optimization provides a 6- to 9-fold increase in expression, allowing the conclusions that the ivermectin-gated channel has an EC₅₀ of 1.2 nM and a Hill coefficient of 1.9. We also confirm that the Y182F mutation in the codon-optimized β subunit results in a heteromeric channel that retains the response to ivermectin while reducing the response to 100 μ M glutamate by 7-fold. The engineered GluCl channel is the first codon-optimized membrane protein expressed in mammalian cells and may be useful for selectively silencing specific neuronal populations in vivo.

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Keywords: GluCl; Codon optimization; Fusion proteins; Electrophysiology; Excitability; EYFP; ECFP

1. Introduction

Comparative genomic studies show that organisms use synonymous codons in a non-random, species-specific fashion (Bernardi, 1985; Nakamura et al., 1996; Kanaya et al., 2001). In *Escherichia coli*, this non-random codon usage appears to parallel expression levels of the cognate tRNAs (Ikemura, 1981a,b). Several authors suggest that high-level expression of genes containing non-optimal codons may be limited by the availability of tRNA for rare codons (Kane, 1995). Codon optimization strategies enable the high-level expression of therapeutically relevant human proteins in *E. coli* (Hernan et al., 1992; Makrides, 1996) and in the yeasts *Pichia pastoris* and *S. cerevisiae* (Woo et al.,

2002). There are fewer examples in which genes from evolutionarily old organisms have been optimized for expression in mammalian cells. Three successful examples include the optimization of green fluorescent protein from the jellyfish *A. porins* (Haas et al., 1996), the tetracycline transactivator from *E. coli* (Wells et al., 1999) and Cre recombinase (Shimshek et al., 2002). To date, there are no publications describing codon optimized membrane bound proteins.

We are developing a strategy that selectively and reversibly silences specific neuronal subpopulations in mammals via expression of the GluCl α 1 and GluCl β genes from *Caenorhabditis elegans* (Li et al., 2002; Slimko et al., 2002); and we seek a high-level expression of these two genes within mammalian neurons. Genes in the GluCl gene family are found in many invertebrates and are part of the nicotinic receptor superfamily, characterized by a large extracellular N-terminus, four transmembrane domains and a large loop between the

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third membrane spanning segment (M3) and the fourth segment (M4) (Cully et al., 1994). Many GluCl receptors are activated by the anthelmintic drug ivermectin (IVM), which is used both in humans and animals to treat a variety of parasitic diseases (del Giudice, 2002; Winnen et al., 2002). Our strategy is to express both GluCl α 1 and GluCl β in specific neurons using tissue-specific promoters in transgenic animals or through local injection of viruses engineered to express these genes. Systematic administration of IVM will then reversibly activate a chloride conductance in the target neurons, reducing their excitability.

We are optimizing this GluCl/IVM strategy. First, the wild type GluCl channel genes seem to express poorly in mammalian neurons. While the weak expression appears partially due to the multi-subgenomic promoter Sindbis virus expression system (Slimko et al., 2002), we asked whether some of the problem arises from sub-optimal codon usage. We have synthesized genes with codon usage similar to a compendium of highly expressed mammalian genes. Because previous work showed that tagging the channels with fluorescent proteins in the M3–M4 loops provides a convenient assay for membrane expression but does not alter the function of the receptor (Gensler et al., 2001; Li et al., 2002), we also incorporated enhanced yellow and cyan fluorescent proteins (EYFP and ECFP) in the wild type and optimized genes. We find that codon optimization alone generates nearly a 6- to 9-fold increase in the fluorescence, indicating a much higher expression level. We also present electrophysiological data that correlates with the fluorescence measurement.

A second GluCl optimization tactic requires minimizing of the endogenous glutamate response of these channels with no decrease in the IVM response. We previously showed that the Y182F mutation to the β subunit (Li et al., 2002) produces the desired abolition of glutamate but not IVM responses in an oocyte expression system. However, robust GluCl responses require IVM concentrations some 100-fold higher in oocyte systems than in mammalian cells. We therefore tested whether these desirable pharmacological characteristics also hold for the codon-optimized Y182F GluCl β channel expressed in mammalian neurons. Indeed, this seems to be the case.

2. Material and methods

2.1. Molecular biology

The codon-optimized cDNAs (optGluCl α 1 and optGluCl β) were synthesized by Entelechon GmbH (Regensburg, Germany) and cloned into pcDNA3.1/TOPO-HIST from Invitrogen (Carlsbad, CA). EYFP was amplified by PCR from pEYFP-N1 from ClonTech

(Palo Alto, CA) with the primers 5'-ACCGGTGA-ATTCCATGGTGAGCAAGGGCGAGGAG-3' and 5'-GCGGCCCGGAATTCTTGTACAGCTCGTCCATGC-3' and cloned into the EcoRI site of optGluCl α 1. ECFP was amplified by PCR from pECFP-N1 from ClonTech with the primers 5'-GATGGTGAGCAAGGGCGAGGAG-3' and 5'-GTCTTGTA-CAGCTCGTCCATGCCG-3' and cloned into the PmlI site of optGluCl β . The wild type fusion genes GluCl α 1-EYFP and GluCl β -ECFP have previously been described (Li et al., 2002). The mutation Y182F of optGluCl β was performed using Stratagene's Quik-Change mutation kit with the following primers: 5'-TGCAACTTCGACCTGGTGTCTTCGCCACAC-CATGAACGACATC-3' and 5'-GATGTCGTTTCATG-TGTGTTGGCGAAGGACACCAGGTCGAAGTTGC A-3'. The sequences for the optimized genes (described in Section 3) have been deposited in GenBankTM, Accession No. AY195802 for optGluCl α 1 and AY195803 for optGluCl β .

2.2. Neuronal culture and transfection

DNA (1 μ g) of each tagged subunit was incubated with 20 μ g of Nupherin-neuron (BioMol Research Laboratories, Plymouth Meeting, PA) in 400 μ l of Neurobasal medium without phenol (Invitrogen), while 10 μ l of Lipofectamine 2000 (Invitrogen) was mixed in 400 μ l of Neurobasal. After 15 min, the two solutions were combined and incubated for 45 min. E18 rat hippocampal neuronal cultures in 35 mm culture dishes, at least 14 days in culture, were incubated in the resulting 800 μ l mixture for 2–4 h and the mixture was then removed and replaced with the original 2 ml medium. Recordings were made 24–48 h later.

2.3. Electrophysiology

The bath solution was (in mM): 110 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 HEPES and 10 D-glucose, pH 7.4, osmolarity 230 mOsm. Patch pipettes were filled with a solution containing (in mM): 100 K gluconate, 0.1 CaCl₂, 1.1 EGTA, 5 MgCl₂, 10 HEPES, 3 ATP, 3 phosphocreatine and 0.3 GTP, pH 7.2, 215 mOsm. For studying glutamate responses, 100 mM CsCl replaced the K gluconate in the pipette solution and 100 μ M CNQX was added to the bath and drug perfusion solution. Whole-cell voltage clamp was maintained using an Axopatch-1D amplifier controlled by a personal computer running pCLAMP 8 software via a Digidata 1200 interface (Axon Instruments, Union City, CA). Data were filtered at 2 kHz and digitized at 5 kHz. Holding potential was -60 mV. Input conductance was measured by a 5 mV hyperpolarizing pulse. 100 μ M glutamate was applied focally to single voltage-clamped neurons using a Picospritzer with a U-

tube system providing the wash (Khakh et al., 1995). Under these conditions, the chloride current measured upon GluCl activation was inward.

2.4. Imaging

After transfection (24 h) with the various combinations of constructs, cells were fixed by a 20-min incubation in PBS with 4% paraformaldehyde and mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Neurons were then imaged with a 100 $\times$ objective using the EYFP filter set XF104-2 (Omega Optical, Brattleboro, VT) or the ECFP filter set XF114-2 and the pixel intensity was averaged over the soma for each neuron.

2.5. Statistics

Pooled data are shown as means $\pm$ S.E.M.

3. Results

3.1. Codon optimization

We used a compendium of 52 highly expressed mouse genes (ribosomal proteins, polymerases, elongation factors and heat-shock proteins) to create the mouse codon usage table shown in Fig. 1 (Sakai et al., 2001). The Codon Adaptation Index (CAI) quantifies the similarity between the codon usage of a specific gene and a species of interest (Sharp and Li, 1987). Table 1 presents the mouse CAI of wild type GluCl α 1, GluCl β and green fluorescent protein (GFP). A CAI value of unity would indicate that each codon of the gene under analysis is optimal. The wild type GluCl α and GluCl β with values of 0.55 and 0.58, respectively score slightly poorer than the wild-type GFP value of 0.6. Because codon optimization has successfully increased the expression of GFP (Haas et al., 1996), we perform analogous modifications to the GluCl genes.

We chose the one or two most prevalent mouse codons for each amino acid (Fig. 1) to generate a nucleotide sequence for both GluCl α 1 and GluCl β . We analyzed the resulting nucleotide sequence with a splice site prediction tool from the Berkley Drosophila Genome Project (Reese et al., 1997), which revealed several potential donor or acceptor sites. We iteratively modified the nucleotide sequence to eliminate these sites. Additionally, we removed and added a number of restriction sites for convenience in cloning. The final optimized cDNAs for the GluCl α 1 and GluCl β genes contain 399 and 352 silent mutations, respectively, leaving the amino-acid sequences identical to the wild type genes. The new sequences are provided in GenBankTM, Accession No. AY195802 for the optimized

GluCl α 1 gene, which we call optGluCl α 1 and Accession No. AY195803 for the optimized GluCl β gene, which we call optGluCl β . Fig. 1 presents the codon usage of the GluCl genes in their native form as well as after our optimization procedure. Codon optimization yielded a significant increase in the calculated CAI, as shown in Table 1, which present comparative data for GFP/EGFP. All three genes have a CAI of roughly 0.6 with their native codon usage and are nearly 1.0 after optimization.

These sequences were synthesized and tagged with fluorescent proteins in the M3–M4 loop. We found that codon optimization yielded a dramatic increase of the fluorescent signal of both GluCl α 1-EYFP and GluCl β -ECFP. Fig. 2 presents images of neurons transfected with the wild type and codon optimized versions of the GluCl α 1 and GluCl β genes. The increase in fluorescence is profoundly noticeable when the transfected cultures are viewed by eye: cells transfected with the wild type genes are barely visible, whereas those expressing the optimized genes are bright. The distribution of all four genes seems to be primarily somatic. There were no difference in localization between the wild type and optimized genes; this point has not been pursued systematically because a somatic localization is adequate for silencing. The average intensity of ten cells transfected with each of the wild type and optimized GluCl α 1 and GluCl β genes is shown in Fig. 3(A). Codon optimization alone has provided nearly a 9-fold increase in expression of the GluCl α 1 gene and a 6-fold increase in the expression of the GluCl β gene. We attempted to achieve further increases in expression by replacing the signal peptide sequence and 5' untranslated region (UTR) of the optGluCl β gene with that of a highly expressed mouse receptor, nAhCR α 4. The result was no further increase in fluorescence intensity (data not shown).

Cotransfection of separate plasmids in this fashion yielded fluorescence signals that are highly correlated. Fig. 4 shows the normalized ECFP fluorescence signal plotted against the normalized EYFP fluorescence signal for each of the 20 cells measured. In data not shown, we verified that there is no cross talk between the two channels. This satisfying result implies that there is roughly the same amount of each protein expressed in an individual cell, eliminating the potential complication that cell populations would contain GluCl channels of widely differing subunit compositions caused by variable expression of the two genes among cells.

We performed whole-cell patch clamping to measure the input conductance of neurons transfected with the heteromeric wild type GluCl and the heteromeric optimized GluCl. Previous data show that an IVM concentration of $\approx$ 5 nM applied to a GluCl-expressing neuron activates GluCl channel conductance sufficient to silence firing due either to glutamate activation or to

	Mouse	Native	Optimized		Mouse	Native	Optimized		Mouse	Native	Optimized		Mouse	Native	Optimized
Ala	GCU 32	36	12	Cys	UGU 47	47	0	Leu	CUU 13	21	0	Ser	UCU 22	13	0
	C 40	24	84		C 53	53	100		C 21	22	1		C 26	11	40
	A 19	28	0						A 6	13	0		A 12	37	0
	G 9	12	4	Gln	CAA 19	71	5		G 42	12	99		G 6	11	3
					G 81	29	95		UUA 4	7	0		AGU 11	19	0
									G 14	25	0		C 23	9	57
Arg	CGU 10	17	0	Glu	GAA 36	50	0	Lys	AAA 32	63	8	Thr	ACU 22	27	3
	C 23	9	85		G 64	50	100		G 68	37	92		C 42	11	93
	A 15	25	0	Gly	GGU 21	23	0						A 22	49	0
	G 21	15	2		C 40	17	97						G 14	13	4
	AGA 16	23	10		A 21	60	0	Pro	CCU 28	18	0	Tyr	UAU 37	49	5
	G 15	11	3		G 18	0	3		C 34	12	98		C 63	51	95
Asn	AAU 41	74	5	His	CAU 40	67	0		A 29	49	0	Val	GUU 18	39	3
	C 59	26	95		C 60	33	100		G 9	21	2		C 24	31	21
Asp	GAU 46	79	0	Ile	AUU 34	63	0	Phe	UUU 38	50	0		A 9	20	0
	C 54	21	100		C 58	21	98		C 62	50	100		G 50	10	76
					A 8	16	2								

Fig. 1. Codon usage of native GluCl α 1 and β subunits compared with the optimized GluCl α 1 and β subunits synthesized in this study. Percentage frequencies of the synonymous codons are shown for each corresponding amino acid. The most prevalent codon is shown in bold.

Table 1
Codon Adaptation Index calculations

Gene	Native CAI	Optimized CAI
GFP	0.604	0.950
GluCl α 1	0.552	0.953
GluCl β	0.585	0.951

current pulses (Slimko et al., 2002); but non-expressing neurons are unaffected by this concentration of IVM. The response of both the wild type and optimized constructs to this IVM concentration is presented in Fig. 3(B). The optimized GluCl genes lead to ≈ 3 -fold greater IVM-induced conductance increase than in experiments with wild-type GluCl. This ratio is probably an underestimate because only the most brightly fluorescent cells expressing nonoptimized subunits are chosen for patch-clamp study; and we believe that the results with fluorescence are closer to an unbiased average.

In a previous study, we were unable to measure accurate dose–response relations for IVM due to variability in the expression levels of the genes. The higher expression level, combined with Nupherin-mediated transfection, enables more systematic study (Fig. 5). The EC₅₀ of the IVM-induced conductance is 1.3 nM, with a Hill coefficient of 1.9.

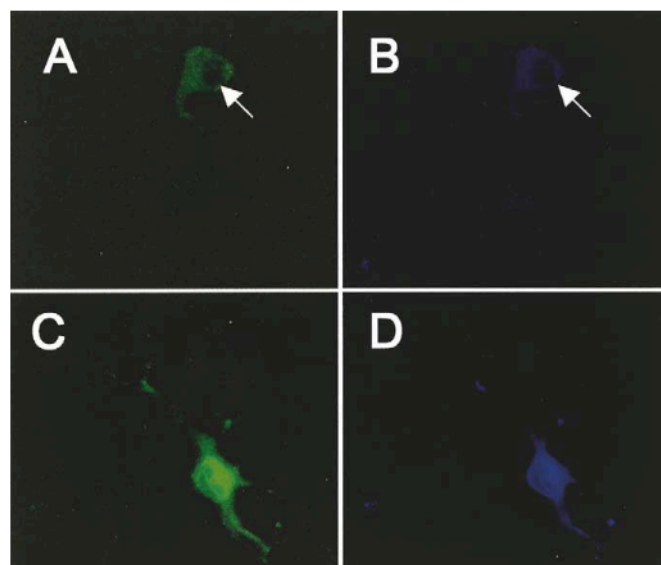


Fig. 2. Increase in channel expression as revealed by fluorescence microscopy. (A, B) Native codon usage GluCl α 1-EYFP fusion and GluCl β -ECFP fusion. The arrow indicates the position of the neuron in each panel. (C, D) Optimized codon usage optGluCl α 1-EYFP fusion and optGluCl β -ECFP fusion. These are representative cells.

3.2. Minimization of glutamate responses

The native ligand for GluCl is glutamate and we have been concerned that unintended GluCl activation by

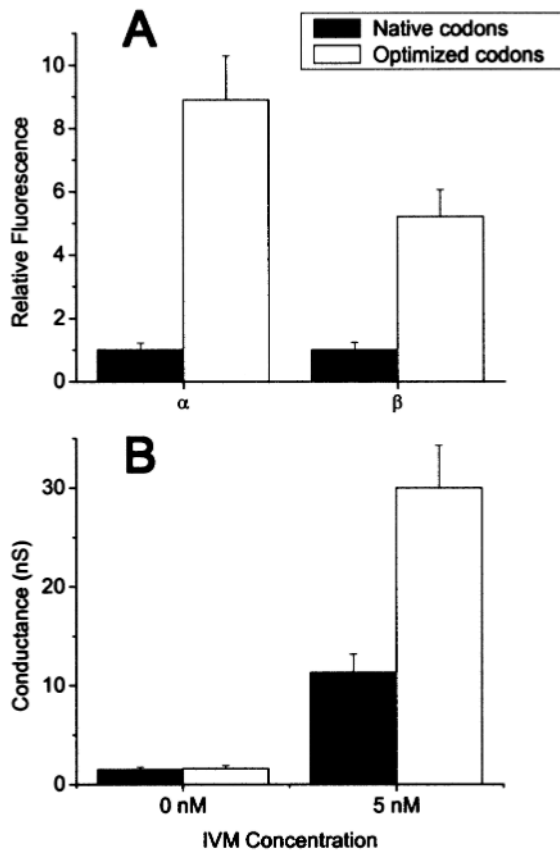


Fig. 3. Increase in expression measured by fluorescence microscopy and electrophysiology. (A) Increase in fluorescence measured by averaging ten cells of both the native codon usage genes and optimized codon usage genes. (B) Conductance of neurons in the absence and presence of 5 nM IVM. Each bar represents the average of ten cells.

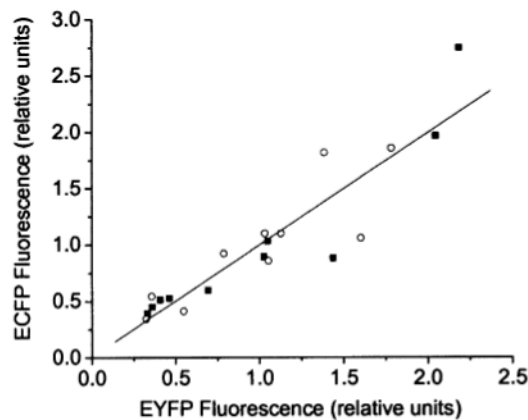


Fig. 4. Correlation of signals in the two fluorescent channels, EYFP and ECFP, for neurons that have been cotransfected with either optGluCl α 1-EYFP and optGluCl β -ECFP (open circles) or wtGluCl α 1-EYFP and wtGluCl β -ECFP (closed squares).

circulating or synaptically released glutamate may also inactivate neurons. Previously data showed that expression of GluCl α 1 and a mutated β subunit, GluCl β Y182F, results in a heteromeric channel that

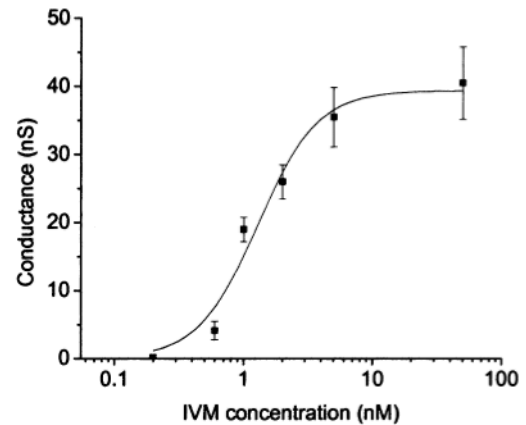


Fig. 5. Dose-response relation for activation of optGluCl channel by IVM. EC_{50} is 1.2 nM and Hill coefficient is 1.9. Each point represents the average of the input conductance of ten cells at the given concentration of IVM.

retains its IVM response with an attenuated glutamate response (Li et al., 2002). We made the Y182F mutation in optGluCl β -ECFP and co-expressed this gene with optGluCl α 1-EYFP in hippocampal cultures. The conductance increase caused by 5 nM IVM is statistically indistinguishable from the unmutated channel (Fig. 6A). However, the maximal response to a 1 s application of 100 μ M glutamate is reduced 7-fold by this mutation, similar to previous *Xenopus* oocyte data (Fig. 6B). In the glutamate response experiments, CsCl was used in the internal pipette solution and endogenous glutamate receptors were blocked with 100 μ M CNQX and untransfected control cells had no response to glutamate.

4. Discussion

Researchers have used codon optimization to increase the expression of a variety of cytosolic proteins. The accompanying increase in expression ranges from modest, such as 1.6-fold for Cre (Shimshek et al., 2002) and 2-fold for rtTA (Wells et al., 1999), to dramatic, such as 40-fold to several hundred-fold for both GFP and gp120 (Haas et al., 1996). This is the first published example of successful codon optimization to increase the expression of a membrane protein in mammalian cells. It is generally thought that membrane-bound proteins are limited in surface expression levels by trafficking, sorting and exporting mechanisms, rather than by translation. However, we find that codon optimization provides a large (6- to 9-fold) enhancement of expression. There was little or no further enhancement when the signal peptide sequence of the optimized GluCl β gene was replaced with the signal peptide of the mouse nicotinic α 4 receptor. The codon optimization procedure may prove useful for other studies in which

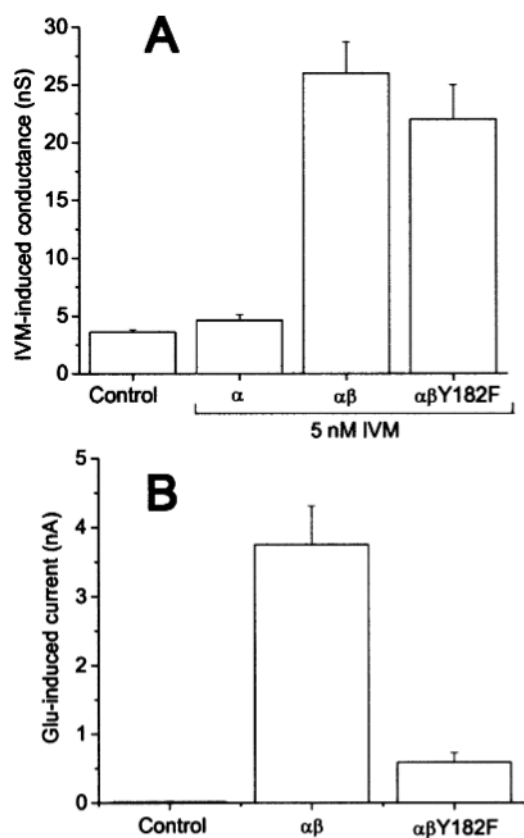


Fig. 6. Effects of optGluCl β Y182F mutation. (A) Input conductance in the presence of 5 nM IVM. The control bar includes three groups of cells: cells that were not transfected and tested in the absence of IVM; cells that were not transfected and tested in the presence of 5 nM IVM; and cells transfected with optGluCl α and optGluCl β or optGluCl β Y182F in the absence of IVM. There were no significant differences among these control groups. IVM (5 nM) was applied to three groups of cells: cells transfected with optGluCl α only (α); cells transfected with optGluCl α and optGluCl β ($\alpha\beta$); and cells transfected with optGluCl α and optGluCl β Y182F ($\alpha\beta$ Y182F). Note that the mutation has no effect on the IVM activation of the channel. (B) The response to 100 μ M glutamate is severely attenuated with the β Y182F mutation. Glutamate (100 μ M) in the presence of 100 μ M CNQX was applied to three groups of cells: untransfected controls; cells expressing optGluCl α and optGluCl β ($\alpha\beta$); and cells transfected with optGluCl α and optGluCl β Y182F ($\alpha\beta$ Y182F).

nonmammalian ion channels, transporters and receptors are expressed in clonal mammalian cell cells, e.g. for screening of antiparasitic drugs.

Several laboratories are developing genetic and pharmacological techniques for selectively silencing specific sets of neurons in vivo with mice or other animals (White et al., 2001; Lechner et al., 2002; Nitabach et al., 2002). Such techniques will presumably enable various experiments that may give us further insight into the system level functioning of the nervous system. The optimized, fluorescent GluCl constructs described here are activated by IVM concentrations of ≈ 1 nM, with little interference from endogenous glutamate responses and with ready verification by fluorescence. This relatively low IVM concentration should help avoid

known non-specific IVM effects (Krusek and Zemkova, 1994; Krause et al., 1998; Khakh et al., 1999; Shan et al., 2001). These GluCl subunits provide a robust system for high-efficiency silencing and we consider that the channels are now appropriate for in vivo use. We are now turning our attention to efficient, specific in vivo expression. The two general strategies are (1) transgenic animals; and (2) local injection of viral vectors.

Acknowledgements

This work was supported by National Institutes of Health (NS 11756 and MH 49176), by the Plum Foundation and by the William T. Gimbel Discovery fund in Neuroscience. We thank David Anderson, Michael Fanselow and Christof Koch for discussion and Sheri McKinney for help with cultures.

References

- Bernardi G. Codon usage and genome composition. *J Mol Evol* 1985;22:363–5.
- Cully DF, Vassilatis DK, Liu KK, Parens PS, Van der Ploeg LH, Schaeffer JM, Arena JP. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* 1994;371:707–11.
- del Giudice P. Ivermectin in scabies. *Curr Opin Infect Dis* 2002;15:123–6.
- Gensler S, Sander A, Korngreen A, Traina G, Giese G, Witzemann V. Assembly and clustering of acetylcholine receptors containing GFP-tagged epsilon or gamma subunits: selective targeting to the neuromuscular junction in vivo. *Eur J Biochem* 2001;268:2209–17.
- Haas J, Park EC, Seed B. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol* 1996;6:315–24.
- Hernan RA, Hui HL, Andracki ME, Noble RW, Sligar SG, Walder JA, Walder RY. Human hemoglobin expression in *Escherichia coli*: importance of optimal codon usage. *Biochemistry* 1992;31:8619–28.
- Ikemura T. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the *E. coli* translational system. *J Mol Biol* 1981a;151:389–409.
- Ikemura T. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. *J Mol Biol* 1981b;146:1–21.
- Kanaya S, Yamada Y, Kinouchi M, Kudo Y, Ikemura T. Codon usage and tRNA genes in eukaryotes: correlation of codon usage diversity with translation efficiency and with CG-dinucleotide usage as assessed by multivariate analysis. *J Mol Evol* 2001;53:290–8.
- Kane JF. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr Opin Biotechnol* 1995;6:494–500.
- Khakh BS, Humphrey PP, Surprenant A. Electrophysiological properties of P2X-purinoreceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J Physiol* 1995;484:385–95.
- Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA. Allosteric control of gating and kinetics at P2X₄ receptor channels. *J Neurosci* 1999;19:7289–99.

- Krause RM, Buisson B, Bertrand S, Corringer PJ, Galzi JL, Changeux JP, Bertrand D. Ivermectin: a positive allosteric effector of the $\alpha 7$ neuronal nicotinic acetylcholine receptor. *Mol Pharmacol* 1998;53:283–94.
- Krusck J, Zemkova H. Effect of ivermectin on gamma-aminobutyric acid-induced chloride currents in mouse hippocampal embryonic neurones. *Eur J Pharmacol* 1994;259:121–8.
- Lechner HA, Lein ES, Callaway EM. A genetic method for selective and quickly reversible silencing of mammalian neurons. *J Neurosci* 2002;22:5287–320.
- Li P, Slimko E, Lester H. Selective elimination of glutamate activation and introduction of fluorescent proteins into a *Caenorhabditis elegans* chloride channel. *FEBS Lett* 2002;528:77.
- Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev* 1996;60:512–38.
- Nakamura Y, Wada K, Wada Y, Doi H, Kanaya S, Gojobori T, Ikemura T. Codon usage tabulated from the international DNA sequence databases. *Nucleic Acids Res* 1996;24:214–5.
- Nitabach MN, Blau J, Holmes TC. Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. *Cell* 2002;109:485–95.
- Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. *J Comput Biol* 1997;4:311–23.
- Sakai H, Washio T, Saito R, Shinagawa A, Itoh M, Shibata K, Carninci P, Konno H, Kawai J, Hayashizaki Y, Tomita M. Correlation between sequence conservation of the 5' untranslated region and codon usage bias in *Mus musculus* genes. *Gene* 2001;276:101–5.
- Shan Q, Haddrill JL, Lynch JW. Ivermectin, an unconventional agonist of the glycine receptor chloride channel. *J Biol Chem* 2001;276:12556–64.
- Sharp PM, Li WH. The Codon Adaptation Index—a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Res* 1987;15:1281–95.
- Shimshak DR, Kim J, Hubner MR, Spergel DJ, Buchholz F, Casanova E, Stewart AF, Seeburg PH, Sprengel R. Codon-improved Cre recombinase (iCre) expression in the mouse. *Genesis* 2002;32:19–26.
- Slimko EM, McKinney S, Anderson DJ, Davidson N, Lester HA. Selective electrical silencing of mammalian neurons in vitro by the use of invertebrate ligand-gated chloride channels. *J Neurosci* 2002;22:7373–9.
- Wells KD, Foster JA, Moore K, Pursel VG, Wall RJ. Codon optimization, genetic insulation, and an rtTA reporter improve performance of the tetracycline switch. *Transgen Res* 1999;8:371–81.
- White B, Osterwalder T, Keshishian H. Molecular genetic approaches to the targeted suppression of neuronal activity. *Curr Biol* 2001;11:R1041–53.
- Winnen M, Plaisier AP, Alley ES, Nagelkerke NJ, van Oortmarssen G, Boatin BA, Habbema JD. Can ivermectin mass treatments eliminate onchocerciasis in Africa? *Bull World Health Org* 2002;80:384–91.
- Woo JH, Liu YY, Mathias A, Stavrou S, Wang Z, Thompson J, Neville DM. Gene optimization is necessary to express a bivalent anti-human anti-T cell immunotoxin in *Pichia pastoris*. *Prot Expr Purif* 2002;25:270–82.