The Development and Application of the Semliki Forest Virus expression system to the study of GABA $_A$ receptors.

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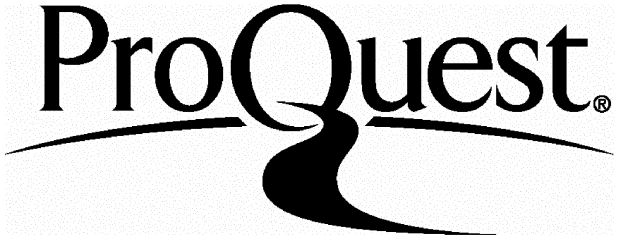

Abstract

$GABA_A$ receptors are presumed to be pentameric hetero oloigomers composed of subunits potentially drawn from five subunit classes with multiple members; α (1–6),β (1–4),γ (1–3), δ, and ε . The mechanisms by which neurones assemble this potentially large number of subunits into functional $GABA_A$ receptors is unknown. Heterologous expression systems have demonstrated that particular combinations of $GABA_A$ subunits assemble to form channels whilst others are selectively retained within the endoplasmic reticulum of the cell.

In order to determine whether the assembly rules which have been defined in such heterologous expression system are representative of the assembly of $GABA_A$ receptor subunits within primary cultures of neurones, a viral expression system was used to express recombinant epitope tagged $GABA_A$ subunits in neurones. The viral expression system which was chosen was based upon the Semliki Forest virus. This particular viral expression system was chosen because of its ability to infect neurones as well as many other cell types with high efficiency.

Recombinant viral particles were produced which could direct the synthesis of the α1 (9E10), β2 (9E10), γ2 L FLAG and γ2 S (9E10) tagged subunits. Using the Semliki viral expression system the assembly of α1 (9E10) and β2 (9E10) subunits in BHK cells was compared to that produced when these subunits were transiently transfected into HEK 293 cells. The results of the viral infections replicated the assembly work performed using conventional transient transfections of HEK 293 cells. Expression of individual α1 (9E10) or β2 (9E10) subunits resulted in their selective retention within the endoplasmic reticulum of the cells whilst on co-expression both the α1 (9E10) and β2 (9E10) subunits could access the cell surface. Whole cell electron microscopy of such cells

demonstrated that the subunits were on the surface of the cell and were preferentially associated with the microvilli of the BHK cells.

The powerful nature of the Semliki viral expression system allowed the demonstration of a biophysical association between α1 (9E10) and β2 (9E10) proteins as demonstrated by changes in the sedimentation coefficients of the two proteins on co-infection of BHK cells by the two viral species. When individually expressed the α1 (9E10) and β2 (9E10) proteins have 5S sedimentation coefficients. On co-infection the sedimentation coefficients of both proteins shifted to 9S.

For the first time, the viral expression system enabled the efficiency of the assembly of the α1 (9E10) and β2 (9E10) subunits to be estimated. It also provided a means to study the stability of assembled and unassembled receptor composed of α1 (9E10) and β2 (9E10) subunits. It was found that only 33% of the available α1 (9E10) and β2 (9E10) subunits became incorporated into the assembled 9S complex. Once formed, this 9S sedimenting species was relatively stable only dropping 10% of its initial signal over a 16 hour period. In contrast the unassembled receptor was quickly degraded, with a 75% drop over 6 hours. The half life of individually virally expressed α1 (9E10) and β2 (9E10) subunits was also investigated and determined to be between 2 to 3 hours.

The stoichiometry of a $GABA_A$ receptor composed of α1 (9E10) and β2 (9E10) receptors was also evaluated using the Semliki expression system. Metabolic labelling in combination with 9E10 immunoprecipitaiton of the assembled 9S receptor allowed a determination of the ratio of α to β subunits after correction for the relative methionine contents of the two proteins. The ratio of α1 (9E10) to β2 (9E10) subunits was 1:1.

The toxicity of viral infection upon SCG neurones was studied by infecting SCG neurones with a recombinant SFV virus engineered to make the Green Fluorescent Protein and then subsequently recording

the resting membrane potential of neurones which could produce green fluorescence. The neurones which produced green fluorescence had normal resting potentials and a normal ability to produce action potentials.

Knowing that the viral expression system had limited toxicity to the SCG neurones and that the expression system was capable of supporting the assembly of the α1 (9E10) and β2 (9E10) subunits within BHK cells the assembly of these subunits was studied in primary cultures of SCG neurones. Infection of such cultures by either the α1 (9E10) or β2 (9E10) virus particles resulted in the retention of the $GABA_A$ subunit within the cell body of the neurone. On co-infection marked surface expression could be demonstrated. This indicated that a common assembly mechanism occurs between neurones and transformed cell lines, with retention within the endoplasmic reticulum and subsequent degradation featuring as a major control mechanism.

The biochemistry, and cell biology of the γ2 subunit was also investigated using the Semliki expression system. The γ2 S (9E10) protein was demonstrated to behave differently to the long isoform on polyacrylamide gel electrophoresis (PAGE) and sucrose gradient sedimentation studies. The size of the long isoform on PAGE was 45KDa which contrasted with the 50KDa size for the γ2 S (9E10). The sucrose gradient studies indicated that the γ2 S (9E10) protein had a sharp profile peaking at 3S whilst the γ2 L FLAG had a much broader profile with its peak nearer 4S and aggregates between 5 and 7S.

Viral expression of the short and long splice variants of the γ2 subunit within BHK cells and SCG neurones produced contrasting cell biology. The short variant was shown to be capable of trafficking to the surface of BHK cells and into the processes of SCG neurones. The short splice variant was also capable of being endocytosed from the surface of BHK

cells. Electron microscopic examination of BHK cells infected with the γ2 S (9E10) virus was used to demonstrate the association of the γ2 S (9E10) protein with coated pits. In contrast the long variant was not able to access the plasma membrane when individually expressed. Expression of the long isoform of the γ 2 subunit within SCG neurones produced only a cell body pattern of expression consistent with the retention of the subunit within the endoplasmic reticulum.

Finally the capability of the γ 2 subunit to be tyrosine phosphorylated within cells expressing high levels of the tyrosine kinase Src was tested. When transiently coexpressed with α 1, β 1 and v-Src both the γ2 L (9E10) and γ2 S (9E10) proteins could be shown to undergo phosphorylation specifically at tyrosine residue 365 and 367 in the short and 373 and 375 in the long .

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Abbreviations

ACh	Acetylcholine
AChR	Acetylcholine receptor
ATP	Adenosine 5'- triphosphate
BIP	Immunoglobulin heavy chain binding protein
BHK	Baby Hamster Kidney cells
BSA	Bovine Serum Albumin
Bmax	Maximum binding sites
CFTR	Cystic fibrosis transmembrane conductance regulator
CHAPS	3-((3-cholamidopropyl)dimethyl-ammonio)-1-propansulphonate
CNS	Central nervous sytem
CHO	Chinese Hamster Ovary cells
DEPC	Diethylpirocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dNTP's	deoxonucleotides triphosphate
DMEM	Dulbecco's Modified Eagle medium
ECL	Ehanced Chemiluminescence
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochroide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
EM	Electron microscopy
ER	Endoplasmic Reticulum
FCS	Fetal Calf Serum
FLAG	FLAG monoclonal antibody

GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
HEK 293	Human Embryonic Kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performeance Liquid Chromatography
Kda	KiloDaltons
KLH	Keyhole Limpet Haemocyanin
LB	L Broth
nAChR	Nicotinic Acetylcholine Receptor
NMDA	N-methyl-D-aspartate
MDCK	Madin-Darby Canine Kidney cells
MHC	Major Histocompatability Complex
(9E10)	(9E10) monoclonal antibody, also referred to as MYC
PBS	Phosphate Buffered Saline
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethlene glycol
PMSF	Phenylmethanesulphonyl fluoride
PFU	Plaque Forming Units
pS	PicoSiemens
RNA	Ribonucleic acid
S	Svedberg Unit (1 S unit $=1x10^{-13}$ sec)
SDS	Sodium Dodecyl Sulphate
TBE	Tris Borate Edta Buffer
TE	Tris Edta buffer
TRIS	Tris(hydroxymethyl)aminoethane

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Chapter 1

Introduction

1.1.1 Gabaergic neurotransmission

γ amino butyric acid (GABA) has been defined as an inhibitory neurotransmitter in the mammalian central nervous system. Indeed it has been shown to be an inhibitory neurotransmitter in the vast majority of multicellular organisms (Nistri and Constantini 1979). The existence of high concentrations of GABA within the central nervous system of mammals was demonstrated in 1950 by Roberts and Frankel. It was then shown that GABA added topically inhibited electrical activity within neurones, (Hayashi and Nagai 1956), and that exogenous GABA could reproduce the inhibition at the crustacean neuromuscular junction produced on stimulating inhibitory axons (Kuffler and Edwards 1958). The inhibitory axons were then shown to contain large amounts of GABA, (Kravitz 1963) which could be released in a calcium dependant fashion (Otsuka 1966). This was later followed by experiments demonstrating that similar reversal potentials could be obtained in cat cortical neurones by inhibitory synaptic activity and iontophoretically applied GABA (Dreifuss et al 1969). ^{3}H GABA could be shown to be taken up by neurones by a high affinity transporter, (Neal and Iverson 1969) and subsequently released in a calcium dependant fashion by depolarisation (Farb et al 1979). The mechanism by which neurones synthesise GABA was then illustrated when ^{3}H glutamate was added to neurones in culture. The cultured neurones were subsequently shown to produce ^{3}H GABA from glutamate via glutamic acid decarboxylase (GAD) (Rando et al 1981). GABA had met the criteria used to define a putative neurotransmitter that is: the existence of a mechanism of synthesis, storage, release, a site of action and a mechanism of removal.

The majority of neurones in the CNS of vertebrates have been shown to be sensitive to the inhibitory effects of GABA. The inhibitory action of GABA is produced by the opening of an integral chloride ion channel, which results in the cell's resting potential being quickly driven to the chloride equilibrium potential. It was shown that this "classical" GABA gated receptor was activated by muscimol but blocked by picrotoxin and bicuculline (Nistri and Constanti 1979). GABA was also demonstrated to produce a slower inhibitory current which was bicuculline insensitive but activated by baclofen instead of muscimol (Hill and Bowery 1981). The "classical" receptors were termed $GABA_A$ and those producing the slower inhibition which was stimulated by baclofen and not inhibited by bicuculline, $GABA_B$. GABA mediated transmission at the $GABA_A$ receptor was also shown to be potentiated by benzodiazepines in the spinal cord, (Schmidt 1971) and in the cuneate nucleus of the brainstem, (Polc and Haefeley 1976). In contrast $GABA_B$ receptors do not show allosteric modulation by benzodiaepines. The $GABA_B$ receptors were shown to be members of the G protein coupled receptor class by experiments describing their capability to modulate adenylcyclase via an interaction with a GTP binding protein (Hill 1985).

The terminology of the GABA receptor has been further updated by the advances generated by the cloning of receptor subunits. The receptors for this neurotransmitter are presently divided into three main classes: $GABA_A$, $GABA_B$ and $GABA_C$. The $GABA_A$ receptor corresponds to a class of ligand gated chloride ion channels mediating fast synaptic inhibition (Schofield et al 1987). The $GABA_B$ receptor is a member of the seven pass receptor class and mediates slower inhibitory events via G protein coupling. This class has recently been cloned and shows similarity to the metabotropic glutamate receptors (Kaupmann et al 1997). $GABA_C$ receptors are ligand gated chloride ion channels which are

bicuculine and baclofen insensitive and are found mainly in the retina. The $GABA_C$ receptors are composed from homoligomers of ρ subunits (1-3) (Cutting et al 1992).

1.1.2 Physiological and Pharmacological importance of Gabaergic transmission.

The critical role of GABA mediated inhibition at the $GABA_A$ receptor in the coherent function of the central nervous system is illustrated when the $GABA_A$ channel is blocked by picrotoxin. Hyper excitability occurs and generalised seizures are produced (Dedeyn et al 1992). The observation that seizures occur when GABA mediated transmission is reduced has provoked the development of new and effective anti epileptic drugs such as Vigabatrin and Tiagabine. Vigabatrin blocks the enzyme GABA transaminase which is responsible for its metabolism (Dichter 1994, Petroff et al 1996, French et al 1996). Tiagabine raises post synaptic levels of GABA by inhibiting the reuptake of the neurotransmitter (Brodie 1995 , Schachter 1995).

The $GABA_A$ receptor represents an important site of pharmaceutical action . The barbiturates and benzodiazepines produce their effects by potentiating the action of endogenous GABA. Benzodiazepines increase the frequency of channel opening, (Rogers et al 1994) whereas barbiturates increase the mean open time of the channel (Porter et al 1992). Recently this dogma has been **challenged** by the discovery that diazepam can increase the conductance of $GABA_A$ channels activated by low concentations (0.5-5μM) GABA (Eghbali et al 1997). The binding of benzodiazepines to $GABA_A$ receptors within the central nervous system has been divided into two classes. The BZ1 class has a higher affinity for the triazolopyridazine CL 218,872 compared to the BZ2 subtype which has a lower affinity for this ligand (Pritchett et al 1989b, Luddens and Wisden 1991). Steroids also alter the response of the channel to GABA.

For example pregnanolone and androsterone cause an increase in mean open time of the channel and frequency of channel opening (Twyman and Macdonald 1992). Volatile anaesthetics such as halothane, enfluorane and isofluorane are known to potentiate GABA mediated currents at clinically effective concentrations (Jones et al 1992 and Harrison et al 1993). It should be noted that although evidence exists which clearly supports the role of anaesthetic agents in potentiating GABA responses the evidence for this causing an increase in inhibitory transmission is not so well defined (Franks and Lieb 1994). The effect of ethanol upon the $GABA_A$ receptor is more controversial. Ethanol has been reported to enhance the inhibitory effect produced by GABA upon cultured spinal cord neurones (Celentano et al 1988). Within the central nervous system the potentiating effects are more variable, 40-60% of neurones from rat and chicken cerebral cortex demonstrated ethanol sensitivity (Reynolds et al 1992). The effect of ethanol was also concentration dependant, higher concentrations (hundreds of mM) diminishing the enhancing effect. However a recent report has described inhibitory effects of ethanol upon GABA currents (Aguayo and Alarcon 1993). Other clinically relevant drugs which modulate GABA neurotransmission include betalactam antibiotics. Penicillin has been reported to reduce the mean open time of the $GABA_A$ channel in a dose dependant fashion (Twyman et al 1992b). This correlates well with its known **pro-convulsant** activity. Cephalosporins are also known to inhibit GABA binding to the $GABA_A$ receptor predisposing the onset of seizures.

1.1.3 Biochemical characterisation of the $GABA_A$ receptor

The biochemical characterisation of the proteins that compose this channel has been extensively investigated. These studies have been aided by the ability to use drugs which have been demonstrated to bind

pharmacologically to the $GABA_A$ receptor, as affinity ligand and photocrosslinking agents. Benzodiazepine affinity purification of this receptor from brain homogenates resulted in the copurification of a high affinity benzodiazepine binding site and a high affinity binding site for the $GABA_A$ agonist ^3H muscimol (Sigel et al 1983). Commassie blue stained SDS /PAGE gels of benzodiazepine affinity purified receptors produced two predominant bands on a PAGE gel. These proteins were termed the α (53 KDa) and β (58 KDa) subunits, (Stephenson 1988). Photo affinity labelling of purified receptor preparations using ^3H flunitrazepam labelled á protein with the same size as the α subunit (Stephenson et al 1986). Photo affinity labelling of receptors using ^3H flunitrazepam from other regions of the brain such as the hippocampus produced multiple peaks of incorporation of signal at 53, 55 and 59KDa (Sieghart and Karobath 1980). More recent studies have demonstrated that the multiple bands associated with the ^3H flunitrazepam labelling correspond to different α subunits (Fuchs et al 1990, Bureau and Olsen 1990).

^3H muscimol was also used to photoaffinity label GABA receptors. Such studies demonstrated that this ligand labelled the 57KDa species, corresponding to the β subunit (Cassalotti et al 1986, Deng et al 1986). More recent studies which involved the microsequencing the ^3H muscimol photoaffinity labelled proteins identified the amino acid phenyalanine residue 64 on the α1 subunit as being the primary residue that is labelled by this ligand (Smith and Olsen 1994).

Monoclonal and polyclonal antibodies have been raised against purified $GABA_A$ receptors. The monoclonal antibody BD17 was demonstrated to be β specific whilst the monoclonal antibody BD 24 was shown to be α specific on western blotting (Schoch et al 1985). The existence of both α and β subunits within the same receptor was supported by the capability

of both these antibodies to immunoppreciptate ^{3}H muscimol and ^{3}H flunitrazepam binding (Schoch et al 1985) .

1.1.4 Cloning of the GABA $_A$ receptor cDNA's

The molecular cloning of GABA $_A$ receptor subunit cDNAs was first reported in 1987. (Schofield et al 1987) This seminal paper described how the first cDNA clones for the $GABA_A$ receptor were isolated. Benzodiazepine affinity purified $GABA_A$ receptors were cleaved using cyanogen bromide and the peptides separated by HPLC. The peptides were then used to derive oligodeoxynucleotide probes. The probes were then used to screen a bovine brain cDNA library. Positive clones were identified and sequenced. The α and β cDNA 's were designated by the presence of the relevant peptide sequences. The sequences were used to make structural and homology studies that indicated that the $GABA_A$ receptor was a member of the ligand gated ion channel superfamily characterised most extensively by the nicotinic acetylcholine receptor (nAChR). Proof that a $GABA_A$ receptor could be produced from the RNA derived from these two cDNA clones was demonstrated by electrophysiological recordings from an oocyte previously injected with both species of RNA (Schofield et al 1987). Further subunits were cloned by homology screening, most importantly the γ2 subunit which was shown to be critical for the formation of a high affinity benzodiazepine binding site when co-expressed with α1 and β1 subunits (Pritchet et al 1989). Recently a new subunit class was identified by searching an expressed sequence tag database with a peptide sequence homologous to other known $GABA_A$ subunits (Davies et al 1997). The cDNA cloned by this technology produced a protein called the ε subunit which when combined with α2 and β1 subunits produced $GABA_A$ receptors insensitive to the modulatory actions of benzodiazepines. The receptors

containing the ε subunit were insensitive to the modulatory effects of pentobarbitone and propofol (Davies et al 1997).

1.1.5 The Structure of the $GABA_A$ receptor.

The cloning studies had revealed that the $GABA_A$ receptor was a member of the ligand gated ion channel superfamily. The most fully characterised member of this family is the nicotinic acetylcholine receptor (nAChR). The structure of this ligand gated ion channel has been demonstrated to be composed of a pentamer of subunits arranged around a central pore, see Fig 1. This has been concluded from direct physical analysis by electron diffraction studies of postsynaptic membranes produced from the *Torpedo* electric organ (Unwin 1993 and Unwin 1995). The $GABA_A$ receptor through homology to the nAChR has been assumed to have a similar pentameric arrangement of subunits.

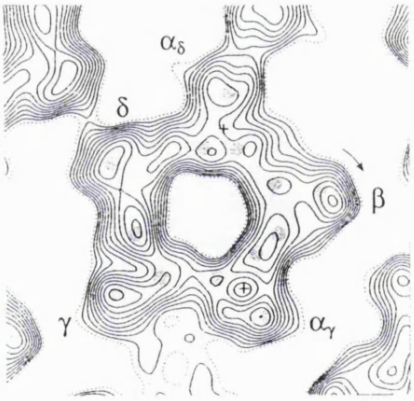


Fig 1. Acetylcholine receptor isolated from the electric organ of the *Torpedo* Ray (Unwin 1995).

Structural analysis such as that performed on the nAChR has not been possible for any other ligand gated ion channel due to the lack of a preparation with equivalent density of receptors. Electron microscopic analysis has been performed on affinity purified preparations of the 5HT3 and $GABA_A$ receptors. These studies did not involve diffraction studies with significant loss of resolution. The purified receptors were

stained with uranyl acetate and the symmetry of the images analysed by harmonic analysis. In both studies a pentameric symmetry was observed (Boess et al 1995 and Nayeem et al 1994).

Biochemical studies performed on the neural nAChR generated on the expression of α4 and β2 indicated a ratio of 2 α subunits to three β subunits. This supports a pentameric arrangement for this particular receptor (Anand et al 1991). Crosslinking studies on another member of the ligand gated superfamily of receptors, the glycine receptor, produced a major adduct of 260KDa which migrated at 8S. It was concluded from this data that the most likely structure was a pentameric arrangement of the subunits (Langosch et al 1988). Sucrose gradient studies in combination with sucrose gradient analysis of solubilised 5HT3 receptors enabled the determination of the molecular weight of the homoligomer to be 248965 Da. This supported the pentameric arrangement seen by electron microscopy (McKernan et al 1990).

The molecular weight of $GABA_A$ receptors purified by benzodiazepine affinity chromatography of bovine brain lysates was determined by gel exclusion chromatography and sucrose gradient ultra centrifugation. The size estimated by these methods was 240,000 (Mamalaki et al 1989). Gel exclusion chromatography of solubilised membranes from a stable cell line expressing α1, β1 and γ2 L subunits produced a comparable result (Hadingham et al 1992). Biochemical studies performed using the methionine labelling of *Xenopus* oocytes microinjected with α1 and β3 cRNA supported an equal ratio of the two subunits in the receptor complex (Kellenberger. et al 1996). This is less compatible with a pentameric model. Tretter et al (1997) recently using a combination of sucrose gradient studies and quantitative western blotting have evidence that supports a pentameric arrangement of subunits. This study concluded that the stoichiometry of a $GABA_A$ receptor composed of

α1β3γ2 subunits was 2 α, 2 β and one γ subunit per receptor (Tretter et al 1997).

1.1.6 The Diversity of $GABA_A$ subunits as revealed by cloning studies.

Five classes of subunits with multiple members have now been isolated by cloning α (1–6), β (1–4), γ (1–3) , δ (1) ε (1). One further layer of complexity exists in the form of alternative splice variants of the α6, β2 and γ2 subunits. The alternative splice variant for the α6 occurs in the N terminus of the subunit between nucleotides 225 and 226 (Whiting et al 1990, Korpi et al 1994). Alternative splicing of the β2 occurs within the the intracellular loop between transmembrane domains 3 and 4 (McKinley et al 1995). The γ2 subunit also has an alternative splice variant which differs by the insertion of eight amino acids in the intracellular loop between transmembrane domains 3 and 4 (Kofuji et al 1991). Within each class there is a 70-80% homology whilst there is only 30-40% homology between classes (Macdonald and Olsen 1994). It is clear that a large number of possible combinations of subunits are theoretically possible.

The diversity of $GABA_A$ receptor subunits and their regional distributions within the central nervous system has been studied using in situ hybridisation and immunohistochemical analysis at the light and electron microscopic level. *In situ* hybridisation studies of different subunit RNA's clearly indicated marked differences in the regional distributions of the subunits, for example the complementary distributions of β2 and β3 subunits but also areas of overlap, for example in the distribution of the α1 and β2 subunits. (Luddens and Wisden 1991)

1.1.7 Immunohistochemical studies of $GABA_A$ receptors in the central nervous system at the light level.

Immunohistochemical studies performed on specimens from the central nervous system of rats using the light microscope have concentrated upon the documentation of the differing distributions of $GABA_A$ subunits within the brain. Colocalisation studies have also been performed using fluorescent microscopy. Such studies have demonstrated the colocalisation of α1 and β2 subunits within neurones and that regions with strong α2 fluorescence also have high levels of β3 (Benke et al 1994). Using a combination of fluorescent microscopy and peroxidase methods it has been demonstrated that the most prevalent combination of $GABA_A$ subunits within the CNS of rats was α1,β2/3 and γ2 (Fritschy and Mohler 1995). These individual subunits had a ubiquitous and similar distribution within the rat brain. Other $GABA_A$ subunits studied had dissimilar distributions, for example the α2 subunit was more common in regions with low levels of the α1 subunit (Zimprich et al 1991, Fritschy and Mohler 1995) . The distribution of these individual proteins mirrored that demonstrated by in situ hybridisation studies (Fritschy and Mohler 1995). The distribution of the alternative splice variants of the γ2 subunit has also been studied. It was found that the overall distributuion of the splice variants was similar, but in some areas the relative intensitiy of the two signals varied. It was found that there was higher γ2 S signal in the hippocampus and cerebral cortex compared to the long splice variant. In contrast the γ2 L splice variant was more abundant in the medulla and cerebellar Purkinje cells compared to the short splice variant. (Guiterrez et al 1994)

The cell topology of $GABA_A$ subunits was also investigated. The α2 and β2/3 subunits were found to have a somatic distribution. Interestingly in the Purkinje cells of the cerebellum the α1 subunit was localised to the

cell soma whilst the α3 subunit had a somatic and dendritic distribution (Fritschy et al 1992).

1.1.8 Ultrastructural analysis of $GABA_A$ receptors in the central nervous system.

Electron microscopic analysis of histological sections taken from the central nervous system of rats using immuno gold labelling techniques have allowed Somogyi's group to demonstrate the synaptic localisation of $GABA_A$ subunits. The differential distributions of the α1 and α2 subunits in hippocampal pyramidal cells has also been illustrated using immuno gold electron microscopy. The α2 subunit being relatively more restricted to the initial segment of the axon, compared to the much widely distributed α1 subunit (Nusser et al 1996). The α6 subunit has also been shown to be detectable only at synaptic sites where as the α1 and β2/3 subunits have both synaptic and extra synaptic distributions in the granule cells of the cerebellum (Baude et al 1992). The distribution of the γ2 subunit in the hippocampus, globus pallidus and cerebellum has also been studied at the EM level. This subunit is concentrated at synapses containing the α1 and β2/3 receptor subunits as demonstrated by double immuno gold colocalisation studies (Somogyi et al 1996).

It is thus clear at the ultra structural level that a differential distribution of $GABA_A$ α subunits occurs in neurones. The mechanisms that produce this selective targeting are not known. Given that the α subunit determines the sensitivity of the receptor for GABA and the type of benzodiazepine pharmacology, (Pritchett et al 1989b, Luddens and Wisden 1991) the differential distribution of receptors with different pharmacology would be expected to have functional significance. However if the α6 subunit is knocked out no major deleterious effects are produced, the mouse retains good co-ordination for example (Jones et al 1997).

1.1.9 The determination of which subunit combinations produce functional $GABA_A$ receptors.

The complexity of the central nervous system and the great diversity of $GABA_A$ subunits that are expressed make it difficult to ask specific questions as to which subunits assemble with which and which combinations of subunits can give rise to a $GABA_A$ receptor. In order to address these questions two different experimental rationals have been used. The first involves the use of subunit specific affinity **columns** to identify co-purifying subunits from brain lysates. The second rational involves the use of heterologous expression systems to identify combinations of subunit cDNA or cRNA that can generate $GABA_A$ receptors.

Antibody studies.

The first approach has depended upon the production of subunit selective antibodies. The production of such antibodies has been facilitated by the determination of the primary amino acid sequences of the $GABA_A$ receptor subunits by cloning studies has facilitated the production of subunits specific polyclonal antibodies.

Many polyclonal antibodies have been produced for example: α1 specific antibodies detect proteins between 50-53KDa, α2 specific antibodies detecting proteins between 52-54 KDa, α3 specific antibodies detecting proteins between 58-61KDa, α4 specific antibodies detecting two proteins one at 51KDa and the other at 67KDa, α5 specific antibodies detect a 55 KDa protein whilst α6 antibodies detect a 57KDa molecule. Anti β1 antibodies detect a 57 KDa molecule, whilst a polyclonal antibody to the β2 subunit recognises a 54-58KDa species. β3 specific antibodies have been shown to recognise two species one at 57 KDa and the other at 59 KDa. Anti γ1 antibodies have been shown to detect a 66 KDa protein whilst γ2 specific antibodies produce a broad range of immunoreactivity

between 43-49KDa. Anti δ antibodies detect a 54 KDa protein (Rabow et al 1995).

Affinity chromatography using columns containing subunit specific antibodies has been used to investigate which combinations of $GABA_A$ subunits exist within the brain. Affinity columns specific for a single α subunit were used to demonstrate that most oligomers only contain one α subunit (McKernan et al 1991). It was found using immunoprecipitation that the β2 subunit preferentially associated with the α1 subunit and with the γ2 subunit (Benke et al 1994). Interestingly it was not possible to detect γ2 or γ3 subunits in $GABA_A$ receptors purified by immunoaffinity chromatography on anti peptide γ1 columns. This suggested that $GABA_A$ receptors only contain a single type of γ subunit (Mossier et al 1994). Immunoprecipitation experiments combined with western blotting of immuopurified $GABA_A$ receptors suggested that the receptor combination α1/β2/γ2 accounted for at least 60% of all $GABA_A$ receptors in the rat brain (Benke et al 1991, Duggan et al 1992, Benke et al 1994, Ruano et al 1994). Immunoprecipitation studies performed selectively upon the population of $GABA_A$ receptors in the cer ebellum demonstrated that the α6 is present in combination with the γ2 and δ subunits. (Quirk et al 1994)

Heterologous expression systems.

Heterologous expression systems have been used to determine which combinations of subunits produce functional $GABA_A$ receptors. Pritchet et al in 1988 demonstrated that GABA gated chloride channels could be recorded from Human Embryonic Kidney 293 cells transiently transfected with α1 and β1 expression constructs. This group could also detect currents when cells were transfected with only the α1 or β1. The currents from homomeric receptors were 10% of those detected with the heteromeric receptors. The authors concluded that homomeric channels

could be generated by either subunit but formed much less efficiently compared to the heteromeric channels. Blair et al in 1988 could also demonstrate the formation of GABA evoked chloride currents in oocytes injected with α1, α2 and α3 RNA. In contrast to these studies Khrestchatisky et al in 1989 demonstrated that robust GABA gated chloride currents were only detectable when mRNA for α1 and a β subunit were co-injected into an oocyte. Siegel et al (1990) also independently found that homomeric channels were not formed when mRNA for α1 was injected into *Xenopus* oocytes. However an anion selective channel was formed on expression of β1 mRNA. This channel was not GABA gated, but could be blocked by picrotoxin. (Krishek et al 1996, Connolly et al 1996b) It was also noted that the formation of this channel could be suppressed by co-injection of α but not γ2 RNA.

It was not known whether the combination of α1 and γ2 could generate GABA gated chloride channels. Studies in *Xenopus* oocytes and HEK 293 cells provided evidence to suggest that such a combination could generate GABA gated channels. (Draguhn et al 1990) and (Verdoon et al 1990). More recently work has indicated that the only combinations of α1,β2 and $\gamma2_L$ that can assemble and form channels are α1β2 and $\alpha1\beta2\gamma2_L$. Single subunits are retained in the endoplasmic reticulum as are the dimer combinations of $\alpha1\gamma2_L$ and $\beta2\gamma2_L$. (Connolly et al 1996a).

Epitope tags were used in this study. The epitopes consist of a small stretch , 10 amino acids, which is recognised by a well characterised monoclonal antibody. The epitope can be introduced into the N terminus of the protein just after the signal sequence. Subsequently the recombinant subunit can be recognised by the antibody enabling it to be more readily studied by biochemical and immunocytochemical techniques. Co-expression studies were also used to study the pharmacological properties of particular subunit combinations.

Receptors formed on the co-expression of α and β subunits were found to respond to GABA and the chloride currents produced by GABA were potentiated by barbiturates (Schofield et al 1987, Levitan et al 1988, Pritchett et al 1988, Sigel et al 1990). Such receptors were blocked by Zn^{2+}(Draguhn et al 1990, Smart et al 1991) and the GABA gated chloride currents were not or very weakly potentiated by benzodiazepines (Schofield et al 1987, Pritchett et al 1989a, Sigel et al 1990). Benzodiazepine senistivity was conferred by the co-expression of a γ2 subunit with the α and β subunits (Pritchett et al 1989a, Sigel et al 1990). This also resulted in the channels becoming resistant to Zn^{2+}(Verdoon et al 1990, Smart et al 1991). Functional studies on heterologous expression systems found that the type of benzodiazepine pharmacology type I or II was determined by the type of α subunit (Pritchett et al 1989b, Luddens and Wisden 1991).

The differential distribution and targeting of $GABA_A$ receptors containing particular subunits to specific regions of neurones is more difficult to address using recombinant studies. However polarised cells such as Madin-Darby Canine Kidney cells (MDCK) can be cultured and used as a model system within which the questions of polarity can be addressed. Such cells are usually grown on semipermeable filters which separates an upper from a lower chamber . Studies have demonstrated that once polarised the basolateral and apical domains of such cells are analogous to the somatodendritic and axonal surfaces of neurones (Dotti et al 1991, De Hoop et al 1995). Using this system Connolly et al (1996b), demonstrated that $GABA_A$ receptors composed of α1β1 subunits did not have a polarised distribution. In contrast, receptors generated from α1β2 and α1β3 combinations were targeted to the basolateral domain of the MDCK cells. Transcytosis of β3 containing receptors from apical to basolateral domains was also reported. It was concluded that the β

subunit was important in determining the distribution of the $GABA_A$ receptor in neurones. This work contradicts work performed by Velazquez and Angelides who reported that the $\alpha1\beta1$ combination was directed to the apical domain and that single $\alpha1$ subunits were targeted to the basolateral surface whilst the single $\beta1$ subunit was targeted to the apical domain of MDCK cells. The interpretation of this work may be confused since the immunohistochemical analysis required the use of detergent (Velazquez and Angelides 1993).

The γ subunit is predicted to have a minor role in the localisation of $GABA_A$ receptors to particular neuronal domains. This is suggested by the results of immunocytochemical studies on neurones harvested from mice which have had their $\gamma2$ subunits knocked out. Such studies apparently show no change in the distribution of their α and β subunits (Gunther et al 1995).

Drawing together immunoctychemical studies, *in situ* hybridisation studies, affinity purification studies and enquiries using heterologous expression systems, the consensus of opinion is that the majority of neural $GABA_A$ receptors are composed of $\alpha1$, $\beta2$ and $\gamma2$ subunits in unknown stoichiometries.

1.1.10 How does a $GABA_A$ receptor assemble?

The assembly process by which heteroligomeric ligand gated ion channels are formed has been most extensively studied for the nAChR. Heterologous expression systems have enabled specific, well controlled experiments to be performed. Since the $GABA_A$ receptor belongs to the ligand gated superfamily, the process by which the $GABA_A$ receptor assembles is believed to be homologous to the nAChR but little information currently exists.

The assembly of the nAChR from its individual subunits has been carefully described utilising pulse chase experiments, sucrose gradient

studies and the temperature dependant oligomerisation of the nAChR of the *Torpedo* electric organ within fibroblasts. This work has used a mouse fibroblast stable cell line expressing the *Torpedo* ACh receptor (Claudio et al 1989, Paulson et al 1991, Green and Claudio 1993). An added advantage of this system is that rapid induction of assembly of the subunits can be produced by dropping the temperature to 20°C. This work identified the formation of α, β and γ trimer complexes as the first species of intermediate to be formed minutes after the initiation of subunit synthesis. The next species to be formed were tetramers by the addition of a delta subunit and then finally the mature pentamer was formed by the addition of a further α subunit, see Fig 2(A). (Green and Claudio 1993)

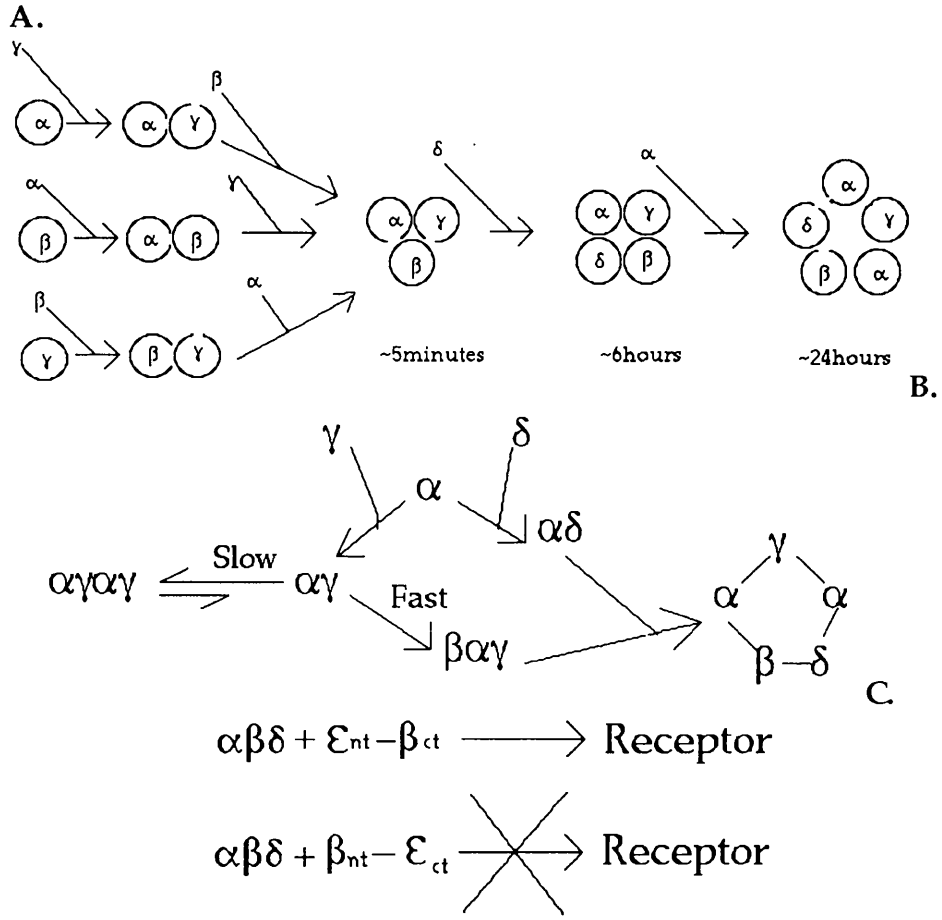


Fig 2 (A) Model of the assembly process of the nACh receptor at the neuromuscular junction (Green and Claudio 1993). Fig 2 (B) Alternative model for nACh receptor assembly (Kreienkamp et al 1995). Fig 2 (C) Chimaera studies of the assembly of the nACh R (Yu and Hall 1991).

It should be noted that this is only one model of the assembly of the nAChR in mouse fibroblasts and is not derived from assembly data from the native electric organ of the *Torpedo* Ray. Assembly of the *Torpedo* nAChR has also been studied in *Xenopus* oocytes (Saedi et al 1991). This studied emphasised αγ and αδ as being distinct early assembly intermediates in contrast to the trimeric species described by Claudio and Green (1993). Analysis of the assembly of the mouse muscle nAChR in a stable fibroblast cell line also described the formation of distinct αγ and αδ complexes. These complexes were retained within the cell's endoplasmic reticulum (Blount et al 1990). The low efficiency of the assembly process has also been demonstrated. Only 20-30% of all α subunits could be chased into the mature functional receptor (Ross et al 1991).

1.1.11 Mechanisms of Assembly.

Domains within the N- terminus of the subunits have been found to be critical for the oligomerisation of the nAChR channel complex (Verrall and Hall 1991). This information was generated by the production of a chimaeric nAChR subunit whose N-terminus was ε and C-terminus was β in nature and vice versa (Yu and Hall 1991). It was found that assembly could only take place if the ε N-terminus was expressed with an α,β and δ subunits whilst no assembly was observed when the α, β, δ and β amino terminus, epsilon C-terminus chimaera were expressed together , see Fig 2 (C).

More recently specific amino acids within the N-terminus of the mouse muscle nACh R γ subunit have been identified which are critical for the assembly of α and γ subunits into tetramers (Kreienkamp et al 1995). It was noted in such experiments that the addition of a δ subunit to cells expressing α and γ subunits did not inhibit tetramer formation nor did the addition of a β subunit to cells expressing α and γ subunits. Only the

expression of both δ and β in cells expressing α and γ subunits suppressed tetramer formation (Kreienkamp et al 1995). An alternative model was suggested, illustrated in Fig 2 (B).

Work performed on the glycine receptor also determined that the N-terminus of the subunits were important in causing subunit oligomerisation and the formation of channels. It was reported that an extracellular domain, between amino acids 22 and 153, of the β subunit determined the stoichiometric assembly of heteroloigomeric i.e. α / β glycine receptors. Utilising a mutagenic strategy assembly boxes in the β N-terminus were delineated which were critical for heteroligomerisation and the inhibition of homoligomerisation (Kuhse et al 1993).

It is assumed that the $GABA_A$ receptor like the nAChR will form a pentameric ion channel and the process of assembly will be dependant on assembly boxes in the N-terminus. Evidence exists which suggests that the N-terminus of the $GABA_A$ subunits may be important for assembly. The α6 subunit has a splice variant where a portion of the N-terminus is deleted. When this splice variant is co-expressed with β subunits no functional channels can be detected (Korpi et al 1994).

The role of chaperone proteins in the assembly process has also been reported for both the $GABA_A$ receptor and the nAChR. The chaperone protein Calnexin has been demonstrated to be associated with individual unassembled α, β and δ subunits of the nACh R but not associated with dimers of α and δ subunits (Keller et al 1996). When individual $GABA_A$ receptor α1, β2 and γ2 L subunits are transfected into HEK cells the chaperone proteins Calnexin and Immunoglobumin heavy chain binding protein (Bip) can be co-precipitated (Connolly et al 1996a). It is imagined that the binding of such chaperone proteins like Calnexin would cause the bound subunit to be retained in the endoplasmic

reticulum of the cell in a similar fashion to the endoplasmic reticulum retention of unassembled T cell receptor subunits which have bound Calanexin (Rajagopalan et al 1994).

1.2.1 Expression of recombinant proteins in neurones.

The expression of recombinant proteins within differentiated neurones is an attractive goal especially if the protein that is being studied has its natural existence within neurones. The difficulty of studying $GABA_A$ receptor cell biology in primary cultures is due to difficulties in transfecting neurones, a paucity of specific antibodies to receptor subunits and the multiplicity of subunits expressed within neurones . Conventional transient transfection techniques such as calcium phosphate precipitation and electroporation are not efficient and or toxic to differentiated neurones. Alternative methods to introduce recombinant proteins into neurones have included the use of microinjection, the bombardment of gold spheres, coated with plasmid directly into the cells, (Lo et al 1994) and the use of Lipofectamine. These methods have only had limited success. In contrast viral transfection methods have been utilised with success to express recombinant proteins in neurones. The most commonly used viral vectors used to express recombinant proteins in non dividing neural tissue include adenovirus, herpes virus and vaccinia virus. Each virus has its advantages as well as its disadvantages.

The vaccinia virus has the capacity to hold 25Kb of recombinant DNA. The large size of its genome, 200Kb precludes the use of plasmids in its propagation and in order to generate a recombinant virus a recombination step is required which necessitates the use of wild type vaccinia virus (Moss 1991). Vaccinia viral vectors have been used effectively to express Betagalactosidase and green fluorescent protein in the central nervous system of frogs and in brain slices from rats (Wu et

al 1995, Pettit et al 1995). The ability to detect long term potentiation and normal synaptic transmission in regions of the hippocampus that had been infected by the vaccinia virus expressing Betagalactosidase was given as evidence of the limited cytotoxicity associated with this vector. Infection of adult animals gave rise to poor expression of recombinant protein, limiting this vector to studies to animals less than three weeks old (Pettit et al 1995).

Vectors based on the Herpes simplex virus can carry 15Kb of recombinant DNA. Recombinant Herpes viral particles are produced using an Amplicon system (Kaplitt and Loewy 1995). There is still a requirement for a Helper virus to package the recombinant viral genome which is a concatomeric arrangement of recombinant DNA. The requirement for Helper virus results in the existence of contaminating Helper virus in the recombinant viral preparation. The broad host specificity of this virus coupled with its capacity to exist in a latent state made it an attractive viral vector. Its capacity to infect post mitotic neurones was demonstrated using a vector that drove the synthesis of Betagalactosidase (Geller and Breakefield 1988). It was also possible to detect the existence of Betagalactosidase two weeks post infection indicating the persistent nature of the expression. The researchers also found low levels of infectious virus two weeks after inoculation and concluded that horizontal transmission of this recombinant virus was very low. The existence of the viral DNA in the neurones was also found two weeks after infection indicating the capacity for this type of viral vector to persist within neurones. The toxicity of the system was not addressed in this study .

The adenovirus system has a much lower size limitation of 6-8Kb. It is based upon a "two component plasmid system". The two plasmids provide essential viral genes which allow packaging expression and

replication (Kaplitt and Loewy 1995). However in order to produce recombinant adenovirus particles one has to infect the cells previously transfected with these recombinant plasmids with adenovirus. The wild type adenovirus used to rescue the recombinant virus has to be purified by caesium chloride centrifugation. The capacity to generate very high titres of this virus 10^7 per µl, a broad host range and an ability to infect post mitotic cells make this an attractive viral vector (Neve 1993). The Food and Drug Administration of the United States have given approval for this vector to be used for gene therapy trials. It has recently been used to deliver normal CFTR to patients who have cystic fibrosis for example, unfortunately with disappointing results (Knoweles et al 1995). Adenoviral vectors have been used successfully to express recombinant proteins such as Betagalactosidase (La Salle et al 1993, Davidson et al 1993, Zhao et al 1996) and Cam Kinase II within neurones from dissociated cultures and in organotypic brain slices (Pettit et al 1994). Toxicity was reported to be associated with expression using high multiplicity's of infection. The kinetics of expression are slower than the other viral vectors Betagalactosidase being first detected one day after infection, however long term expression of Betagalactosidase up to two months post infection (La Salle et al 1993).

One other viral vector The Semliki Forest virus had recently been developed, (Liljestrom and Garoff 1991) and was also commercially available. (Gibco Ltd)

1.2.2 The Semliki virus expression system.

The Semliki forest Virus is a member of the Alphaviridae. It has a single stranded RNA genome of positive polarity which is infectious on entry to the cell cytoplasm. The virus particle is 69nm in diameter. It is an envelope virus and this envelope contains two glycoproteins E1 and E2 each approximately 50 KDa in size. The E1 and E2 proteins form

heterodimers and it is three of such heterodimers that give rise to one of the viral spikes on the surface of the virus. The glycoproteins form an icosohedral lattice with the formation of 80 spikes. The capsid protein interacts with the envelope glycoprotein heterodimers in a one to one ratio. The alpha virus genome is divided into two major regions the non structural, replicase proteins and a structural section which encodes the capsid and envelope proteins (Strauss and Strauss 1994).

Semliki is an arbovirus (arthropod borne) commonly transmitted by mosquitoes and has birds as its primary host. Migratory birds are thought to increase its dispersal. It has a very broad host cell range having the capacity to infect cells from insect salivary gland cells to mammalian neurones. Within their host the virus can replicate in a great variety of cells including neurones, glia, striated and smooth muscle cells, synovial cells and endothelial cells (Strauss and Strauss 1994).

Infection occurs through receptor mediated endocytosis. The virus probably has the potential to bind to multiple receptors. Evidence exists which supports the MHC class 1 molecule as being one receptor which Semliki binds to (Helenius et al 1978). However cells which do not express these proteins for example CNS neurones are capable of being infected by this virus. The laminin receptor has been put forward as an alternative mammalian receptor (Wang et al 1992). The virus then enters the cells endosome. In the endosome of the cell the pH falls and this gives rise to a conformational change in the E2-E1 heterodimer exposing the fusion domain of E1 to the cell's endosomal membrane, which promotes fusion of the viral envelope with the endosomal membrane. The nucleocapsid is released into the cell cytoplasm and then disassembles promoted by an interaction with the large ribosomal subunit. Upon release of the viral RNA genome into the cell viral

replicase proteins are translated which can then duplicate the RNA genome many times (Strauss and Strauss 1994).

Host protein synthesis is inhibited at approximately three hours post infection, after this synthesis of protein is dedicated to viral proteins. Host cells become resistant to super infection between 15 and 60 minutes after infection (Strauss and Strauss 1994).

Semliki is not associated with mortality among human beings (Hanson et al 1967) although one report of a fatality following a laboratory infection exists (Willems et al 1979). It can in its wild type form cause an encephalitis, myalgia, arthralgia and fever (Mathiot et al 1990). A related virus the Western Encephalitis virus is associated with mortality (Strauss and Strauss 1994).

Studies on a related virus, Sinbis virus, which is also a member of the Alphaviridae genus has been shown to induce cell death by apoptosis. This process can be inhibited by the expression of Bcl-2 (Levine et al 1993). Immature neurones when infected by this virus also undergo apoptosis but mature neurones are resistant to viral induced cell death (Griffen et al 1994a and b). The non neurovirulent strains however can undergo genetic changes which can confer a more virulent phenotype. In such cases apoptosis is not blocked by the over expression of Bcl-2 and mature neurones also undergo apoptosis (Ubol et al 1994).

1.2.3 The Semliki virus as an expression vector.

In the early 90's Liljestrom and Garrof pioneered the adaptation of this virus as a heterologous expression system. Plasmid constructs were generated which allowed the substitution of a cDNA in place of the DNA which encodes the structural proteins (Liljestrom and Garoff 1991). To use the Semliki virus as a vector a full length cDNA cloning vector is required which has the structural 26S RNA domain deleted and replaced with a cloning site. This is commercially available from Gibco

Ltd. The first step requires the sub cloning of the cDNA of interest into this vector. The information in the cloning vector is enough to produce large amounts of viral RNA, 10^5 copies per cell, but is insufficient to package the newly formed viral genome. This requires the structural proteins which are produced using a second construct, the Helper vector. RNA is then made in vitro from linearised recombinant and helper vectors using SP6 RNA polymerase. The two RNA species are co electroporated into BHK cells which then synthesise both structural and recombinant proteins.

Fig 3 A. Recombinant GABA subunit RNA species.

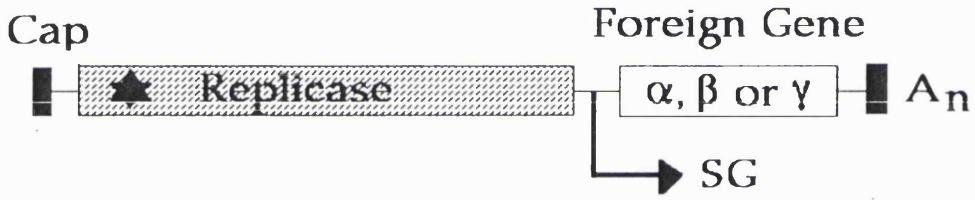
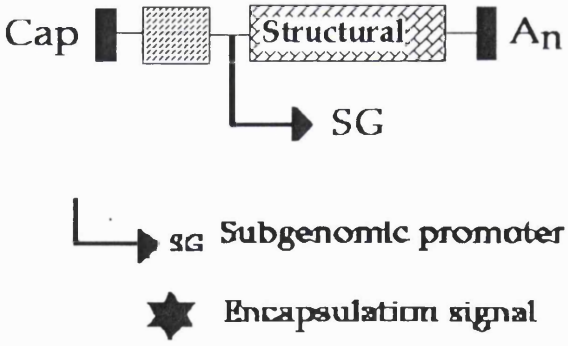


Fig 3 B. Helper RNA species.



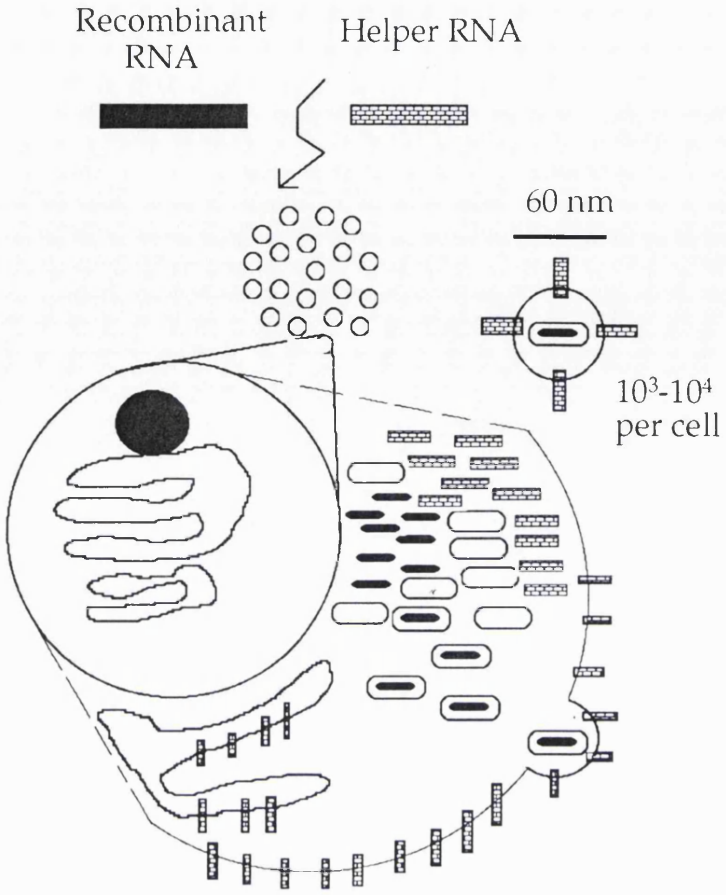


Fig 4. Synthesis of recombinant Semliki Forest virus particles.

Recombinant virus buds off into the culture medium from which it is harvested. Approximately 10^3 - 10^4 virions are produced from each cell. No virus is generated that contains capsid or coat protein RNA . This is due to the lack of recruitment of the structural RNA species by the capsid protein produced on transfection of the helper RNA. This occurs due to a deletion in a region within the capsid gene which is required for recruitment by the capsid protein. RNA splicing could generate a species that had the capacity to produce coat and capsid proteins however these particles are prevented from re infecting by a second safety measure. This involves the Helper 2 construct which has a mutation in one of the coat proteins which requires it to be exposed to chymotrypsin before it can undergo fusion and thus infection (Berglund et al 1993). Since there is no chymotrypsin in culture medium re-infection is inhibited. Thus the

virus that is produced can only infect cells once and should not produce a lytic infection as no budding will occur.

Recombinant virus buds off into the culture medium which is subsequently harvested. This can be titred and frozen into aliquots and stored at -70°C for prolonged periods. Typical titres of virus are about 10^8 infectious particles per ml of culture medium (Liljestrom and Garoff 1991).

1.2.4 Current Applications of the Semliki Expression system.

The Semliki expression system since its development has been applied to the investigation of a diverse range of topics including cell biology, immunology and pharmacology.

This system has been used to express recombinant proteins in polarised cultures of primary neurones in order to study protein trafficking. The immunoglobulin receptor used for transcytosing IgA in epithelial cells was expressed in polarised hippocampal cultures using Semliki forest virus. Trancytosis from the dendritic domain to the axonal domain was demonstrated for this receptor in polarised neurones (Dehoop et al 1995). The capability of the Semliki system to express recombinant proteins in neurones was used to study the neuronal processing of the Amyloid Precursor Protein. This protein is believed to be an important contributory agent in the pathogenesis of Alzheimer's disease. The delivery of this protein first to the axon and then to the dendrites of a neurone was illustrated using the Semliki expression system (Simons et al 1995). Mutant forms of this protein known to be associated with increased prevalence of the disease were also expressed in neuronal cultures. Increased intracellular accumulation of the mutant proteins was observed (Destrooper et al 1995). The neurobiology of the Presenelin proteins has also been studied using the Semliki expression system. The

Presenelins are also thought to be important in the pathogenesis of Alzheimer's disease (Cook et al 1996).

The Semliki expression system has not only been used as a tool to study the cell biology of protein but it has also been applied to the production of immunity. The application of this system to vaccine development was demonstrated by the production of a good immune response on direct injection of naked RNA coding for the nucleoprotein of the influenza virus in to rats (Zhou et al 1994, Atkins et al 1996).

The efficiency and reproducible nature of this expression system has attracted pharmacologists to use it to produce receptors for drug screening and possibly structural analysis. Both G protein coupled receptors and ligand gated ion channels have been expressed using the Semliki expression system.

G protein linked receptors such as the neurokinin 2 and dopamine D 3 receptors have been expressed by the Semliki system. The neurokinin 2 receptors were capable of stimulating calcium fluxes illustrating the ability of a virally expressed receptor to couple to G proteins (Lundstrom et al 1995). A mutation in the D3 receptor Ser 9 to Gly, which is associated with a two fold rise in frequency of schizophrenia was expressed using the Semliki expression system and demonstrated to have a higher binding affinity for dopamine (Lundstrom and Turpin 1996). The prostaglandin E 4 receptor has also been expressed using this expression system and the ^{3}H prostaglandin E 2 has been demonstrated to bind to membranes from cells expressing this receptor (Patel et al 1996).

Ligand gated ion channels have also been produced using this expression system. The 5 HT_3 receptor which is a homoligomeric protein was expressed at high levels , 53 pmol/mg protein . These receptors were capable of generating whole cell currents in response to 5HT. The human purinoreceptors P2x has also been expressed in Chinese Hamster

Ovary cells (CHO) also gave large whole cell responses to ATP , 4-6μA. Using fermenter technology 15mg of His tagged 5HT3 receptor has been produced by this expression system (Lundstrom et al 1997). The capability to produce large amounts of recombinant membrane proteins using the Semliki expression system will promote structural analysis , possibly crystalography of a ligand gated ion channel. Structural information gained from such studies would perhaps allow the development of more specific drugs.

1.2.5 Reasons for using the Semliki expression system

The decision to use the Semliki expression system was made because:

The expression system did not require the use of a wild type virus for packaging purposes. The virus was not associated with serious morbidity. Individuals were using the expression system within the institute. The expression system was plasmid based and did not involve recombination steps. It was known to be capable of infecting neurones (Olkkonen et al 1993). The reagents to make recombinant virus were readily available and Home Office approval to use this viral expression system already existed.

1.3.1 Aims of the thesis.

The objectives of this thesis include:

1. The demonstration that the Semliki expression system can be used to produce heteroligomeric $GABA_A$ receptors in cells.

The use of this viral expression system had only been applied to the expression of homomeric proteins and it was unknown whether this viral expression system would support heteroligomerisation.

2. The determination of the efficiency of $GABA_A$ receptor assembly and the stoichiometry of a $GABA_A$ receptor composed of α1 (9E10) and β2 (9E10) subunits.

The reproducible transfection of cells at high frequency and the rapid production of high levels of recombinant proteins make the Semliki expression system a powerful biochemical tool. This enabled the questions of assembly efficiency and stoichiometry to be addressed.

3. The identification of whether assembly rules defined in transformed cell lines are applicable to the assembly of $GABA_A$ receptors in neurones.

The assembly data for the oligomerisation of the nAChR and for the $GABA_A$ receptor has been defined using heterologous expression within transformed cell lines and *Xenopus* oocytes. It is not known whether the same restrictions on the assembly of subunits defined in transformed cell lines or oocytes apply within neurones where specific cellular machinery critical for the assembly of ligand gated ion channels and their trafficking may exist. The Semliki virus's capability to infect a broad range of cells , was used to express epitope tagged $GABA_A$ subunits in primary cultures of neurones. This made it possible to study the cell biology of the $GABA_A$ receptor in cells that normally express these proteins. It also enabled unambiguous immunohistochemical studies to be performed using monoclonal antibodies to determine the distribution of the virally expressed epitope tagged proteins.

4. To analyse the significance of the splicing of the $\gamma 2$ subunit. Do the $\gamma 2$ S and $\gamma 2$ L forms have differing functional properties in terms of cell surface targeting and phosphorylation by protein tyrosine kinases?

Chapter 2

Materials and Methods.

Unless otherwise stated all chemicals and reagents were purchased from Sigma Chemical Ltd. Restriction enzymes and molecular biological reagents were purchased from New England Biolabs unless otherwise stated.

Oligonucleotides were purchased from the Oswel DNA service, Department of Chemistry, University of Edinburgh.

Radionucleotides were purchased from Amersham Ltd.

Specialised bacterial media components were obtained from Difco Laboratories Ltd.

The majority of molecular techniques are taken from Sambrook et al (1989).

2.1 Molecular Biology.

2.1.1 Bacterial strains

Site directed mutagenasis required the use of the E.coli strain CJ236 (F' cat (=pCJ105; $M13^{s}Cm^{r}$) /dut ung 1 thi-1 relA1 spoT1 mcrA (Raleigh et al 1989).

Subcloning into the SFV palsmid were performed using the E.coli strain XL 1- Blue (F':Tn 10 $proA^{+}B^{+}$laclq Δ(lacZ)M15/recA1 endA1 gyrA96(Nalr) thi hsdR17($r_K^{-}m_K^{+}$) supE44 relA1 lac (Bullock et al BioTechniques 1987).

Standard subcloning and large scale preporation of PRK 5 plasmids was performed using the E.coli stain MC 1061 (F-araD139 Δ(ara-leu)7696 galE15 galK16Δ(lac)X74 rpsL(Str^{r}) hsdR2($r_K^{-}m_K^{-}$)mcrAmcrB1 (Raleigh et al 1989).

2.1.2 Growth Media and Agar Plates.

Bacteria were grown in Luria Broth (LB) containing 10g bacto-tryptone, 5g yeast extract and 10g NaCl per litre or on LB-agar plates (LB + 30g bactoagar/litre). Solutions were autoclaved at 15lb/sq.in. for twenty minutes and then stored at room temperature. When required the plates and broth were supplemented with Ampicillin, final concentration 100µg/ml and or with, Chloramphenicol 30µg/ml, final concentration , or with Kanamycin, final concentration 70µg/ml. All antibiotics were added to sterilised solutions once they had cooled to below 55°C. Agar plates were stored at 4°C and air dried before use. The E.coli were grown at 37°C and liquid cultures were grown with continuous agitation in a rotating shaker.

2.1.3 Competent cell preparation.

Electrocompetent cells were produced by inoculating 1 litre of L broth from a 10ml overnight culture of the relevant strain of E.coli. The bacteria were then grown until an O.D. $_{600}$ of 0.6 was reached. The cells were centrifuged for 15 minutes at 3000rpm and resuspended in 1 litre of ice cold sterile water. The centrifugation step was repeated. The bacteria were resuspended in 500ml of ice cold sterile water. The centrifugation step was repeated and the bacteria resuspended in 20mls of ice cold sterile water. Finally the bacteria were centrifuged for 15 minutes at 3000 rpm and resuspended in 2.5mls of sterile ice cold 10% glycerol. The bacteria were then snap frozen in liquid nitrogen and stored in liquid nitrogen until required.

2.1.4 Transformation of bacteria with plasmid DNA by electroporation.

Electrocompetent cells were thawed on ice. 1µl of plasmid DNA was then added to 40µl of electrocompetent cells. The mixture was then placed in a Biorad 0.2 cm electroporation cuvette. The cuvette was kept on ice prior to the electroporation. The cuvette was wiped clean of moisture and

loaded into the electroporator. One electroporation was given with the settings of 2.5KV, 200Ω and 25μF. The bacteria were immediately resuspended in 1ml of L broth which had been prewarmed to 37°C. The cells were incubated for at least 15 minutes before they were plated out onto a selective plate and incubated at 37°C overnight with the plates facing downwards.

2.1.5 Ethanol precipitation of DNA.

0.2 volumes of 10M Sodium acetate were added to the solution of DNA followed by 2 volumes of 100% ethanol. The solution was mixed by inverting the eppendorf tube at least twice. The tube containing the sample was placed at -20°C for 10 minutes to promote the precipitation of the DNA. If small amounts of DNA were being precipitated (less than 1μg) a microlitre of glycogen (1mg/ml) was added prior to the addition of salt or ethanol to act as a carrier. The precipitated DNA was pelleted by centrifugation at 13000rpm for 5minutes. The pellet was then washed in 70% ethanol and dried using a Speed Vac.

2.1.6 Phenol/Chloroform extraction.

DNA was extracted using an equal volume of a 50:50 mixture of phenol, (Appligene Ltd) and chloroform /isoamylalcohol 24:1 ratio. The phenol was equilabrated with 0.5M Tris pH 8 before it was used for extracting DNA. The DNA and phenol /chloroform solutions were mixed by shaking and then the aqueous phase isolated by centifugation at 13000 rpm for 5 minutes. The upper aqueous phase was removed and re-extracted until the interface of the two phases was clear.

2.1.7 Restriction digests.

DNA was digested for 1 hour at the optimal temperature for the restriction enzyme using appropriate buffers for the particular enzymes used. As a general rule restriction enzymes were diluted at least ten fold.

2.1.8 Electrophoresis of DNA.

Agarose gels were made by melting 0.4-1.5% w/of agarose in 1x TAE . Once melted the solution was supplemented with 10µl of ethidium bromide solution(10mgs/ml) were run as described in Sambrook et al (1989) in 1x Tris Acetate buffer (TAE) 50x stock containing 242g Tris base, 57ml glacial acetic acid and 100ml of 0.5M EDTA pH 8, per litre. DNA samples had loading buffer (10x stock contains 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol and 25% w/v ficoll-400) added prior to being run in horizontal submarine agarose gels. The DNA was then visualised by placing the gel on a UV transilluminator.

2.1.9 Subcloning; preparation of Insert to be subcloned.

After performing a restriction digest on the parental vector the fragment to be subcloned was separated from the parental vector by running the product of the restriction digest (after phenol extraction and ethanol precipitation) on a low melting point agarose gel. The agarose containing the insert was then carefully cut out and added to a tube containing approximately five volumes of 20mM Tris pH 8 1mM, EDTA pH 8. The solution was then raised to a temperature of 65 degrees centigrade for 5 minutes to melt the gel. The solution was then cooled slowly to room temperature and then an equal volume of phenol, equilibrated with 0.1M Tris pH 8, added and the solution shaken. The cloudy solution was then centrifuged at 13000rpm for five minutes and the aqueous upper phase retained and re- extracted with a phenol / chloroform solution until the interface was clear. The aqueous phase was then ethanol precipitated using a µl of glycogen as a carrier, 0.2 volumes of 10M ammonium acetate and 2 volumes of 100% ethanol. The precipitate was collected by spinning at 13000 rpm for 5 minutes. The DNA was then resuspended in 20µl of TE and the quality of the preparation ascertained by running a µl on an agarose gel.

2.1.10 Preparation of Vector into which the insert is to be cloned.

25µg of the pSFV1 vector was cut with BamHI . The digest was first checked on an agarose gel to ascertain if full digestion had occurred. If acceptable the product of the digest was then extracted with phenol chloroform , ethanol precipitated, and resuspended in 90µl of water. The digested vector was then phosphatased with calf intestinal phosphatase using the relevant buffer and 0.2 units of phosphatase. The incubation was at 37° C for 30 minutes. The phosphatase was inactivated by heating at 75°C for 10 minutes before being extracted with phenol chloroform and ethanol precipitated. The preparation was then gel purified away from uncut vector on a low melting point agarose gel and the parental, now phosphatased vector, isolated as above.

2.1.11 Ligations.

Ligations were performed using 1 unit of T4 DNA ligase per reaction. In order to optimise the ligation a range of vector to insert ratios were used. Vector only and insert only controls were also performed simultaneously. The relevant buffer plus 1mM final concentration ATP was added and the mixture which had a final volume of 10µl incubated at 17°C overnight. Note controls with vector alone and insert alone were also included in the ligations. The products of the ligations were extracted with phenol chloroforn and precipitatated with sodium acetate, 1µl of glycogen as a carrier and ethanol. The precipitate was washed in 70% ethanol and dried in a speed vac before being resuspended in 10µl of water. The ligations were then electroporated into XL1 Blue electrocompetent bacteria and plated out on Ampicillin LB agar plates. The ligations were screened by enzyme digestion if an enhancement above background was apparent.

The orientation of the clones was then determined by a double restriction digestion with Sma I and EcoRV. A clone with the correct

orientation was grown up and a caesium banded preparation of DNA produced.

2.1.12 Mini preparation of DNA.

Individual colonies from transformed bacteria were grown overnight in 5 ml cultures. 1.5mls of the culture were then pelleted by spinning the sample for 5 minutes at 6000rpm. The pellet was then resuspended in 250µl of solution 1 (25mM Tris pH8, 10mM EDTA , 50mM Glucose). 400µl of fresh solution II (0.2M NaOH/1%SDS) was then added to the tube and carefully mixed by inversion. This mixture was kept on ice for five minutes before 300µl of solution III (5M Potassium acetate pH 4.8 , produced by adding 29ml of glacial acetic acid , 50 ml water and pH'd to 4.8 with 10M KOH and made up to 100ml with water). The solution was then incubated on ice for a further 5 minutes . The solution was then spun at 13000rpm for 10 minutes in the cold room and the supernatant retained. This was extracted twice with phenol/chloroform. The aqueous phase was added to an equal volume of isopropyl alcohol and incubated on ice for 10 minutes. The pellet was washed with 70% ethanol and resuspended in 40µl of TE (50mMTris CL pH7.6, 10mM EDTA).

2.1.13 Maxi preparation of plasmid DNA by Caesium Chloride Banding.

A 250ml culture of transformed bacteria which had been grown overnight was harvested by centrifugation using 3000rpm for 15 minutes. The bacteria were then resuspended in 10mls of solution I (see above). 20mls of Solution II (see above) was then added and the solutions gently mixed by inversion. The mixture was then left on ice for 5 minutes. 15 mls of solution III (see above) was then added and the solution mixed well and then left on ice for a further 5 minutes. After this period the mixture was spun at 3000rpm for 20 minutes and the supernatant retained. One volume of isopropyl alcohol was added to the supernatant and the mixture inverted several times. This was left on ice

for 5 minutes. The mixtures was then spun at 3000rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 10ml of 10x TE (500mMTris CL pH7.6, 100mM EDTA) and precipitated with one volume of 5M Ammonium Acetate and 2 volumes of 95% ethanol. This was then spun at 3000rpm for 10 minutes and the pellet resuspended in 8mls of 10xTE. 1g of Caesium Chloride per ml of supernatant was added followed by 100µl of ethidium bromide (10mgs/ml). The solution was mixed well and then used to fill quick seal tubes (Beckman Ltd). The tubes were heat sealed and then loaded into a TLN 100 rotor (Beckman Ltd) and then spun at 100000rpm for at least 4 hours. The tubes were then removed from the rotor and the super coiled plasmid (lower band) identified and removed by aspiration. The ethidium was then removed by extraction using water saturated butanol. The DNA was then ethanol precipitated and recovered by centrifugation. The pellet was resuspended in TE and ethanol precipitated once again. The DNA was then extracted once with Phenol Chloroform and finally ethanol precipitated and washed in 70% ethanol. The DNA was then resuspended in 1ml of TE and the yield estimated by absorbance at 260nm

$$\text{Nucleic acid concentration(mg/ml)} = \frac{A_{260} \times \text{Epsilon} \times \text{Dilution factor}}{10000}$$

Epsilon is the extinction coefficient for the absorbance of DNA at 260nm. This is 50 for double stranded DNA at 20°C.

2.1.14 Gel Purification of Oligonucleotides.

The oligonucleotides were dried down in a vacuum drier and then resuspended in 200µl of distilled water. Forty percent of the oligonucleotide was then added to an equal volume of 100% formamide and then heated for five minutes at 55°C. The oligonucleotide was then applied to a 14% polyacrylamide gel containing 7M urea in a TBE buffer. Once the gel had run the oligonucleotide was identified by UV

shadowing over a thin layer chromatography plate. The top band was cut out of the gel and sliced up several times before it was added to a 15 ml conical tube. 5ml of 0. 5M ammonium acetate was added and the tube incubated overnight at 37°C in a shaking incubator. The oligonucleotides were then purified by using a Sep pak column (Waters/Millipore). In order to isolate the oligonucleotide the column had to be prepared by passing 10ml of HPLC grade acetonitrile through it using a 10 ml syringe. This was followed by 10ml of HPLC water and then 2ml of elution buffer (0.5M ammonium acetate). The sample was then added followed by 10 ml of HPLC water. The oligonucleotide was then eluted in three times 1 ml fractions of 60% methanol 40% water. The fractions were individually collected and dried in a vacuum drier. The oligonucleotides were then resuspended in 200µl of water and the OD estimated at 260nm.

2.1.15 Site Directed Mutagenesis.

CJ 236 were transformed with the relevant plasmid and plated on chloramphenicol and ampicillin plates. Uracil bases were substituted for thymidine as a result of using this strain and conditions. Colonies were picked and grown in the presence of ampicillin for one hour prior to the addition of 5µl of 35mg/ml kanamycin and 5µl of M13KO7(10^{10} PFU) phage. The cultures were grown overnight and single stranded DNA isolated by PEG precipitation the following day. The oligonucleotides used to introduce the mutation into the cDNA were commercially manufactured (Oswell). The oligonucleotides were then gel purified as above.

The oligonucleotides (200pMoles) were then kinased at there 5' end using polynucleotide kinase (NEB) with relevant buffer and additional ATP (1mM). The reaction was conducted at 37 degrees centigrade for one hour and then the kinase was inactivated at 70°C for 10 minutes. The

reaction products were extracted once with phenol/chloroform and then ethanol precipitated with the addition of a microloitre of glycogen to act as a carrier. The kinased oligonucleotide was then resuspended in a convenient volume of water usually 20µl. 10pMoles of mutagenic oligonucleotide was then annealed to 300 pMoles of template DNA by heating to 65°C in a hot block for 5 minutes and then allowing to cool to room temperature slowly. (10x annealing buffer 200mM Tris pH 7.4, 20mM Magnesium Chloride, 500mM Sodium Chloride). The double strand was then synthesised by adding 1µl of synthesis buffer, 1µl of T4 DNA ligase and 1µl of a 1:5 dilution of T4 DNA polymerase (Biorad) in ice cold polymerase dilution buffer. The synthesis buffer contained : 0.4 mM of each dNTP, 0.75mM ATP, 17.5mM Tris pH 7.4, 3.75 mM $MgCl_2$ and 1.5mM DTT. The dilution buffer contains 100mM potassium phosphate pH 7, 5mM DTT, and 50% glycerol. The synthesis was commenced by incubating on ice for 5 minutes then at room temperature for five minutes then at 37°C for 90 minutes. The reaction products were extracted with phenol/chloroform and then precipitated using ethanol and 1µl of glycogen as a carrier. The mutagenasis products were resuspended in 10µl of water and subsequently elctroporated into XLI blue bacteria which do not tolerate uracil containing DNA. The parental strand is replaced by sequence complementary to the new mutagenic strand. The bacteria were plated onto plates containing 100µg/ml ampicillin and left ovenight before colonies were picked . Mini preps of ten colonies were screened either by sequencing if a point mutation was being generated, or by enzyme digestion if a new restriction site was being introduced. Once mutants had been identified by the screening methods caesium chloride stocks of plasmid DNA from the mutant clones were produced and stored at -20 °C.

2.1.16 In vitro transcription.

25µg of a pSFV 1 construct was cut with enzyme Nru 1 (This linearised the DNA.) A fiftieth of the linearised DNA was checked on an agarose gel to ascertain whether the plasmid had completely been linearised. The linearised DNA was then extracted once with Phenol/chloroform and then precipitated with sodium acetate and ethanol. The DNA was then washed with 70% ethanol and then resuspended in 5µl of TE. The in vitro transcription was then set up :

5µl of SP6 10x buffer (Promega), 5µl of Rnase free BSA (1mg/ml) (Pharmacia) , 5µl of 10mM m7G(5')ppp(5')G, 5ul of 50mM DTT (Promega), 5µl of rNTP mix (10mM ATP, 10mM CTP, 10mM UTP, 5mM GTP) (Pharmacia), 18.5µl DEPC treated H_2O, 1.5µl RNasin (50 units)(Promega), 30 units of SP6 RNA polymerase (Promega), and add the 5µl of linearised plasmid DNA at the end. The reaction was incubated for 60 minutes at 37 degrees centigrade. The production of RNA was then determined by running 2µl of the reaction on an agarose gel. Formamide was added to the sample to denature the RNA . The reaction products were then frozen at -80^{o}C. The same procedure was performed for the helper construct except that it was linearised with Spe I rather than Nru I.

2.1.17 Sequencing.

The sequencing method utilised the United States Biochemical sequencing kit using the Sequenase enzyme. The DNA to be sequenced was first denatured with alkali. This was performed by adding 8µl of 1M NaOH (freshly made) to 32 µl of DNA (5-10 µg). The solution was left for 5 minutes before the DNA was precipitated using 16µl of 5M Ammonium Acetate and 200µl of ethanol. The DNA was then isolated by centrifugation. The pellet was then washed with 70% ethanol and then vacuum dried. The DNA was then resuspended in 7µl of distilled

water and then 1µl of primer at a concentration of 21ng/µl added. Additionally 2µl of 5x sequenase buffer was incorporated. The mixture was heated to 70°C and allowed to cool to room temperature slowly. The termination reactions were then set up in a multiwell Nunc plate. In the adenosine termination well 2.5µl of a mixture containing: 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 50mM NaCl plus 8µM **dd**ATP was added, whilst in the guanosine termination well the ddATP was substituted with ddGTP.

In order to incorporate radioactive label into the growing DNA molecule a labelling reaction was set up:

A five fold dilution of 1.5µM dGTP,dCTP, dTTP , radioactive 35S labelled dATP , annealed DNA, diluted sequenase, 10mM DTT. The mixture was incubated at room temperature for 5 minutes. The termination wells were prewarmed at 37°C prior to the addition of the labelling reaction mixture. The labelling mixture was equally divided between the four termination lanes and the termination reactions mixed and incubated for a further five minutes at 37°C. The reactions were stopped by the addition of formamide and heated to 75°C for 2 minutes prior to loading onto a sequencing gel.

2.1.18 List of Adaptors used for subcloning.

EcoRI/BamHI adaptors

5'AATCGCGGCCGCG3'

3'GCGCCGGCGCCTAG5'

EcoRI/Bgl II adaptors

5'AATTCGCGGCCGCA3'

3'GCGCCGGCGTCTAG5'

2.1.19 List of Oligonucleotides used for Mutagenasis.

Insertion of Bam HI site at 5' of γ 2 L and S cDNA in PrK 5:

TCTGCAACCCAGAGGGATCCCGAGAGGCGAGAGGA

Insertion of Bam HI site at 3' of γ 2 L and S cDNA in Prk5:

TTTTATTGATAGGGGGATCCTGTTATTCGCTGAAT

Mutation of tyrosine's in the intracellular loop between transmembrane domains 3 and 4 of the γ 2 S (9E10) and long subunits :

5' CAACACTCAAAGCCAAATTCTTCATCCCT 3'

Mutants were identified by sequencing, using the sequencing primer:

5' GGCAATCGAATATGTAT 3'.

Mutation of serine 65 of the GFP protein to threonine was performed using the oligonucleotide:

5' CACTACTTTCACTTATGGTGT 3'.

The sequencing primer used to screen the clones was:

5' AGTGCCATGCCCGAAGG 3'

Mutation of a 5' BamH I site plus Kozak consensus sequence into the serine 65 mutant GFP mutant was performed using the oligonucleotide:

5' TTCTCCTTTACTCATGGTGGATCCCTTTTATTCGTG 3'

Mutation of a second subgeonomic promoter into the 3' end of the previously mutagenised γ2 S (9E10) cDNA, (This had been mutated to introduce a second BamH I site at 3' end to facilitate subcloning) was conducted using the oligonucleotide:

5'ATCCCCCTATCACCAATCTAGGACCGCCGTAGAGGTATAAAACCC ATA. 3'

The mutants were screened by sequencing using the sequencing primer:

5'TTCTTCCCTACCGCCTT3'

2.1.20 List of Vectors.

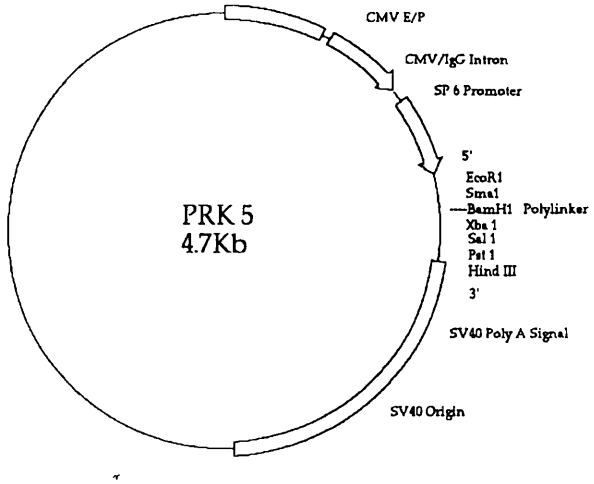
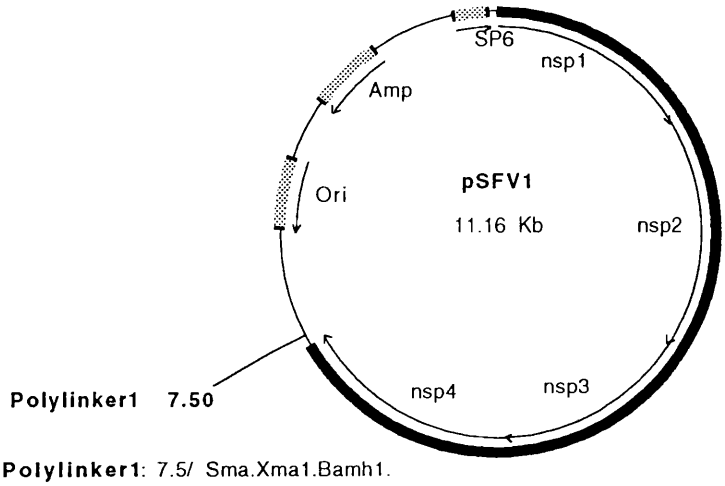


Fig 5 Vectors that were routinely used.



Polylinker1: 7.5/ Sma.Xma1.Bamh1.

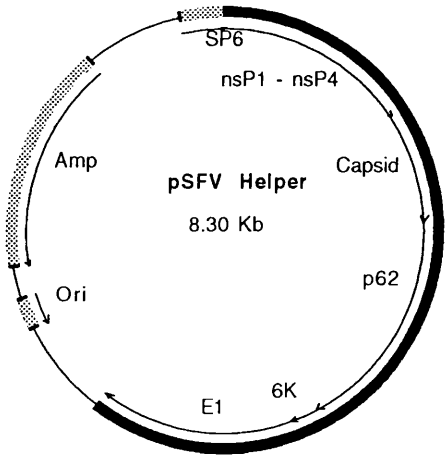


Fig 5 Vectors that were routinely used.

PRK 5 v-src, PRK 5 α1, β1 and γ2 L (9E10) double tyrosine mutant were kindly provided by Dr Stephen Moss.

PRK 5 α1 (9E10), β2 (9E10), γ2 S (9E10) , γ2 L (9E10) and γ2 L FLAG were kindly provided by Dr Stephen Moss, Dr Chris Connolly and Mr Bernard MacDonald.

PRK 5 GFP Gift fro Dr Beartrice Durand MRC Laboratory of Molecular Cell Biology.

2.1.21 Engineering of viral constructs.

1. Creation of α1 (9E10), β 2 (9E10) and γ 2 long flag tagged viral constructs.

Each of the cDNA's were in the EcoRI site of PRK5. The α1 (9E10) cDNA was subcloned into pSFV1 using the EcoRI Bgl II adaptors. The β2 (9E10) and γ2 L FLAG tagged cDNA's were subcloned into pSFV1 using the EcoRI BamH I adaptors.

2. Creation of γ2 S (9E10) viral construct.

Subcloning of γ2 S (9E10) into pSFV1 was performed by introducing BamH1 sites at the 5' and 3' ends of the cDNA by site directed mutagenasis.

3. Creation of the GFP viral construct.

GFP c DNA was mutagenised to change serine 65 to threonine.

This was then re-mutagenised to introduce a Kozak consensus site and a BamH I site at the 5' end of the GFP cDNA. This was then subcloned into pSFV1 as a BamH I fragment.

4. Creation of γ2 S and L (9E10) double tyrosine viral constructs.

BamH I sites were introduced at the 5' and 3' ends of the γ2 S and L (9E10) cDNA's via site directed mutagenasis. The mutants were remutagenised to replace the tyrosines in the intracellular loop with phenylalanines. These mutants were then subcloned as BamH1 fragments into pSFV1.

5. Creation of γ2 S (9E10) GFP dicistronic construct.

The GFP in PRK5 which had been mutated to introduce a Kozak consensus sequence, a Bam HI site at the 5' end and a change of serine 65 to a threonine was subcloned into pSFV 1 as a BamH I fragment. This was digested with Sma1 which removed the 3' BamH I site and religated. The γ2 S (9E10) double BamH I mutant was then subcloned into the pSFV1 GFP vector at the 5' BamH I site.

6. Creation of γ2 S (9E10) GFP double subgeonomic promoter construct.

The γ2 S (9E10) which had the two BamH I sites introduced by site directed mutagenasis was remutagenised to introduce a viral subgeonomic promoter at the 3 ' end of the cDNA after the stop codon and before the 3' BamH I site. This was then subcloned as a BamH I fragment into the pSFV GFP vector at the 5' BamH I site.

2.2 Cell Biology.

2.2.1 Cell Culture.

BHK 21 cells were grown at 37°C with 5%CO_2 in complete BHK 21 medium. (BHK medium (Gibco G-MEM), 5% Fetal Calf Serum (Gibco), 10% tryptose phosphate broth (Gibco), 20mM HEPES (Sigma), 2mM

Glutamine (Gibco), 0.1u/ml penicillin (Sigma), 0.1µg/ml streptomycin (Sigma). The cells were passaged every three days.

HEK 293 cells were grown at 37°C with 5% CO_2 in DMEM containing 10%FCS volume by volume, 0.1u/ml penicillin (Sigma), and 0.1µg/ml streptomycin (Sigma). The cells were passaged every three days.

2.2.2 Transient transfection of HEK 293 cells.

1x 10^6 HEK 293 cells were resuspended in 0.5ml of OPTIMEM medium (Gibco) and 10µg of plasmid DNA added to the cells . The cells were then added to a 0.5cm Biorad electroporation cuvette . The cuvette was electroporated using the settings of 400Volts , 250µF and infinity Ohms. The electroporated cells were then removed from the cuvette and placed in their normal medium and incubated for at least 24 hours at 37°C before experiments were performed.

2.2.3 Production of Virus.

Baby hamster kidney cells were grown to late log phase in a 10cm diameter Falcon dish. The cells were trypsinised and then washed twice with DEPC treated PBS. The cells were resuspended to a concentration of some 10 million per ml. The RNA made from the helper construct and the RNA made from the recombinant construct were mixed together with the cells and then added to a 0.2 cm width Biorad electroporation cuvette. The cells were then pulsed twice with a discharge from the electroporator with settings: voltage of 1.5KV, 25uF and infinity ohms. The cells were then plated out onto a 10 cm diameter culture dish and 10 mls of normal BHK medium added. The cells were incubated at 37°C for 36 hours at 5% CO2. Virus was shed into the culture medium which was subsequently harvested. Debris was removed from the viral stock by a 4 minute spin at 1500rpm. The virus was then aliquoted and stored at -80°C.

2.2.4 Determination of the viral titre.

The virus was titred by determining the dilution of virus required to produce a 66% infection frequency. This was performed by placing glass coverslips in twenty four well falcon culture dishes and loading approximately 50000 cells per well. The cells were allowed to attach to the coverslip and then virus was added to each well of one row of six wells. The virus added to each well was at a ten fold lower dilution than the adjacent well. This was achieved simply by performing a serial dilution of a 50 µl volume of virus into 450µl of binding medium. The virus was incubated for 60 minutes before being removed. The cells in the control row of six wells were trypsinised and then counted so as to determine the number of cells in each well. The following day Immunofluorescence was performed upon the cells exposed to the varying dilutions of virus and the frequency of infection was estimated at the different dilutions. The data was graphed with percentage infection against dilution and the dilution of 50µl of virus that coresponded to 66% infection estimated. At this dilution the number of cells on the coverslip corresponds to the number of virus particles.

2.2.5 Preparation of Infection medium.

5mls of 10x RPMI was placed in a 100ml beaker and 44 mls of sterile water added and then 0.5mls of 2M HEPES. This solution was then pH 'd to 6.8 with a calibrated pH meter. Then 0.5ml of 20% BSA was added and the solution was filter sterilised with 0.2µm Millipore filter.

2.2.6 Infection of BHK cells.

BHK cells were infected by incubating the cells in infection medium which contained virus for one hour at 37°C. After this period the infection medium was replaced with complete BHK medium.

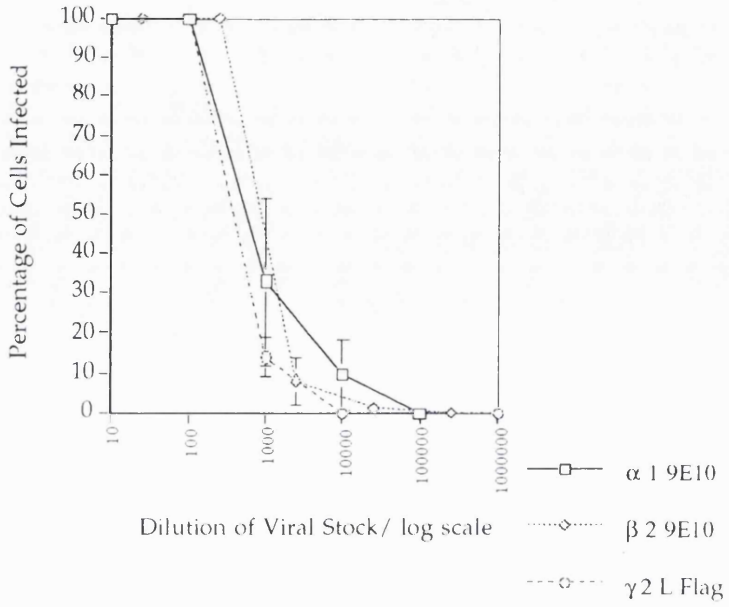


Fig 6. Viral titres. Example of graphs used to determine viral titres. The three virus preparations exhibited above had titres of approximately 3 x 10^8 viral particles per ml.

2.2.7 Infection of SCG neurones.

SCG primary neurone cultures were performed by Mrs Yvone Vallis. The neurones were incubated in viral binding medium (RPMI pH 6.8, 20mM HEPES, 20mM BSA) containing a one in ten dilution of a viral stock of a Betagalactosidase Semliki forest virus (produced by Dr Ronke Brone), for 60 minutes at 37°C. The cells were then washed with SCG culture medium and incubated for varying periods of time after which the Beta galactosidase content of the cells was assayed by staining and microscopy. Both neuronal and non neuronal cells were counted separately in the final experiment of this type.

2.2.8 Staining for Betagalactosidase.

The virally infected cell were fixed for 5 minutes at room temperature with a solution containing 0.27ml of 37%formaldehyde and 80µl of 25% gluteraldehyde made upto a total volume of 5mls with PBS. The cells were then washed with PBS and the stain (2.5mg of X-gal in 25µl of

DMSO, 10µl of 1M MgCl2 , 8mg of Potassium ferricyanide, 10.5mg of Potassium ferrocyanide made up to 5mls with PBS.) added and incubated at 37°C for one hour.

2.2.9 Propidium Iodide staining of neurones.

SCG neurones were infected with the Betagalactosidase virus and cultured for 24 hours before being fixed as described for Betagalactosidase staining. The cells were then exposed to acetone / ethanol (1:1) at -20 °C for thirty seconds. The cells were then washed with PBS . Then the cells were incubated in phosphate buffered saline containing RNAse at 20µg /ml and 20µg /ml propidium iodide for fifteen minutes at 37°C. The stained cells were then washed with PBS to remove excess stain. Betagalactosidase activity was then assayed as previously described. The cells were then mounted on slides and viewed using a confocal microscope with both transmission and fluorescence capabilities.

2.2.10 Immunofluorescence.

BHK cells or neurones were fixed with 3% paraformaldehyde (Sigma) made up in PBS, by incubating them in this solution for 10-20 minutes. After this period excess paraformaldehyde was washed off with PBS before being quenched by incubating the cells for two 10 minute periods in 50mM Glycine , made up in PBS. Once quenched the cells were blocked in a solution of 10% horse serum (Gibco) and 0.5g per 100mls bovine serum albumen made up in BS. If permeabilised specimens were required the block solution was supplemented with 0.1% NP40 (Calbiochem) or 0.1% saponin (Sigma). The blocking process was performed by incubating the cells for two 10 minute periods in the block solution. The primary antibody was then added to the block solution at the relevant dilution , 1:200 for FLAG (approx. 5µg per ml) and 1:2 for the 9E10 hybridoma supernatant. The primary antibody was applied to the cells for 1 hour. The cells were then carefully washed with block solution

to remove unbound antibody. This was performed at least three times before the secondary antibody was applied. The secondary antibodies were produced by Jackson immunochemicals. Anti mouse fluorescene or anti mouse rhodamine were commonly used as was anti rabbit fluorescene or rhodamine. The secondary antibodies were used at a dilution of 1:200 in block solution. The secondary antibodies were applied to the cells for 45 minutes. After this had elapsed the cells were washed at least three times with block and finally once with PBS before being mounted onto glass slides. The cells were then viewed with an MRC 1000 confocal microscope or Zies Axiophot fluorescence microscope.

2.2.11 Multiple infection followed by immunofluorescence.

BHK cells were plated out on coverslips in 24 welled falcon plates. Varying dilutions of α and β virus particles were incubated with the BHK cells for one hour. Normal BHK medium was then reapplied and the cells incubated overnight. The cells were then fixed with 3% paraformaldehyde and immunofluorescence performed. The cells were not exposed to detergent during this procedure in order to observe only cell surface fluorescence.

2.2.12 Infection of SCG neurones by the GABA subunit virus particles.

SCG neurones harvested from 14 day old rat pups were infected with virus as previously described. The cells were fixed the following day after infection and immunofluorescence performed.

2.2.13 Infection of cortical cultures made from a γ 2 knock out mouse.

These neuronal cultures were kindly provided by Dr Bernard Luscher's group. The knock out cultures were identified by a PCR based screen of multiple cultures taken from E 15 animals. The cortical cultures were left in culture for at least one week before being infected as previously described. The neurones were infected by the γ2 S (9E10) virus for one

hour and then after an overnight incubation were either fixed with 3%PBS or subjected to electrophysiological whole cell recording. Immunoflourescence was performed on the fixed cells to identify the location of the γ2 S (9E10) protein in the neurones. Whole cell recordings were performed by Dr Jack Benson.

2.2.14 Antibody feeding experiments.

BHK cells were infected with Semliki forest virus particles which had been designed to drive the expression of the γ2 S (9E10) protein and the double tyrosine mutant of this protein as previously described.

The cells were infected for one hour, as previously described with either fifty microlitres of the mutant γ2 S (9E10) or the wild type version, in 550 µl of binding medium. The cells were then left for sixteen hours (over night), after this period the cells were incubated in 9E10 supernatant for a period of one and half hours. Once this incubation period had elapsed the cells were carefully washed with PBS and then fixed with paraformaldehyde. The cells were permeabilised using 0.1%NP40 and then subjected to immunofluorescence

The 9E10 antibody that had bound to the pool of γ2 S (9E10) present on the surface of the BHK cells was detected by exposing the fixed and permeabilised cells to a fluorescene conjugated anti mouse secondary antibody.

2.2.15 Quantification of Fluorescence.

The level of surface membrane 9E10 staining on cells was quantified using confocal microscopy. BHK cells were fixed at differing time points post infection. Surface receptor populations were then detected simultaneously via immunofluorescence using the monoclonal antibody 9E10 without permeabilising the cells. Individual cells at each time point were selected at random and the cell surface fluorescence signal analysed using the area command using identical gain and iris

diameter settings. The settings of iris diameter and gain were chosen so that the assay was performed in the optimal dynamic range for the specimens. The average of intensities from at least five cells were converted to numerical readings (pixels).

2.2.16 Electron microscopy.

BHK cells were infected with γ2 S (9E10) virus or co-infected with α1 (9E10) and β2 (9E10) virus particles using a multiplicity of infection of 10. Fourteen hours after infections the cells were fixed for 15 minutes in 3% paraformaldehyde in PBS. They were then quenched using 0.27% ammonium chloride and 0.37% glycine in PBS for 2 periods of 10 minutes. The cells were blocked with 1% BSA in PBS for 30 minutes and then incubated in 1:2 dilution of 9E10 supernatant for 1 hour. The cells were then rinsed three times with PBS /1% BSA and then incubated with a 1:40 dilution of an anti-mouse gold conjugated antibody (British Biocell International) for 1 hour. The cells were then rinsed 5 times in block before being fixed with 4% gluteraldehyde in 0.1M sodium cacodylate for 15 minutes. This was followed by fixation in 1% w/v reduced osmium tetroxide in water at 4°C for 1 hour. The cells were then rinsed in 0.1 M sodium cacodyalte and dehydrated in ethanol. The specimens were then exposed to critical point drying using an "Agar Scientific" critical point drying apparatus. The cells were then carbon -platinum rotatory shadowed at 45° and then carbon coated at 90° using a Baker FDU 010 coating unit. The coated cells were then floated off coverslips using 8% hydrofluoric acid and digested for 2 hours with 10 M sodium hydroxide. The cells were rinsed with distilled water and mounted on 200 square mesh copper grids. The specimens were then examined using a Philips CM12 transmission electron microscope.

Ms Adele Gibson performed immunogold elecron microscopy on whole mount specimens of BHK cells co-infected with α1 (9E10) and β2 (9E10)

virus particles. Immuno gold electron microscopy on whole mount specimens of BHK cells which had been infected by the γ2 S (9E10) virus were also performed.

2.3 Biochemistry.

2.3.1 SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE sample buffer (80mM Tris-HCL pH 7.8, 100mM DTT, 10% glycerol (v/v), 2% SDS (v/v) and 0.1% bromophenol blue) was added to protein samples prior to loading onto a 1mm thick large polyacrylamide gel (20cmx20cm) (4% stacking gel with 0.1% SDS in 0.375M Tris-HCL pH 6.8 and an 8% separating gel with 0.1% SDS in 0.125M Tris-HCL pH 8.8) containing acrylamide: bisacrylamide in a ratio of 30:1. The samples were boiled for 5 minutes prior to loading. Running buffer contained 0.192 M Glycine, 0.1%SDS, and 25mM Tris base and gels were electrophoresed at 70 to 80 Volts. Proteins were then stained and fixed with 1% Comassie blue in 10% acetic acid/10% methanol and destained in 10% acetic acid/ 10% methanol. The gels were then amplified in 1M sodium salicylate for 30 minutes before being dried down using a Biorad gel drier. Alternatively, the protein gel was directly transferred to nitrocellulose without prior fixation. Biorad low range markers were used as standards for molecular weights.

2.3.2 Western Blotting.

The polyacrylamide gel was carefully placed onto 3MM Whatman filter paper. The filter paper had been moistened in transfer buffer prior to the application of the gel. Moist nitrocellulose (Amersham) was then placed on top of the gel. Another layer of damp filter paper was applied and the "sandwich" of paper, nitorcellulose and gel placed into the cassette of a Biorad western blotting tank. The cassette was positioned so that the positive electrode faced the side of the gel nearest to the nitrocellulose. The tank was filled with transfer buffer and run overnight at 30V. The

nitrocellulose was then stained with Penceau S (Sigma) and the location of the molecular weight markers marked with a biro. To allow orientation of the blot one cut away the bottom right corner. The nitrocellulose was then blocked with PBS containing 5g per 100ml of "Marvel" . The PBS / Marvel mixture also contained 0.05% Tween 20 (Sigma). After blocking for one hour at room temperature the nitrocellulose was incubated with the primary antibody for one hour with constant agitation. This incubation took place in the block solution, the (9E10) monoclonal being used at 25µg per ml, FLAG at 10µg/ml. After this period had elapsed the nitrocellulose was washed five times with block solution and then incubated with the relevant secondary peroxidase conjugated antibody (Pierce) for one hour at room temperature. The secondary antibody was then washed five time in block and finally in PBS twice. The nitrocellulose was then exposed to the enhanced chemi luminescent (ECL, Amersham) substrate for one minute after which it was wrapped in Sarran rap and placed in a film cassette.. Kodak biomax film was then exposed to the nitrocellulose for varying exposure lengths.

2.3.3 BHK membrane solubilisation of αβ heteroligomers by the detergents CHAPS and Deoxycholate.

The membranes that had been generated for the binding assay were also used to identify which detergent was more effective at solubilising the GABA subunits and secondly to elucidate how effective solubilisation is, that is what percentage of protein reaches the soluble fraction.

90µl of membranes from α1 (9E10) and β2 (9E10), α1 (9E10) alone, or β2 (9E10) alone virally infected cells were mixed on ice with either 0.5% Deoxycholate or 2% CHAPS. The membranes were left on ice for 30 minutes. The mixtures of membranes and detergents were then spun at 100000 rpm / 4°C in a Beckman bench top ultracentrifuge for 15 minutes.

The supernatant was collected from each tube and an equal volume of reducing Lamelli sample buffer added (total volume 150µl). The pellet in each tube was re suspended in 50µl of sample buffer. Both samples were then boiled for 10 minutes. The samples were then analysed by PAGE. Half of the pellet sample i.e. 25µl and half the supernatant fraction i.e. 75µl were loaded to separate lanes. Western blot analysis of the gel was then performed using the mouse monoclonal 9E10 to the 9E10 peptide.

2.3.4 Whole cell lysate experiments demonstrating suppression of host biosynthesis.

BHK cells were plated out onto 3cm diameter falcon petri dishes. At 8am the virus (3.5×10^6 infectious units) was added to the cells (approximately 1.6×10^5) . After 90 minutes the virus was removed by washing with medium. Subsequently at 2,4,8,10,12 and 24hours after virus inoculation 0.5mCi of 35 S methionine was applied to the dishes and the cells incubated in its presence for some 30 minutes. Before the hot incubation took place the cells were starved of methionine by incubating them in methionine free medium for thirty minutes. The dishes were carefully washed with methionine free medium prior to starvation of methionine in order to remove all traces of methionine. The culture medium containing the radioactive methionine was retained after each incubation so that it could be reapplied at the next time point.

After the 30 minute incubation in ^{35}S methionine medium the cells were carefully washed with ice cold PBS three times before being lysed on ice using a 200µl volume of lysis buffer containing 2% NP40 and protease inhibitors. The lysate was left on ice for ten minutes before the nuclei were removed by a 5 minute spin at 13000 rpm. The supernatant was taken and stored at -20°C.

Each time point was analysed by running a quarter of the total lysate, approximately 40000 cells (a 25th of a 9cm diameter plate), on an 8% PAGE gel under reducing conditions. The gel was then fixed, amplified and dried before exposing it to film.

2.3.5 Immunoprecipitation.

BHK cells were infected with virus and subsequently incubated in methionine free medium for thirty minutes before ^{35}S methionine translabel was added to the cells. The infected cells were then incubated in the presence of the label for varying periods of time . Before the cells were lysed the radiolabel was carefully removed. Label not incorporated was washed off the cells with PBS. The cells were then lysed in non denaturing lysis buffer.

This buffer contained 6mM disodium hydrogen phosphate, 4mM sodium dihydrogen phosphate, 5mM EDTA, 5mM EGTA, 1mM sodium orthovanadate, 5mM sodium pyrophosphate, 50mM sodium fluoride and 50mM sodium chloride. The buffer was pH'd to 7.4 and supplemented with deoxycholic acid to 0.5% and NP40 to 2% weight by volume. Protease inhibitors were added to the lysis buffer on the day of the experiment. They included; 0.1mM PMSF, 10µg/ml of leupeptin, antipain, pepstatin and 0.1mg/ml of aprotinin.

Cells were lysed in this buffer on ice and after ten minutes were spun at 13000 rpm for five minutes to pellet the nuclei. 25 µl of a 50% solution of protein G sepharose (Pharmacia) in lysis buffer was added to the supernatant and subsequently incubated on a rotating wheel at four degrees centigrade for one hour. The solution was then spun at 13000 rpm for 30 seconds and the supernatant carefully removed leaving a pellet of protein G sepharose. 10µg of monoclanal antibody, 9E10 or FLAG was then added to the precleared supernatant. This mixture was then placed on a rotating wheel for one hour at 4°C. After this time had

elapsed 50µl of a 50% solution of protein G sepharose made up in lysis buffer was added to the antibody /supernatant mixture and again incubated at 4°C with rotation. Once this incubation had finished the protein G sepharose was pelleted by a 30s spin at 13000 rpm . The supernatant was discarded and the pellet washed three times in lysis buffer involving three consecutive centrifugations to pellet the protein G sepharose. Before the final wash the pellet was transferred to a new eppendorf tube. After the final wash the pellet was resuspended in SDS PAGE reducing sample buffer. Before loading the immunoprecipitate onto a PAGE gel it was in some cases boiled for ten minutes. Finally the sample was spun for 30s at 13000rpm and loaded onto the gel.

2.3.6 Inhibition of N linked glycosylation by Tunicamycin.

BHK cells were infected with virus and after a period of four hours the infected cells were placed into methionine free medium containing 5µg/ml tunicamycin. After a thirty minutes had elapsed 500µCi of ^{35}S methionine translabel was added. The cells were incubated in the presence of the radiolabel and the tunicamycin for two hours . After the two hours had elapsed the cells were lysed in non denaturing lysis buffer. The GABA subunits were then immunoprecipitated with the appropriate monoclonal antibody . A control immunoprecipitation was performed using Mouse IgG in place of the monoclonal antibody. The immunoprecipitates were run on an 8% PAGE gel and subsequently the gel was fixed, amplified with 1M sodium salicilate, dried and exposed to film at -70°C.

2.3.7 Half life experiments.

BHK cells were infected by the α1 (9E10) or the β2 (9E10) virus. The cells were then incubated in methionine free medium containing 400μCi/ml ^{35}S methionine for 20 minutes. The labelling occurred four hours after the infection. The cells were then chased for 0, 20 minutes, 1 hour, 2 hours, 4 hours and 6 hours with an excess of cold methionine in BHK medium. The cells were lysed in non denaturing lysis buffer , the nuclei removed by a 5 minute spin at 13000 rpm and the supernatant snap frozen. The lysates for the time points were stored at -80 ^{o}C. For one particular time course all the time points were immunoprecipitated at the same time using the same reagents. The immunoprecipitate was run out on SDS PAGE and the gel fixed, dried and quantified using a Biorad phosphorimager. Background was subtracted using the same volume that was used to integrate the subunit signals. The signals for the immunoprecipitated subunits were plotted against the chase time. This experiment was repeated three times.

2.3.8 Sucrose Gradient Fractionation.

Virally infected BHK cells were incubated for some 24 hours before they were lysed on ice, in non denaturing lysis buffer containing 2% NP40 and 0.5% deoxycholate, and protease inhibitors. The lysate was rotated for 10 minutes before being centrifuged at 13000 rpm for 5 minutes. Subsequently the supernatant was removed and layered carefully onto the top of a sucrose gradient.

The sucrose gradient was generated by loading a linear gradient maker with 5% and 20% sucrose solutions, weight by volume of lysis buffer containing protease inhibitors. The 5% sucrose solution was placed in the chamber nearest the outlet and the 20% sucrose solution furthest from the outlet.

A linear gradient was generated by turning on the peristaltic pump (so as to deliver between 50 and 100 µl per second) and opening the two taps on the gradient maker, making sure that a stirrer was mixing the solutions at the outlet of the gradient maker. After filling the Beckaman tube to 4mm below the top the peristaltic pump was stopped and the delivery tube was then removed from the centrifuge tube containing the gradient. The tubes containing the linear gradients were then weighed to ensure that the centrifuge was balanced. The sample was then carefully layered onto the top of the gradient. The maximum amount of sample for a 5ml tube was 250µl.

A Beckman Sw 55 Ti rotor was set up to spin at 40, 000 rpm for some 14 hrs 14 minutes at 4 degrees centigrade. Final $\omega^2 t$ equaled $9x10^{11}$ After the centrifugation had taken place the gradients were fractionated into fourteen 350µl fractions.

2.3.9 Calibration of sucrose gradients.

In order to calibrate the sucrose gradient sedimentation a second gradient was run in parallel. This was used to sediment a sample which contained protein standards. The standard proteins used to calibrate the gradients were : bovine serum albumen, aldolase, and catalse . Each of these proteins consist of multimers apart from BSA. When denatured BSA, runs at 68KDa, aldolase runs at 40KDa and catalase runs at 57.5 KDa. The sedimentation coefficient of BSA is 4.3 S , aldolase is 7.4 S and catalase is 11.2 S

This gradient containing the standards was spun in the same way as the gradient containing the virally expressed protein ,made with the same sucrose solutions, and fractionated in the same fashion.

Reducing sample buffer containing SDS was applied to the fractions from the standard gradient and 100 µl of each fraction run on an eight percent PAGE gel. The PAGE gel which contained the standards was

fixed and stained with commassie blue in order to identify which fractions of the gradient contained the peak concentrations of the three standard proteins.

The fractions from the gradient used to sediment the virally expressed protein were either subjected to immunoprecipitation or run directly on a PAGE gel and western blotted.

2.3.10 Pulse Chase analysis of GABA $_A$ receptor assembly.

BHK cells were plated at a density of one hundred thousand cells per three cm diameter well . Twelve wells in total were used, each time point corresponding to three wells of cells. The cells were allowed to attach over night . The cells were infected with 500µl of α 1 (9E10) virus and 500µl of β2 (9E10) virus in 12 mls of binding medium. Each well was infected by 1 ml of binding medium containing the above dilution of virus. The cells were incubated for one hour and then normal BHK medium was applied. The cells were incubated for three hours before being starved of methionine for thirty minutes prior to the addition of ^{35}S methionine at a concentration of 150µCi per ml. The label was removed after an hour, and free label carefully washed off with normal medium. The first time point was immediately after this labelling corresponding to 0 hours of chase. Cells were lysed in non denaturing lysis buffer. The infected cells were chased for 0, 6 and 20 hours . After each time point the lysates were snap frozen and stored at -80°C. The lysates for all three time points were then sedimented on 5-20% linear sucrose gradient as previously described. The gradients were then fractionated into fourteen 350µl aliquots. These aliquots were immunoprecipitated with 10µg of affinity purified 9E10 antibody. The precipitates were then run on an 8% PAGE gel . The gel was fixed and once dried exposed to a phsophorimager screen for 48 hours. The

amount of total α and β proteins that were present at the 6 and 20 hours was compared to that at the 0 time point.

2.3.11 Stoichiometry of a $GABA_A$ ligand gated ion channel.

BHK cells were infected simultaneously with α1 (9E10) and β2 (9E10) SFV virus particles. The cells were subsequently labelled with ^{35}S translabel at the four hour time point post infection. The cells were incubated in the presence of this label overnight or pulse chased. The infected cells were carefully washed in PBS prior to lysis to remove any free label. The lysate was then incubated at four degrees on a spinning wheel for ten minutes The lysate was then centrifuged at 13000rpm for five minutes to pellet insoluble matter. The supernatant was then layered onto the top of a 5 to 20% sucrose gradient and centrifuged in an SW 55 Ti rotor for 14.14 hours at 40000rpm. The gradient was then fractionated into 14, 350 µl volumes. Fractions 8, 9 and 10 were pooled and subjected to immunoprecipitation with the 9E10 monoclonal antibody. The immunoprecipitate was then analysed by SDS PAGE and quantified by a Biorad phosphorimager and cutting out the radioactive bands corresponding to the individual subunits and counting them using a scintillation counter. Figs were corrected for the differing methionine content of the two proteins.

2.3.12 Protein Assays.

Protein assays were performed with BSA standards using a Biorad protein assay kit as described in the manufacturers instructions.

2.3.13 Antibodies:

Polyclonal anti-phosphotyrosine.

Rabbit polyclonal

Gift from Professor Huganir, John Hopkins University, Baltimore.

Polyclonal anti α1 C terminus

Gift from Dr Stephenson, London School of Pharmacy.

Rabbit polyclonal raised against a synthetic peptide corresponding to the C terminus of the α 1 subunit (amino acids 413-429) (Pollard et al 1993). Used in immunofluorescence at 5μg/ml.

Polyclonal anti α 1 Intracellular loop.

Raised against a synthetic peptide corresponding to the intracellular loop of the murine α 1 subunit (amino acid residues 326-343) (Macdonald and Olsen 1996, Krishek et al 1994).

BD 17.

This is a mouse monoclonal that detects the extracellular domain of β 2 and 3. (Schoch et al 1985) Used at 10μg/ml for fluorescence.

Polyclonal anti β 2.

Gift from Dr Benke, Institute of Pharmacology, University of Zurich.

Rabbit polyclonal raised against a synthetic peptide corresponding to amino acids 335-341 of the β 2 subunit (Benke et al 1994). Used at 10μg/ml for immunoprecipitation experiments

Polyclonal anti GFP.

Gift from Dr Ken Sawin ICRF Laboratories, Lincoln Inns Fields, London.

Myc monoclonal antibody (9E10).

The 9E10 is a mouse monoclonal antibody that recognises the epitope, EQKLISEEDL, (Evan et al 1985). The hybridoma cell line was kindly given to the Laboratory from Dr Evan, ICRF Laboratories, Lincoln Inns Fields, London. The hybridoma was grown in DMEM supplemented with 10% FCS incubated at 37 degrees centigrade and at 5% CO2. The cells were grown up in large Falcon roller bottles and after approximately four weeks the supernatant was harvested. The hybridoma cells were separated from the supernatant by a 4000 rpm spin and the supernatant was also filtered through a 0.2μm filter. A portion of the supernatant was used for immunoflurescence the rest was affinity purified using a protein A sepharose column. The protein in the (9E10) supernatant was

precipitated with ammonium sulphate. Whilst stirring constantly saturated ammonium sulphate solution (pH'd to neutral with HCL) was added slowly to the supernatant to bring the final concentration to fifty percent (i.e. an equal volume). The solution was kept at four degrees centigrade overnight. The precipitate was centrifuged at 3000rpm for 30 minutes and the supernatant subsequently discarded. The pellet was then resuspended with 0.1 of the starting volume of PBS . The solution was then dialysed against PBS overnight. The solution was removed from the dialysis tubing and then centrifuged to remove debris. This solution was then subjected to affinity purification.

The antibody solution was adjusted to a NaCl concentration of 3.3M and a one tenth volume of 0.5 M sodium borate pH 8.9 also was added. The antibody solution was then added to the protein A column. The column was then washed with ten column volumes of 3.0 M NaCl, 50mM Sodium Borate pH 8.9, followed by ten column volumes of 3M NaCl, 10mM Sodium Borate pH 8.9. The column was then eluted with a solution containing 100mM glycine pH 3.0 and 100mM NaCl. The elutant was collected in an eppendorf tube containing 50 microlitres of 1M Tris pH 8.0. As the fractions were collected they were carefully agitated to mix with the Tris and elevate the pH of the solution. The immunoglobulin containing fractions were identified by spectroscopy at 280nm.

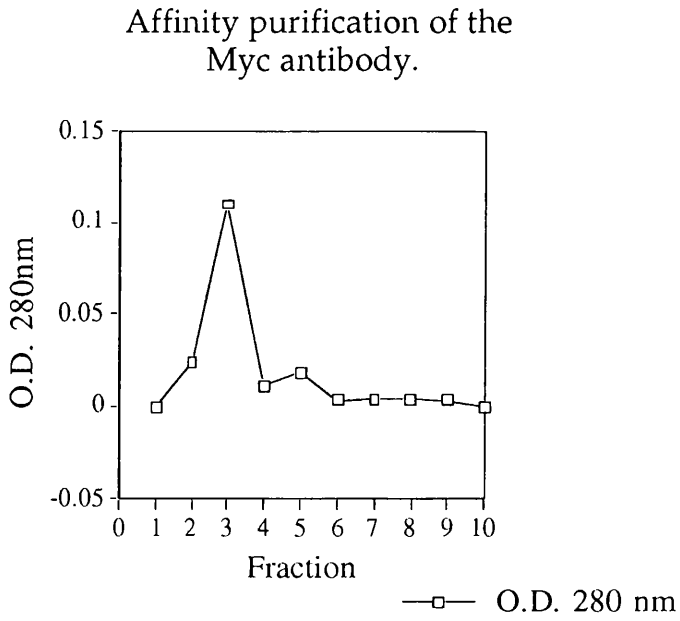


Fig 7. Affinity purification of 9E10 monoclonal antibody.

This represents a typical affinity preparation of the 9E10 monoclonal antibody.

Immunoprecipitations using 9E10 were conducted using 10µg/ml and western blots using this antibody were conducted using 25µg/ml of affinity purified antibody.

FLAG M2 Antibody.

This is a mouse monoclonal antibody that recognises the epitope, DYKDDDDK. (Hopp et al 1988) The affinity purified antibody was purchased from IBI ltd This antibody was used at 6µg/ml for immunoprecipitation experiments and for western blotting.

Production of Phosphotyrosine Antibodies.

The peptide LQERDEE-Y(P)-G-Y(P)-E was synthesised by Immune Systems Ltd and as can be seen was chemically phosphorylated at the two tyrosine residues. This peptide was coupled to Keyhole Limpet Haemocyanin (KLH) manufactured by Pierce. This was achieved using the following method.

One millilitre of sterile PBS (pH 7.4) was injected into a twenty milligram vial of KLH. This vial was then incubated overnight at room temperature in a rotatory mixer in order to solubilise the KLH. The following morning, 10mg of synthetic peptide was dissolved in 1ml of sterile PBS and the pH adjusted to 7.5. The molar ratio of peptide to KLH was 600. The peptide solution was added to the KLH solution and then 2mls of a freshly made 0.2% solution of gluteraldehyde was added slowly to the vial containing peptide and KLH, stirring constantly. This solution was then left stirring for one hour at room temperature. 1 ml of a 1M glycine solution (pH 7.2) was then added to the vial and the solution incubated for a further hour. The mixture was then aliquoted and stored at -20 degrees centigrade. This conjugate was then used to immunise animals, which was performed by Froxfield farms UK ltd.

Animals were boosted every month for three months and before bleeding out a test bleed was conducted to ascertain whether further immunisations were required.

This was performed by first conjugating the peptide onto BSA using gluteraldehyde and then dotting out 500ng of conjugated peptide onto nitrocellulose. Ten fold serial dilutions of serum were then assayed by a modified western blot protocol utilising ECL.

Once the titre of antibody had reached an adequate level the serum from the animal was collected and the antibodies to the immunogen were isolated by affinity chromatography.

Affinity Purification of Anti Phosphotyrosine Guinea Pig Antibodies.

An affinity purification column was created by the following method.

The peptide used to immunise the Guyana Pigs was covalently coupled to free carboxy groups on the ECH sepharose 4B from Pharmacia. This reaction utilised the Carbodimide EDC from Pierce (1-Ethyl-3-(3-Dimethylaminopropyl)carbidomide), to facilitate the conjugation of

peptide to the carbon spacer projecting from the sepharose beads. 1ml of ECH sepharose was washed with 80 ml of 0.5M Na Cl, and then washed with 50ml of water pH 4.5. Then 8mg of peptide was dissolved in 1ml of water and the pH of the solution adjusted to 4.5. Fifty mg of EDC was dissolved in 5ml of water and the pH adjusted to 4.5. The gel and 1ml of the EDC solution together with 1ml of the peptide solution were mixed over night in an end over end shaker.

The column was then washed with 15mls of water pH 4.5 .The uncoupled sights were subsequently blocked using a 0.4M solution of ethanolamine and a 10 mg per ml solution of EDC, both solutions being at a pH of 4.5. 1ml of each solution was combined with the ECH sepharose and incubated at room temperature for 2 hrs.

The column was then washed with 20 volumes of 10mM Tris HCl pH 7.5 followed by 10 volumes of 100mM glycine pH 2.8, followed by 10 volumes of Tris HCl pH 8.8, then 10 volumes of 100mM triethylamine pH 11.5, and then finally 10 volumes of 10mM Tris HCl pH 7.5.

The anti sera was spun at 3500 rpm for 10 minutes and the supernatant taken and diluted tenfold with 10mM Tris HCl pH 7.5. The diluted serum was then applied to the column twice.

The column was then washed with 20 volumes of 10mM Tris HCl pH 7.5, followed by 10 volumes of 100mM Tris HCl containing 500mM NaCl. The bound antibody was then eluted off the column using five volumes of 100mM glycine pH 2.8, and five volumes of 100mM triethylamine pH 11.5, separated by a wash step using 10 volumes of 10mM Tris HCl pH 8.8. The eluted fractions were collected in a container which had a tenth volume of 1M Tris HCl pH 8.0. The column was then washed with Tris HCl pH 7.5 and stored in this solution containing 0.02% azide.

The eluted fractions were pooled and dialysed overnight against phosphate buffered saline pH 7.4 . The absorbance at 280 nm was taken of a tenfold dilution and the yield of antibody calculated.

2.4 Pharmacology and Electrophysiology.

2.4.1 ^{3}H muscimol binding experiments performed on whole BHK cells co-infected with the α 1 (9E10) and β 2 (9E10) virus particles.

BHK cells were seeded out onto 10cm diameter tissue culture plates at a density of 800, 000 per plate. Ten plates were made at this density. The cells were allowed to adhere and then infected with a 1: 1 ratio of α1 (9E10) to β 2 (9E10) virus, 2mls of each viral stock in a total volume of 40 mls. After washing the cells once with binding medium 8mls of virus mixture was applied to each of five plates. These were incubated for an hour at 37^{o}C and then the infectious medium applied to the second set of five plates which were also incubated for one hour at 37^{o}C. The cells were then incubated overnight before being harvested the following morning. This was conducted by washing the cells with ice cold PBS containing 5mM EDTA and then gently blowing them off the dish. The cells from all ten dishes were pooled and centrifuged at 3000rpm for 5 minutes. The pellet was then resuspended into a final 4 ml volume of ice cold PBS and counted in a haemocytometer.

The cells were then used to perform a whole cell binding assay. 100µl aliquots of cells, corresponding to 100,000cells, were used for each point 10nM , 20nM, 30nM, 50nM, 70nM, 90nM, 110nM and 150nM of ^{3}H muscimol being applied at each point. The cells were incubated on ice for one hour before being harvested by fast filtration onto GFIIB Whatman filters that had been fire polished, using a Brandell harvester. The filters were then removed from the harvester and placed into scintillation vials and 5 mls of liquid scintillant added. The vials were then counted after 24 hours to ensure all the material had dissolved into the

scintillant. Each point was performed in triplicate, non specific binding being estimated by the incorporation of 100μM GABA for each concentration of muscimol. The total counts for each concentration of ^3H muscimol was estimated by the addition of the same amount of ^3H muscimol used for each concentration to a scintillation vial and counted. A protein assay was performed upon an aliquot of cells to determine the amount of surface protein per binding experiment.

2.4.2 Electrophysiology.

This was conducted by Professor Trevor Smart based at the London School of Pharmacy. BHK cells were co-infected with α 1 (9E10) and β 2 (9E10) virus particles . BHK cells were also singly infected by the α1 (9E10) or β2 (9E10) virus. The following day whole cell recordings were taken from the doubly and singly infected cells to ascertain if GABA gated chloride currents could be elicited in the co-infected but not in the singly infected cells.

Whole cell recording . (This methodology is taken from the methods section of a paper published by Krishek et al in 1994.) Experiments on BHK cells were performed using a List EPC7 amplifier in a whole cell recording mode. Patch electrodes were fabricated from capillary tubes made from borosilicate glass. The electrodes contained, 140mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 11mM EGTA, and 2mM ATP. The cells were viewed under phase contrast optics and continuously perfused with Kreb's solution containing 140 mM NaCl, 4.7mM KCl, 1.2mM $MgCl_2$, 2.5mM $CaCl_2$, 10mM HEPES and 11 mM glucose.

Results.

Chapter 3.1.

Characterisation and development of the Semliki expression system to the study of $GABA_A$ ligand gated ion channel.

The mechanism that controls the assembly of $GABA_A$ receptors within neurones of the central nervous system are unknown. Five classes of $GABA_A$ subunit have been discovered and within each class multiple subunits exist (Macdonald and Olsen 1994, Davies et al 1997). The large number of receptors that could form by random combinations of subunits are not observed in the central nervous system. Only a limited number of subunit combinations have been proven to occur by biochemical and immunocytochemical analysis (Benke et al 1994,Quirk et al 1994, Fritschy and Mohler 1995, Connolly et al 1996a). Such observations would suggest that specific rules underlie the assembly process. Heterologous expression systems, where defined combinations of subunits can be expressed, have been used to determine the combinations of subunits that can give rise to $GABA_A$ receptors. It is not known whether the assembly "rules" which have been defined in transformed cell lines are applicable to neurones. This question was addressed by using the Semliki forest virus system to introduce epitope tagged $GABA_A$ subunits into differentiated neuronal cultures. Before such experiments could be conducted the viral expression system had to be characterised.

The Semliki expression system's ability to produce recombinant protein in neurones had been published. The IgA receptor, (De Hoop et al 1995) amyloid precursor protein, (Simons et al 1995) and the enzyme Betagalactosidase had been expressed in differentiated neurones using this expression system (Olkkonen et al 1993). It was important to reconfirm the ability of the virus to infect differentiated neurones and

further investigate its ability to express proteins in brain slice preparations.

Reports existed that the Semliki virus rapidly inhibited the host cell's biosynthetic machinery, (Liljestrom et al 1991) and a related virus , Sindbis virus, induced apoptosis in neurones harvested from neonatal mice (Griffen et al 1994a). It was therefore important to determine what deleterious effects the Semliki expression system had upon neurones. In order to accurately assess the toxicity associated with viral infection a virus that expressed the Green fluorescent protein (GFP) (Prasher et al 1992) was engineered. The fluorescence produced by this flurophore was normally weak. In order to enhance its fluorescence and thus make observations easier to perform a mutation was introduced at codon 65, substituting a serine with a threonine. This had been reported to enhance the GFP's fluorescent signal six fold (Heim et al 1995). Living cells which had been infected by GFP mutant virus could have their electrophysiological characteristics measured and compared to control cells. The simplicity of identifying infected cells by their green fluorescent signal without the need to perform elaborate staining or substrate feeding protocols enhanced the accuracy of the observations.

The Semliki expression system had been used to express serpentine receptors such as the Neurokinin 2 , Dopamine D3 and prostaglandin Ep4 receptors and the ligand gated ion channel 5HT3,(Lundstrom-K et al 1995; Patel-K et al 1996; Werner-P et al 1994). It was not known whether the Semliki viral expression system was capable of generating heteroligomeric ligand gated ion channels. This was an important pre-requisite to studying the assembly of the $GABA_A$ receptor using this system. In order to address this, combinations of virally expressed $\alpha 1$ (9E10) and $\beta 2$ (9E10) subunits were examined using a combination of biochemical, immunohistochemical, pharmacological and

electrophysiological methods. The choice of a heteroligomer composed of α1 (9E10) and β2 (9E10) subunits was made because neither of these subunits is capable of forming homomeric channels (Connolly et al 1996a Krishek et al 1994, Angelotti and Macdonald 1993, Sigel et al 1990). Secondly a receptor composed from α and β subunits is the most simple generally recognised $GABA_A$ receptor (Macdonald and Olsen 1994).

Recombinant studies existed which supported the production of a GABA $_A$ ligand gated ion channel only when an α1and a β2 subunit were co-expressed in HEK 293 cells . When the α1 (9E10) or β2 (9E10) subunits were expressed individually no GABA gated currents could be elicited and the individual subunits were retained in the endoplasmic reticulum of the cells. (Connolly et al 1996a). The capability of this expression system to reproduce such experiments was tested. This involved the determination of the presence or absence of $GABA_A$ receptor at the plasma membrane by immunohistochemical methods. Whole cell ligand binding studies were also performed using ^3H muscimol. This allowed a more quantitative analysis of the expression of receptors at the plasmamembrane. Whole cell recording of cells co-infected with the α1 (9E10) and a β2 (9E10) virus particles was also performed in order to determine whether the channels that were expressed were functional.

The question of triple infection was relevant since in order to produce a $GABA_A$ gated ion channel that could be allosterically modulated by benzodiazepines required the co-expression of an α, β and a γ subunit. In order to address this question the co-infection of GFP virus, α1 (9E10) virus and β2 (9E10) virus was performed and the ability to produce green cells which also had cell surface fluorescence for the GABA subunits was evaluated by confocal microscopy.

A further objective of this work was to use the Semliki forest virus to introduce wild type and mutated subunits into neural networks from animals in which defined subunits had been deleted by homologous recombination. Given that low levels of infection may occur it is desirable to co-express a marker protein. The marker to be effective would be detectable with the minimum of intervention. The Green fluorescent protein was chosen since no fixation or substrates are required for this protein to be detected. It was therefore attractive to engineer a viral vector which could produce not only a $GABA_A$ subunit but also GFP simultaneously.

3.1.1 Confirmation of the generation of virus expressing the desired subunits by immunocytochemistry and biochemical analysis.

Immunofluorescence Data.

BHK cells were infected separately with α1 (9E10), β2 (9E10), γ2 L FLAG and γ2 S (9E10) virus particles. The γ2 L differs from the γ2 S by having an eight amino acid insertion within the predicted intracellular loop between residues 337 and 338 of the mature protein (Kofuji et al 1991). The BHK cells were infected at a multiplicity of 10:1, that is 10 virus particles per BHK cell. The cells were then examined immunohistochemically, 14 hours post infection using the relevant antibody , either FLAG or 9E10. Strong fluorescence signals were detected for each infection. The distribution of the α1,(9E10) β2 (9E10) and γ2 L FLAG proteins within the cells was endoplasmic reticulum in morphology with no antigenicity detectable when immunofluorescence was performed on intact cells, in the absence of detergent see Fig 8 (E) and (F) This reticular pattern of fluorescence with staining of the nuclear membrane is characteristic of the endoplasmic reticulum (ER). This is supported by immunocytochemical studies on proteins that are retained

in the endoplasmic reticulum due to the presence of an ER retention signal

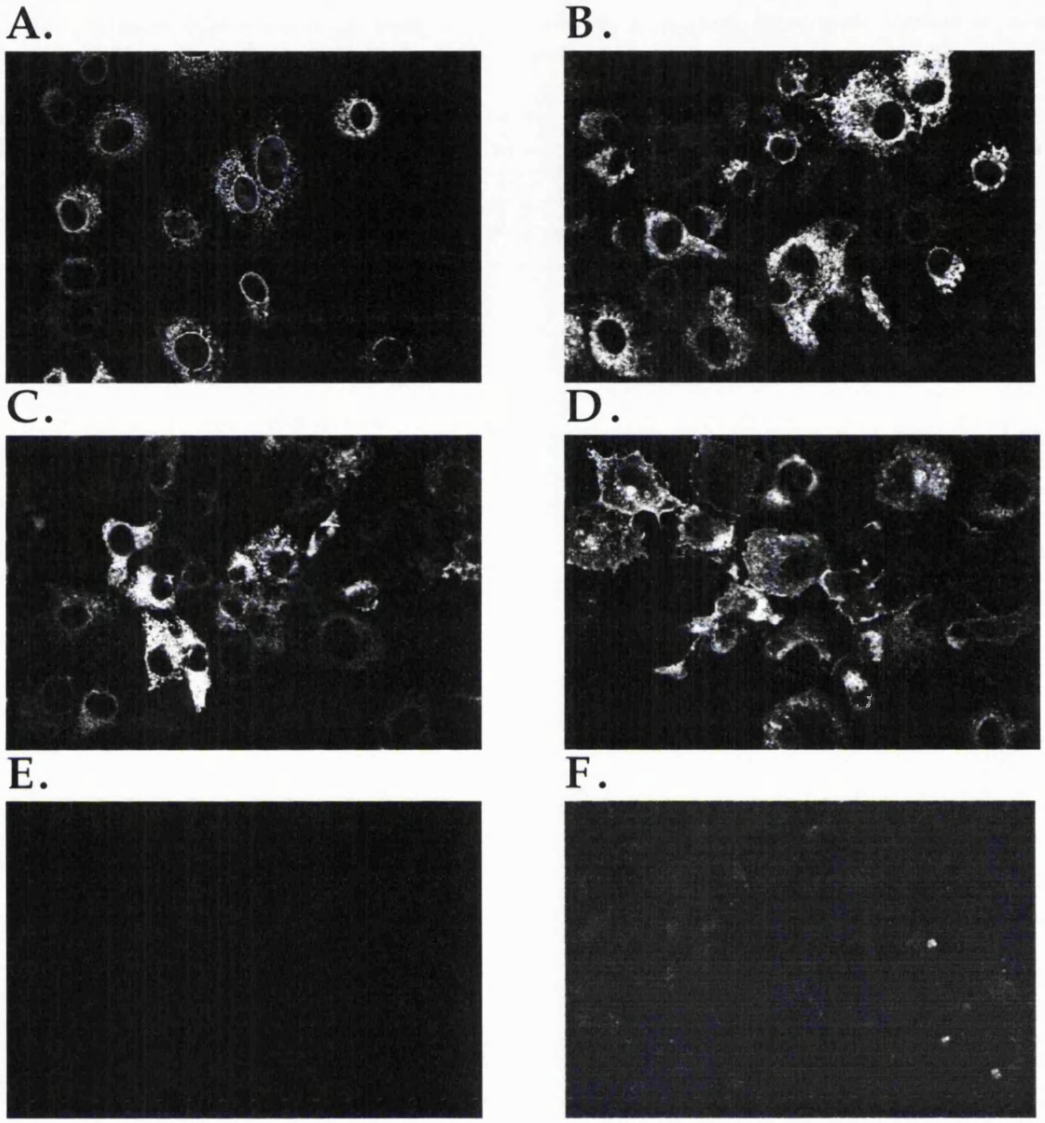


Fig 8. Expression of $GABA_A$ subunits by the Semliki viral expression system in BHKcells. Immunofluorescence performed on BHK cells infected with α1 (9E10) (A.) and (E), β2 (9E10) (B.) and (F), γ2 L FLAG (C.) and γ2 S (9E10) (D.) 20 hours post infection. The cells were permeabilised with 0.5%NP40 in panels A., B., C., and D. Fluorescence on intact BHK cells infected by α1 (9E10) virus is shown in panel E. Fluorescence on intact BHK cells infected with the β2 (9E10) virus is shown in panel F. Immunofluorescence was conducted using the 9E10 monoclonal antibody except for panel C, where the FLAG monoclonal was used. Images were collected using an MRC 1000 confocal microscope. The scale bar represents 50μm.

such as KDEL (Connolly et al 1994). These results compared well to previous immunohistochemical analysis of cells expressing only the α1, β2 and γ2 long proteins (Connolly et al 1996a). In contrast the γ2 S (9E10) protein was not restricted to the endoplasmic reticulum and signal at the plasma membrane was also visible. In some of the cells a distinctly punctate pattern can also be observed, see Fig 8 (D). The cell surface pattern and the internal staining is more characteristic of a receptor that undergoes constitutive endocytosis such as the transferrin receptor (Bretscher 1983).

3.1.2 Biochemical Data Characterising the Virus particles.

The capability of the α1 (9E10) and β2 (9E10) viral constructs to produce proteins with the correct molecular weight as defined by polyacrylamide gel electrophoresis was performed by running whole cell lysates of ^{35}S labelled BHK cells that had been exposed to virus at increasing time points after infection. The results of this experiment ilustrated in , Fig 9 (A and C), not only indicated that a protein of the correct molecular weight was being produced but it clearly demonstrated the rate at which host protein synthesis is inhibited in favour of the synthesis of viral proteins. As can be seen in Fig 9 (A) and (B), after approximately 3 hours host protein synthesis is effectively stopped. Since other viral proteins for example the viral replicase, were also being labelled it was important to confirm the presence of the $GABA_A$ subunit by imunoprecipitation, this can be seen in Fig 9 (C) and (D). and is shown alongside the whole cell lysate experiment for comparison. The 9E10 immunoprecipitation confirmed that the $GABA_A$ subunits were being produced for the α1 (9E10) and β2 (9E10) constructs as demonstrated by the formation of distinct bands of 52 and 56KDa respectively.

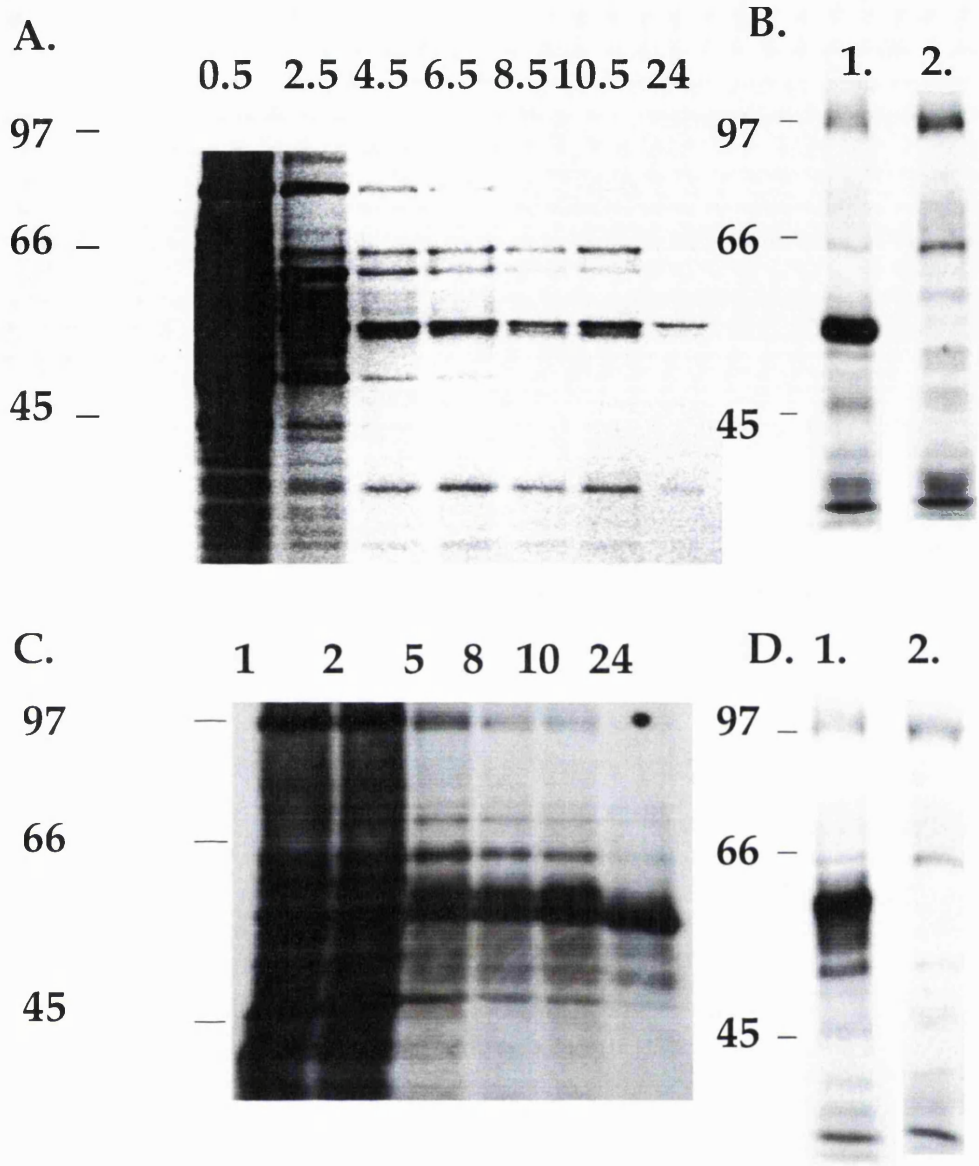


Fig 9. Biochemical characterisation of the $GABA_A$ receptor α1 (9E10) and β2 (9E10) subunits produced on Semliki Forest virus infection of BHK cells. BHK cells were infected with either the α1 (9E10) virus (A.) or the β2 (9E10)virus (C.). The cells were labelled for 30 minutes with 500μCi/ml ^{35}S methionine at defined periods post infection, the units shown are hours. After the labelling period the cells were lysed and the labelled proteins resolved by SDS PAGE followed by autoradiography. The subunits were also immunoprecipitated from the lysate as shown in panels B. and D. for the α1 (9E10) and β2 (9E10) infections. The migration of protein standards (Biorad) are shown , the units are KDa, (KiloDaltons).

The sizes of these proteins is consistent with previous experiments on the expression of recombinant proteins (Connolly et al 1996a, Moss et al 1995) and endogenous proteins detected by western blotting (Rabow et al 1995). The γ2 L FLAG subunit 's synthesis by the viral expression system was confirmed by western blotting lysates of infected BHK cells at various time points after infection. In Fig 10 (A) the production of a specific band of 45 KDa can be observed which increases with time post infection.

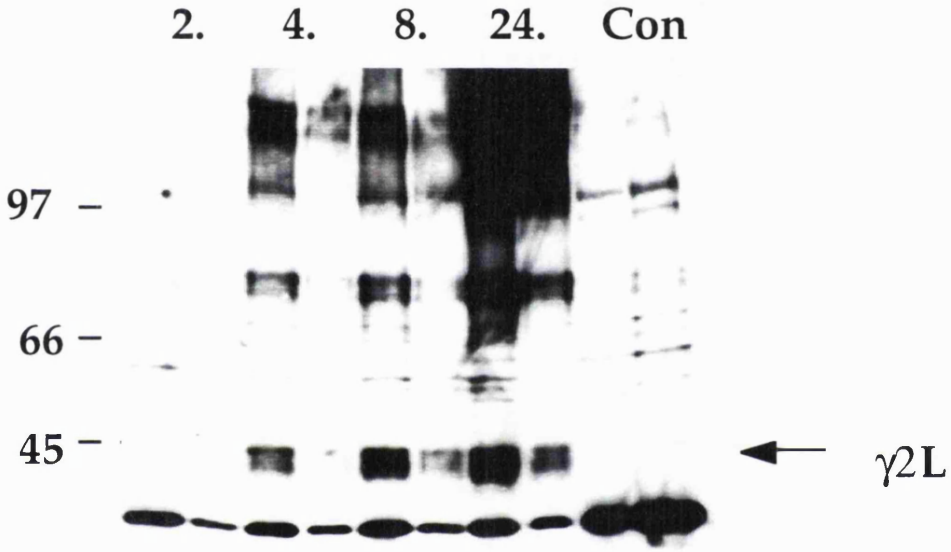


Fig 10 A. The γ2 L FLAG protein is expressed after viral infection of BHK cells by the γ2 L FLAGvirus. BHK cells were infected by the virus and after defined time points the cells were lysed. The proteins in the lysate were resolved by SDS PAGE and FLAG western blotting of the gel. The time points post infection are in hours. Control BHK cells not infected by the γ2 L FLAG virus were lysed and also western blotted with the FLAG antibody. These were run in the control lanes. (Con) The two lanes for each time point correspond to a 50% dilution of the sample and no dilution. Migration of molecular weight standards is indicated. The units are KiloDaltons.

BHK cells were infected by the γ2 S (9E10) virus and incubated for 14 hours at 37°C before being lysed in reducing sample buffer and sonicated. BHK cells which had not been infected were also lysed and sonicated. The infected and control lysate were subjected to SDS PAGE and subsequently western blotted using the monoclonal antibody 9E10. Fig 10 (B) clearly demonstrates the production of two species of protein one

with a molecular weight of 50KDa and the other with a size of 45 KDa. The sizes of these two proteins are in agreement with studies using the heterologous expression of epitope tagged γ2 subunits (Moss et al 1995, Connolly et al 1996a) and the detection of endogenous γ2 subunits using subunit specific antibodies (Khan et al 1994, San Juan et al 1995).

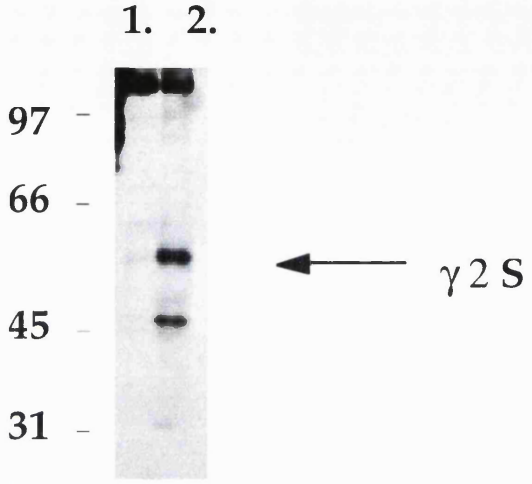


Fig 10 B. The γ2 S (9E10) protein is expressed in BHK cells after their viral infection by the γ2 S (9E10) virus.BHK cells were infected by the γ2 S (9E10) virus and after twenty hours were lysed in reducing sample buffer. The proteins in the lysate were run on an 8% SDS PAGE gel. The proteins were transferred to nitrocellulose . The nitrocellulose was then western blotted with the 9E10 monoclonal antibody. Control BHK cells not infected by the virus were also lysed and run on the same gel. Lane 1 corresponds to the control unifected cells and lane 2 corresponds to the γ2 S (9E10) infected cells. Migration of molecular weight standards is indicated, units are KiloDaltons.

3.1.3 The $GABA_A$ subunits produced by the Semliki expression system are glycosylated.

It was unknown whether this system would glycosylate the recombinant proteins. In order to test this and discover the unglycosylated molecular weight of the $GABA_A$ subunits produced by this vector experiments were conducted in the presence or absence of tunicamycin, a known inhibitor of N-linked glycosylation. BHK cells were infected with recombinant virus for one hour and then at four hours post infection cells were incubated in methionine free medium containing 5μg/ml tunicamycin for half an hour. After this period had elapsed 500μCi of ^{35}S

methionine were added and the cells were incubated in the presence of the radioactive label and the tunicamycin for a period of two hour before being lysed. Half the lysate was subjected to immunoprecipitation with the monoclonal (9E10) and the other half immunoprecipitated with mouse IgG to form a control. It is clear that proteins produced by the Semliki expression system are glycosylated. In Fig 11 (A), a clear decrease in the size of the immunoprecipitated α1 (9E10) and β2 (9E10) proteins can be observed when tunicamycin is present. The α1 (9E10) subunit has a glycosylated molecular weight of 53 KDa and a non glycosylated molecular weight of 47 KDa. In contrast the β2 (9E10) subunit has a glycosylated molecular weight of 56 KDa and a non glycosylated weight of 50KDa. The decreases in molecular weight observed when infected cells are treated with tunicamycin are similar to the results performed using transiently transfected HEK 293 cells with expression plasmids containing α1 (9E10) and β2 (9E10) cDNA (Connolly et al 1996a). These figures agree with molecular weight determinations made using subunit specific antibodies. The α1 protein has been reported to be 50, 51 or 53 KDa in size, (Benke et al 1991, Duggan and Stephenson, 1989, Duggan et al 1991, Endo and Olsen, 1991, McKernan et al 1991, Sato and Neale, 1989, Stephenson et al, 1989).The β2 protein was reported to be between 54 and 58 KDa in size (Machu et al 1993). Similar experiments performed on the γ2 proteins are described in chapter 3.3.

3.1.4 Solubilisation of virally expressed proteins.

In order to analyse the virally expressed proteins by techniques such as sucrose gradient sedimentation, proteins produced by the viral expression system it was important to determine if the virally expressed $GABA_A$ subunits could be solubilised. BHK cells were infected with the α1 (9E10) and β2 (9E10) virus particles . The cells were collected and incubated in PBS containing either 0.5% deoxycholic acid or 2% CHAPS

for thirty minutes. The cells were centrifuged at 100,000 rpm for 15 minutes and the supernatant and pellets collected for each of the detergent treatments. Fig 11 (B) illustrates the results of a 9E10 western blot of the pellet and supernatant fractions for the deoxycholic acid and CHAPS solubilisation. It is clear that the virally expressed α1 (9E10) andβ2 (9E10) subunits can be solubulised and that 0.5% deoxycholic acid is more efficient at solubilising the subunits than 2% CHAPS.

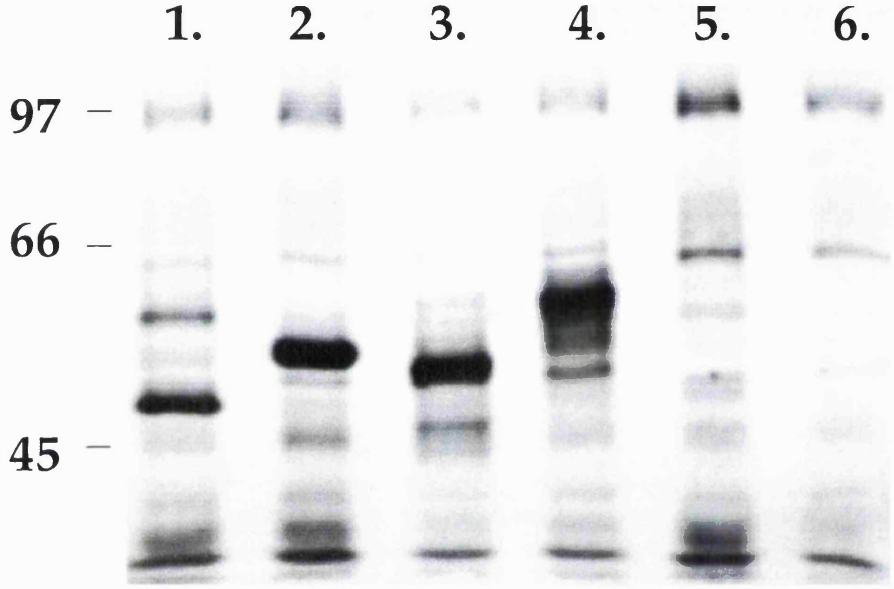


Fig 11 A. The virally expressed α1 (9E10) and β2 (9E10) proteins are glycosylated. BHK cells were infected by the α1 (9E10) or β2 (9E10) virus particles and 4 hours post infection were labelled with ^{35}S methionine for 2 hours in the presence (+) or absence (-) of 5μg per ml of tunicamycin. The virally expressed proteins were then immunoprecipitated with the (9E10) monoclonal antibody or mouse IgG as a control. lane 1 α1 (9E10) +, lane 2 α1 (9E10) - , lane 3 β2 (9E10) +, lane 4 β2 (9E10) -, lane 5 α1 (9E10) + mouse IgG, lane 6 β2 (9E10) + mouse IgG. The migration of molecular weight standards is indicated , units KiloDaltons.

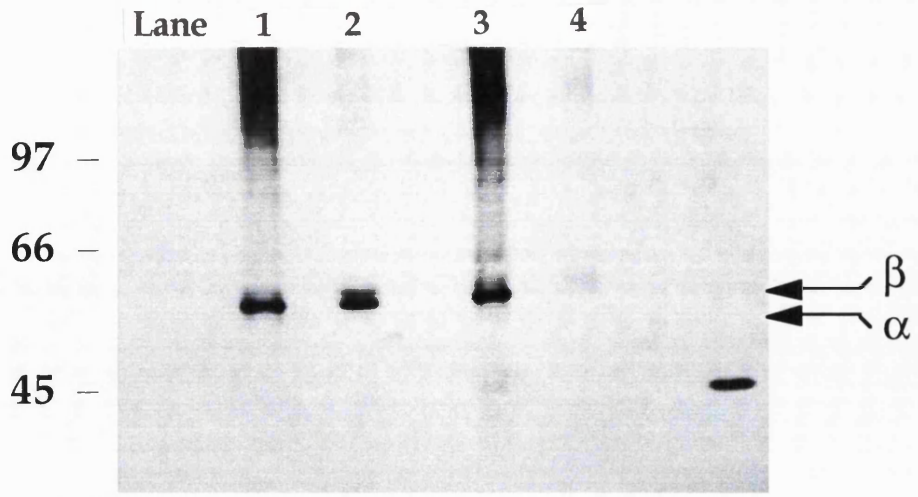


Fig 11 B. The virally expressed proteins can be solubilized by detergents. BHK cells were co - infected by α1 (9E10) and β2 (9E10) virus particles and after 20 hours the cells were lysed for thirty minutes on ice using Deoxycholic Acid or 2% CHAPS or 0.5%. The lysate was then spun at 100,000 rpm for 15 minutes and the supernatant and pellet seperated for each detergent. Reducing sample buffer was then added to the two different fractions. The samples were then resolved by SDS PAGE and western blotting using the 9E10 monoclonal antibody. Lane 1 is the pellet fraction and lane 2 is the supernatant fraction of the deoxycholic acid solubilisation. Lane 3 is the pellet fraction and lane 4 is the supernatant fraction of the CHAPS solubilisation. Lane 5 is non infected BHK cell whole cell lysate as a control for the 9E10 western blotting. The migration of molecular weight standards is indicated in KiloDaltons.

3.1.5 The Semliki expression system can express recombinant protein in primary cultures of neurones and organotypic brain slices.

It had been reported that this virus was capable of infecting primary neurones. Betagalactosidase had been shown to be expressed in hippocampal neurones using Semliki forest virus (Olkkonen et al 1993). More recently the immunoglobulin A receptor has been expressed in hippocampal neurones (De Hoop et al 1995). The amyloid precursor protein has also been expressed in hippocampal neurones using Semliki forest virus (Simons et al 1995). The capability of this expression system to produce recombinant protein in differentiated neurones was confirmed by infecting cultures of superior cervical ganglion neurones (SCG) with a Semliki virus that expressed the enzyme Betagalactosidase. This allowed the rapid detection of infected cells. Primary superior

cervical ganglion cultures from P14 rat pups were infected and at time points post infection the cultures were fixed and stained for the presence of Betgalactosidase. The cultures contained neurones and fibroblasts. The frequency of infected neurones and the total frequency of cellular infection, which included neurones and fibroblasts was estimated. Fig 12 demonstrates that the frequency of neurones infected over the time course averages at 50%. This figure also demonstrates that initially a high percentage of the cells in the culture are infected but this rapidly drops between the second and third day after infection. In contrast the frequency of neurones infected remains relatively constant. This discrepancy is due to the death of infected supporting fibroblast cells and the repopulation of the culture from fibroblasts which had not initially been infected.

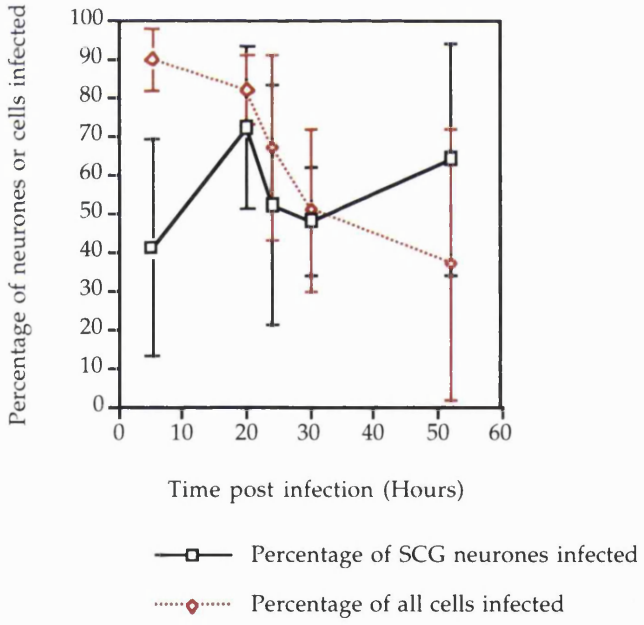


Fig 12 , SCG cultures of neurones were infected with the Semliki virus engineered to express the enzyme Betagalactosidase. The cultures were fixed at time points post infection and stained for the presence of the enzyme Betgalactosidase. The percentage of neurones expressing the enzyme at time points after infection was evaluated as well as the total percentage of cells expressing the enzyme . (n = 10 for each mean)

The ability to introduce recombinant proteins into complex arrays of neurones that were more representative of the neuronal array within the central nervous system of animals was an attractive objective. Organotypic brain slices of the hippocampus were carefully prepared by Mrs Yvonne Vallis. These were infected wih the Semliki forest virus engineered to produce Betagalctosidase (virus titre 10^8/ml) in a global fashion and by the application of virus in a local fashion via a 1μm patch pipette to the CA 3 region of the hippocampus. The production of Betagalactosidase is demonstrated by the production of a blue pigment. This can be seen in Fig 13 (A) and (B). The focal application of a Semliki virus producing the Green Fluorescent Protein (GFP) was also performed (virus titre 10^8/ml). The production of green fluorescent cells in the CA 3 region of the hippocampus can be seen in Fig 13 (C). The greatest intensity of signal corresponds to the cell bodies of the neurones. The focal application experiments were conducted by Dr Jane Haley. These results illustrate the capacity of this system to infect neurones in complex networks both in a global fashion and in a localised manner.

3.1.6 Investigation of the toxicity of the Semliki virus expression system upon primary neurones using recombinant SFV vectors expressing the Green Fluorescent Protein and Betagalactosidase .

The potential toxic side effects of using the Semliki expression system in neuronal cultures, was investigated by infecting neurones that produced the enzyme Betagalactosidase SCG neurones were infected by the virus and fixed at varying time points, up to 50 hours after infection. The neurones were fixed and stained for the presence of Betagalactosidase. Even at some 50 hours post infection SCG neurones were clearly still adherent to the substratum and retained good morphology, see Fig 14 (A). The nuclear integrity of the infected neurones was examined in order to determine if the infected neurones were undergoing apoptosis.

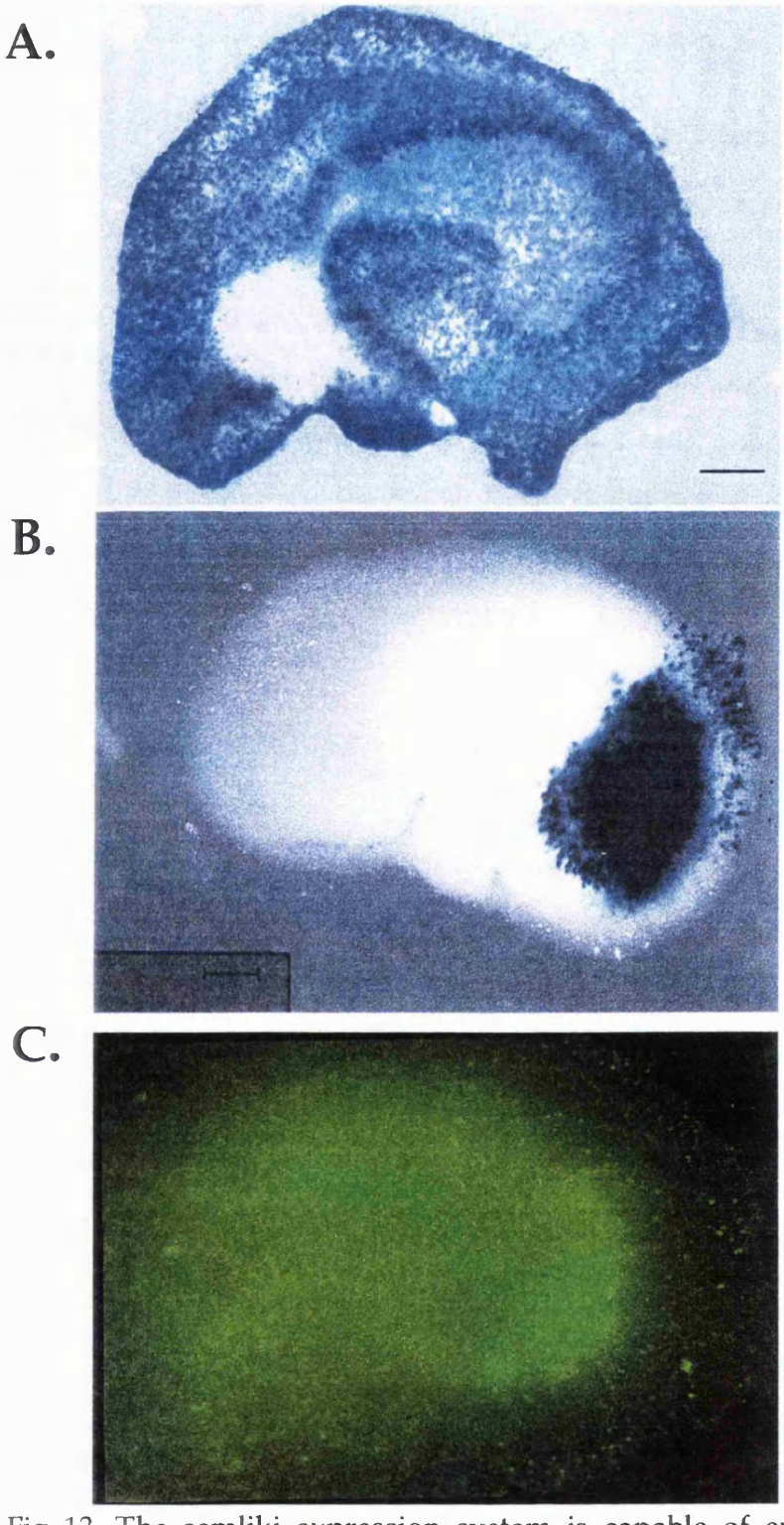


Fig 13. The semliki expression system is capable of expressing recombinant proteins in organotypic brain slices. Panel A. illustrates the global infection of a brain slice of the hippocampus by Semliki forest virus particles engineered to express the enzyme betagalactosidase. The blue stain indicates the presence of the enzyme. Panel B. illustrates the focal application of virus particles to the CA 3 region of the hippocampus via a micropipette. Panel C. is a similar experiment , using a virus engineered to express GFP. Scale bars represent 50μm.

This had been reported to occur in neuronal preparations that had been infected by the Sindbis virus, which is also an RNA virus and a member of the Alphaviridae (Griffen et al 1994a). Propidium iodide staining of cells has been used to determine the existence of apoptosis (Barres et al 1992 and Coles et al 1993). Propidium iodide staining of neurones virally expressing Betagalactosidase was performed at 17 hours post infection. In contrast to apoptotic cells, which have condensed chromatin resulting in a smaller denser nucleus the neurones expressing Betagalactosidase had a reticular meshwork of chromatin with no evidence of pyknosis, see Fig 14 (B).

In order to study the potential toxic effects of viral infection in more detail it was decided to make a virus that expressed a protein whose detection did not require the addition of exogenous substrates and could be detected in intact living cells. This would allow the determination of physiological characteristics such as the resting membrane potential of infected neurones to be determined. The protein chosen was the green fluorescent protein (GFP). The cDNA for the protein had been cloned from the jelly fish *Aquora*-Victoria (Prasher et al 1992). A mutated version of GFP was used which produced a brighter fluorecent signal. (Heim et al 1995)

Superior cervical ganglion neurones were infected by the GFP producing virus and subsequently at 24 and 48 hour time points the neurones expressing GFP as identified by green fluorescence, had their resting membrane potential recorded. The results of four seperate experiments are summarised in the graph of Fig 14 (C). The viral infection does not alter the resting potential of the neurones. The electrophysiology in these experiments were performed by Dr. Jane Wiley. Similar results were also independently reported by Professor Trevor Smart (Personal communication).

A.

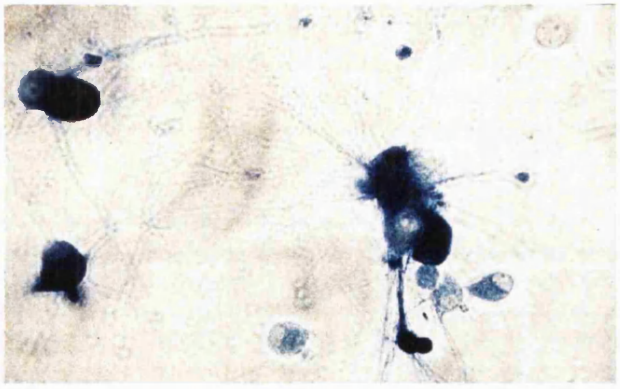


Fig 14 A. SCG neurones were infected by the betaglactosidase Semliki forest virus and stained for the presence of the enzyme 48 hours after infection, indicated by the blue colour.

B.

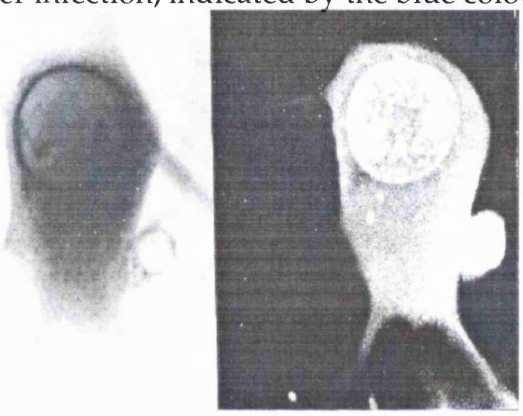


Fig 14 B. SCG neurones were infected by the betagalactosidase expressing virus and fixed 18 hours after infection. The cells were stained for the presence of the enzyme and with propidium iodide.The image on the left is a transmission image of an SCG expressing betagalactosidase, indicated by the dark pigmentation, on the right is a fluorescence image of the same cell illustrating the propidium iodide staining of the cell's nucleus.

C.

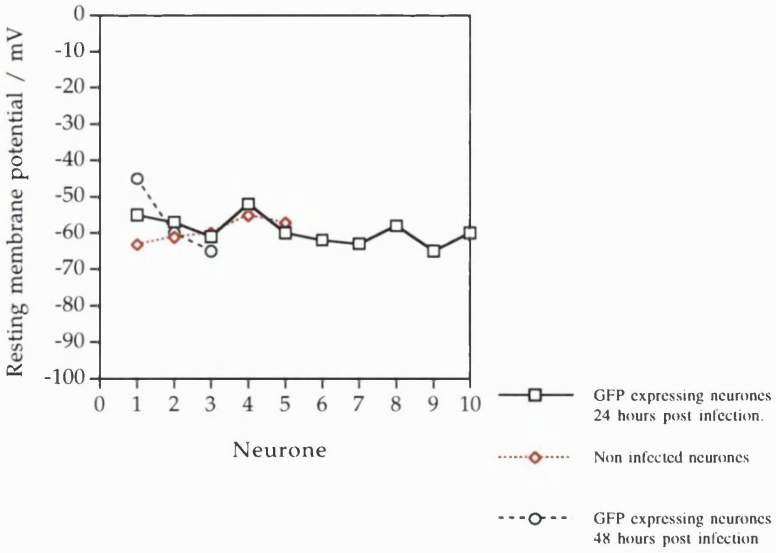


Fig 14 C. SCG neurones were infected by the GFP Semliki forest virus and at 24 to 48 hours post infection resting membane potentials recorded.

3.1.7 Two proteins can be made simultaneously by a vector containing a second subgenomic promoter.

The potential of utilising this viral vector to introduce a recombinant protein into neuronal cultures was attractive, however the frequency of expression was not 100%. It was felt necessary to try and incorporate a marker of infection, that could be detected in living cells. A viral construct was engineered that was designed to express both γ2 S (9E10) and Green Fluorescent Protein as described in material and methods. BHK cells were infected by virus produced from this construct. The expression of the GFP was week and to further analyse its expression a rabbit polyclonal antibody raised against GFP was used to detect its expression. As can be seen from Fig 15 (C) and (D), it was possible to detect GFP in most cells but less so for the γ2 S (9E10). Expression of both proteins was detected. Unfortunately high levels of GFP corresponded to low levels of γ2 S (9E10) and vice versa see Fig15 (C) and (D). This could be due to the preferential production of one subgeonomic RNA message in preference to the other. In order to try and increase the GFP signal a second subgeonomic promoter was engineered into the construct The results produced by this construct are shown in Fig 15 (A) and (B). As can be seen by the green and red signals two proteins were produced, the GFP, probably being produced with greater abundance compared to the bicistronic construct. Unfortunately, the GFP signal could only be detected using the rabbit polyclonal to GFP. It is interesting that when double constructs such as those described above are used to make two proteins simultaneously the efficiency of producing the second protein, in this case GFP is less than that produced when a dedicated GFP virus and dedicated α1 (9E10) and β2 (9E10) virus particles are used together see **Fig 18.** In this latter triple infection no GFP antibody was required to identify the GFP.

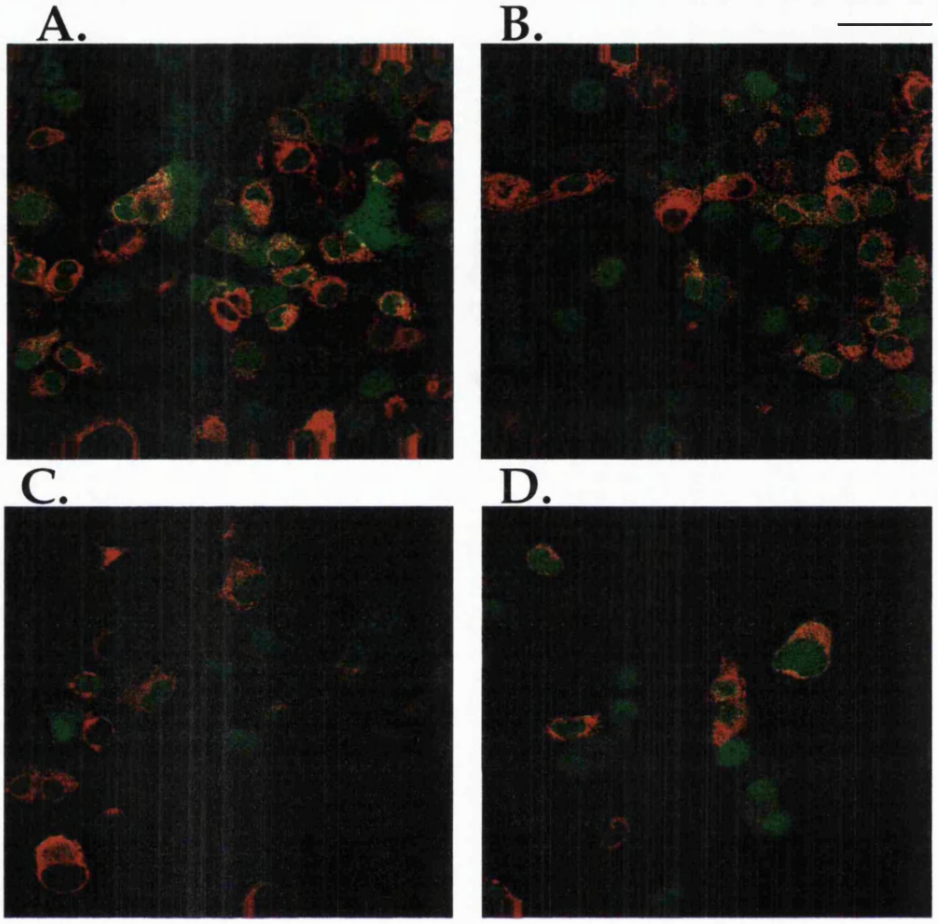


Fig 15. Two proteins can be made from a viral construct containing two cDNA's. Panel A and B illustrate the infection of BHK cells by a virus which was engineered to contain two subgeonomic promoters driving the production of two proteins,γ2 S (9E10), and GFP. The GFP was detected using immunofluorescence on permeabilised cells using a rabbit polyclonal to GFP and a fluorescien anti rabbit secondary.The γ2 S (9E10) was detected using 9E10 and a rhodamine anti mouse secondary. Panels C and D illustrate the infection of BHK cells by a virus engineered to produce γ2 S (9E10) and GFP using only one subgeonomic promoter, (a bicistronic format). The scale bar represents 50μm.

3.1.8 Proof of the capability of this expression system to support double and triple infection.

The ability for this transient expression system to express more than one recombinant protein in a single cell was unknown. In order to determine whether this was possible BHK cells were infected by binding medium containing α1 (9E10) and β2 (9E10) virus. Double infection was then assayed by the ability of the (9E10) tag to be detectable in intact cells. Previous work had determined that α1 (9E10) and β2 (9E10) subunits need to be co-expressed in an individual cell in order for either protein to be detectable at the plasma membrane. If either protein was expressed on its own it was retained within the endoplasmic reticulum of the cell (Connolly et al 1996a). To test whether the viral expression system obeyed these rules BHK cells were infected either by the α1 (9E10) or the β2 (9E10) virus. The cells were fixed after 18 hours of infection and subjected to immunofluorescence in the presence or absence of detergent. As can be observed in Fig 16 (A) and (B) high levels of protein could be detected immunohistochemicaly and the pattern of fluorescence was consistent with an endoplasmic reticulum (ER) morphology. In contrast when the fluorescence was conducted in the absence of detergent no signal could be observed for the β2 (9E10) or the α1 (9E10), as illustrated in Fig 16 (C). In keeping with the observations made using conventional transient transfections of HEK 293 cells with eukaryotic expression plasmids, (Connolly et al 1996a) 9E10 antigenicity was detected in the absence of detergent as seen in Fig 16 (D), when BHK cells were co-infected by α1 (9E10) and β2 (9E10) virus particles. In order to demonstrate the colocalisation of the α1 (9E10) and β2 (9E10) proteins on the surface of the BHK cells double immunofluorescence was performed using a rabbit

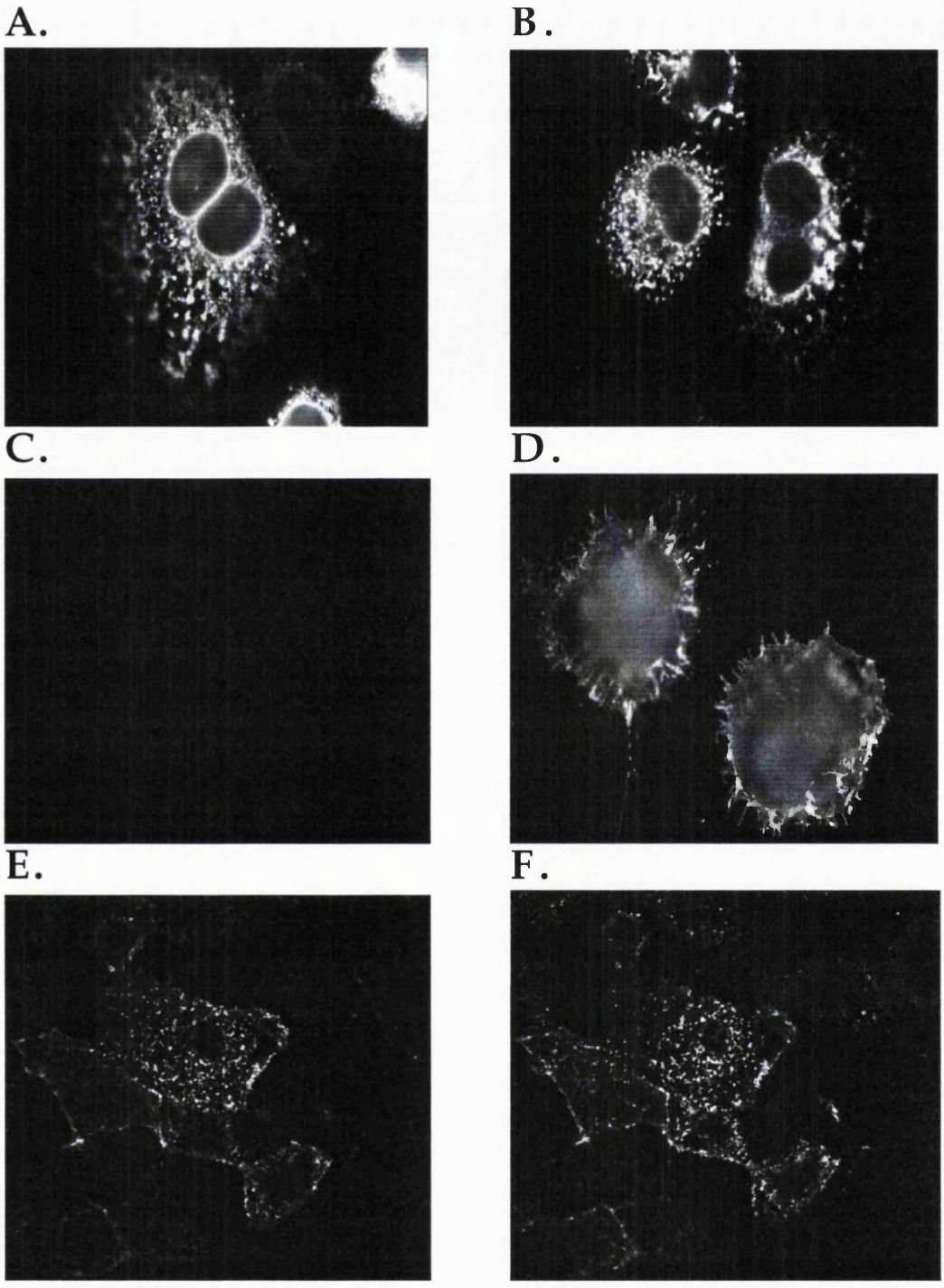


Fig 16. Surface expression of $GABA_A$ receptor subunits in BHK cells. BHK cells were infected with either single α1 (9E10) or β2 (9E10) virus or coinfected with both and expression determined by immunofluorecence with (+) and without (-) permeabilisation at 20 hours post infection. Images were collected by confocal microscopy. A; α1 (9E10), detected by 9E10 monoclonal (+), B; β 2 (9E10), detected by 9E10 monoclonal (+), C; α 1 (9E10) , 9E10 monoclonal (-), D; α1 (9E10) β2 (9E10) coinfection,, 9E10 monoclonal (-). Double fluorescence using a polyclonal antibody to α1 and

a monoclonal antibody to the β2 subunit (BD17) of co infected cells was also performed. Panel E coresponds to the fluorescein signal for the α1 subunit and panel F coresponds to the rhodamine signal for the β2 subunit. Scale bar represents 50μm.

polyclonal α1 C terminal specific antibody (Pollard et al 1993) and β2 specific mouse monoclonal antibody BD 17 (Schoch et al 1985). Both antibodies detected extracellular domains of the proteins. As can be seen from Fig 16 (E) and (F) there is good colocalisation of the two proteins.

When BHK cells are co-infected by α1 (9E10) and β2 (9E10)virus particles the two subunits can be demonstrated to oligomerise by co-precipitation. BHK cells were infected by both virus species at a multiplicity of infection of 10. At four hours post infection the cells were labelled with 300μCi/ml ^{35}S methionine for one hour and after one hour of labelling the cells were lysed in non denaturing lysis buffer. The sample was divided in to two and one half immunoprecipitated with 10μg/ml of rabbit IgG and the other half of the sample immunoprecipitated with 10μg/ml of polyclonal β2 antibody (Benke et al 1994).

The immuoprecipitate was then subjected to SDS PAGE and the gel fixed, dried and exposed to film. As can be seen in Fig 17 the β2 specific immunoprecipitation not only precipitates the β2 subunit but a second band is also visible. Neither of the two signals are present in the control lane. Also shown in Fig 17, for comparative purposes, are results from a separate experiment where cells have only been infected by either the α1 (9E10) or the β2 (9E10) virus particles, ^{35}S methionine labelled and immunoprecipitated with the monoclonal antibody (9E10). The second band immunoprecipitated with the β2 specific antibody corresponds to the migration of the α1 (9E10) protein. The oligomerisation of α1 (9E10) and β2 (9E10) subunits has also been demonstrated imunopprecipitation

studies performed on transient transfections of HEK cells (Connolly et al 1996a).

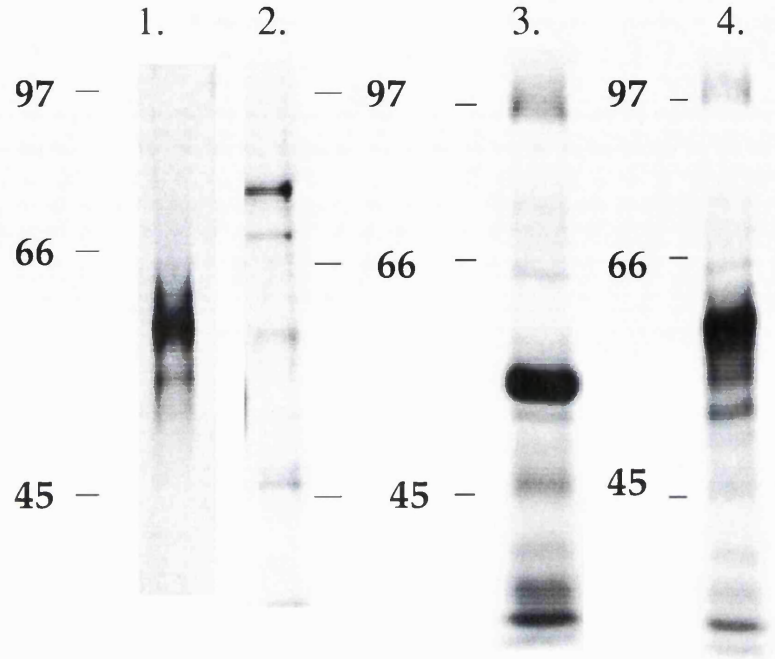


Fig 17. BHK cells were co-infected with α1 (9E10) and β2 (9E10) virus particles. The cells were labelled with ^{35}S methionine 4 hours after infection for a period of 2 hours. The cells were then lysed in non denaturing lysis buffer. The β2 subunit was immunoprecipitated with a rabbit polyclonal antibody. Lane 1 illustrates the co-immunoprecipitation of the α1 (9E10) protein. Lane 2 is a sham precipitation with rabbit IgG. Lane 3 and 4 corresponds to 9E10 immunoprecipitations from α1 (9E10) or β2 (9E10) infected cells to demonstrate the sizes of the two proteins.

In conclusion this system is capable of double expression, but could it support triple expression? By co- infecting BHK cells with α1 (9E10), β2 (9E10) and GFP virus one could find cells expressing GFP, α1 (9E10) and β2 (9E10) simultaneously. Such intact cells have a green interior, since GFP is a cytosolic protein and a red border, since the (9E10) epitope is present at the plasma membrane only when α1 (9E10) and β2 (9E10) are both present. This can be observed in Fig 18 (A) and (B). It was concluded that triple infection was indeed possible. However, when triple infections of varying ratios of α1 (9E10), β2 (9E10) and γ2 L FLAG were performed it was not possible to detect Flag antigenicity in intact cells. This contrasted with the cell biology demonstrated for triple transient

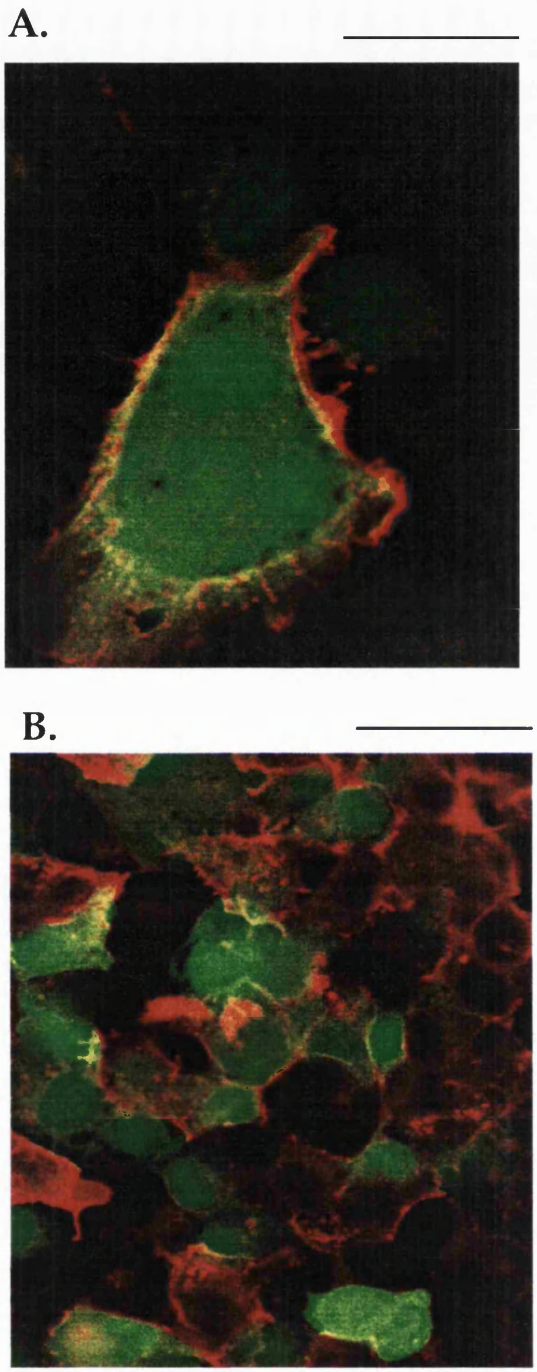


Fig 18. Triple infection of BHK cells with α1 (9E10), β2 (9E10) and GFP virus particles. BHK cells were infected by a mixture of virus particles engineered to separately express the GFP, α1 (9E10) and β2 (9E10) proteins. Immunofluorescence was performed on intact cells. The GFP signal was detected directly using fluorescein filters, whilst the 9E10 tagged proteins were detected using the 9E10 monoclonal and a secondary anti mouse rhodamine conjugated antibody. Panel A and panel B are confocal pictures of intact BHK infected by the mixture of virus particles, panel is at a higher magnification. Upper scale bar represents 25μm lower scale bar represents 50 μm.

transfections of HEK 293 cells with α1 (9E10), β2 (9E10)and γ2 L FLAG cDNA's where FLAG antigenicity could be observed in the absence of detergent (Connolly et al 1996a).

3.1.9 nA chloride currents can be produced when GABA is applied to Baby Hamster Kidney cells which have been co-infected by α1 (9E10) and β2 (9E10) virus particles.

BHK cells were co-infected with α1 (9E10) and β2 (9E10) virus at a multiplicity of infection of 10 to 1. BHK cells were also individually infected with α1 (9E10) and β2 (9E10) virus as controls. The cells were then incubated overnight before electrophysiological analysis was performed. Whole cell recording was performed by Professor Trevor Smart on cells which were co-infected or individually infected with either the α1 (9E10) or β2 (9E10) virus particles., As can be seen from this Fig 19 (A), GABA gated currents could only be recorded from cells that were infected by both species of virus. The dose response curve, shown in Fig 19 (B), indicated that the receptors expressed by this expression system had an EC 50 for GABA of 1.5μM. this compares well with previous data (Macdonald and Olsen 1994). The dose response curve also demonstrates that a maximal whole cell current of 3nA could be generated from cells infected by both virus particles. This is significantly higher (10x) than that produced by conventional transfection of α1 (9E10) and β2 (9E10) subunits (Connolly et al 1996a). The currents were inhibited by 10μM Zn^{2+} but were not enhanced by flunitrazepam. This is characteristic of cells which do not contain a γ subunit (Draguhn et al 1990, Smart et al 1991, Verdoorn et al 1990). Cells infected with either the α1 (9E10) or β2 (9E10) virus particles were not sensitive to GABA (1mM) or pentobarbitione (2mM). The latter was used since homomeric β1 and β3 ion channels are sensitive to pentobarbitone in preference to GABA (Sigel et al 1989, Krishek et al 1996 and Connolly et al 1996b).

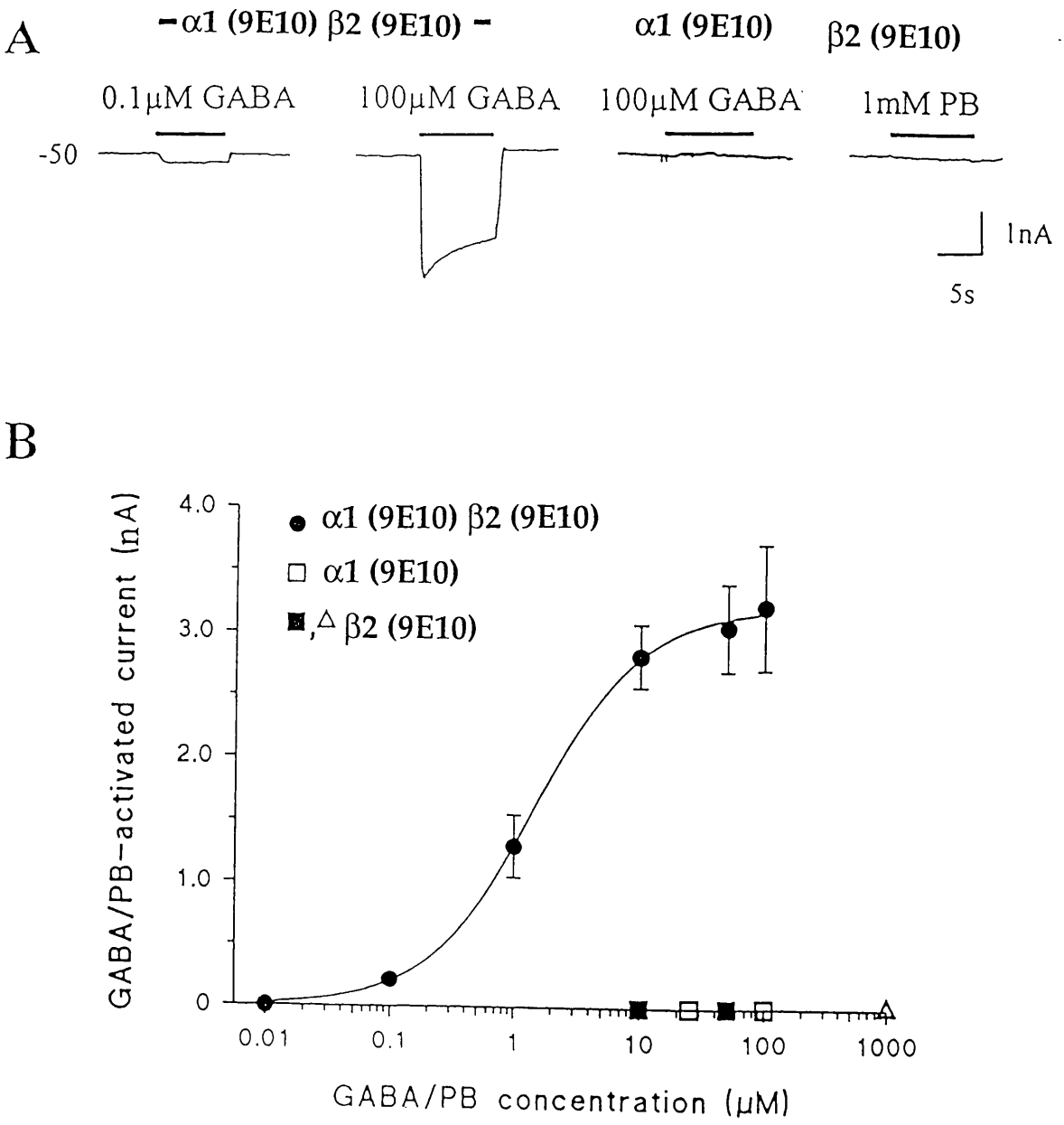


Fig 19. Co-infection of α1 (9E10) and β2 (9E10) virus particles can give rise to GABA gated chloride currents. A. Whole cell recordings from BHK cells infected with Semliki Forest viruses expressing the α1 (9E10) and β2 (9E10) subunits in combination or individually. Detection of homomeric β2 (9E10) channels was assayed by the application of pentobarbital (PB.) The duration of the ligand application is indicated by the solid line. The holding potential was -50mV.

B. GABA and PB equilibrium concentration responses for BHK cells expressing α1 (9E10) and β2 9E10 or single subunits. Data was fitted by $I/Imax=1/(1+((A) / EC_{50})^n)$ where I and I max represent GABA activated and maximally activated current, n is the Hill coefficient and A the GABA concentration . EC 50 is 1.47+/- 0.12 μM, n is 1.0 +/- 0.08. Points are mean +/- s.e.m. This experiment was conducted by Professor T. Smart.

It is clear that the viral system can produce GABA gated chloride ion channels when double infection of these cells occurs but not when cells are singly infected by α1 (9E10) or β2 (9E10) virus particles. Pentobarbital was also used since it has been reported that homomeric β receptors respond to high concentrations of barbiturates. (Krishek et al 1996, Connolly et al 1996b)

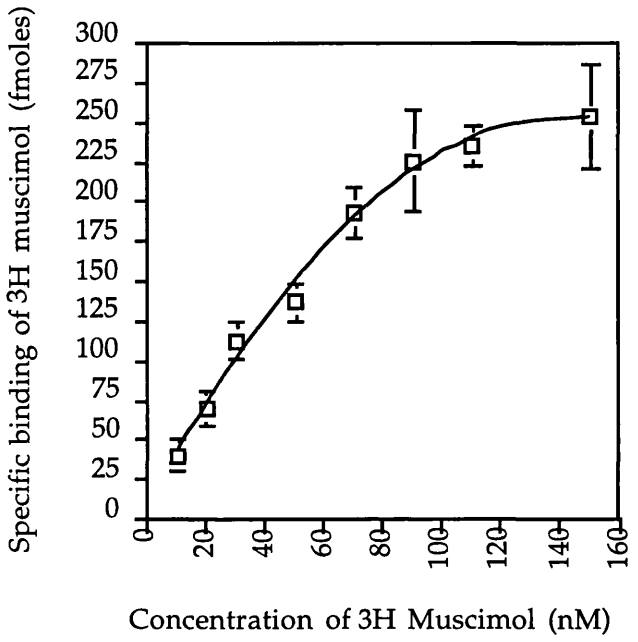
3.1.10 Specific and saturating ^{3}H muscimol binding can be detected in intact BHK cells co- infected with α1 and β2 (9E10) virus particles.

In order to demonstrate the existence of cell surface $GABA_A$ receptors whole cell ligand binding was performed. Four million BHK cells were co-infected by α1 (9E10) and β2 (9E10) virus particles at a multiplicity of infection of 10 to 1. The cells were incubated overnight and then harvested in phosphate buffered saline. 100,000 cells were used per assay, each point was performed in triplicate. The protein content of a representative sample of cells, used for each assay, was measured by a Bradford protein assay kit from Biorad. This was estimated to be 25μg. Non specific binding for each concentration of the ^{3}H muscimol was determined by the addition of 1mM cold GABA. Concentrations of 10 - 150nM ^{3}H muscimol were used to perform the binding assay. The samples were incubated at four degrees for one hour and then harvested by fast filtration using a Brandell cell harvester, onto GF II b filters. The filters were then inserted into scintillation vials and 5mls of Emulsifier Safe (Packard Bell) scintillant added. The vials were then counted in a scintillation counter immediately and after 24 hours. Estimation of total counts for each data point was determined by the addition of the same amount of ^{3}H muscimol used for each of the concentrations directly to a scintillation vial. The means and the standard deviation of each of the data points were calculated and graphed see Fig 20 (A) and (B). Scatchard analysis of this data produced high a Kd of 100nM, with a Bmax of 450

fmoles of ^{3}H muscimol per assay. The value for the dissociation constant is similar to previously determined values for recombinant and endogenous GABA $_{A}$ receptors. Each assay contained 25µg of protein, corresponding to 100,000 cells. Thus approximately 16 pmoles of ^{3}H muscimol bound per mg of protein or 4.5 pmoles/10^{6} cells. This produces a receptor density at least 2 fold higher than the Baculovirus expression system (Pregenzer at al 1993). and is 40 fold higher that produced by conventional transfection methods using a CMV promoter (Moss et al 1991).

Saturation binding studies performed on endogenous $GABA_A$ receptors using ^{3}H muscimol have determined that this ligand binds to high affinity (10nM) and low affinity sites (500nM), (Agey and Dunn 1989). Interestingly ^{3}H muscimol saturation binding studies on freshly isolated brain membranes, which had not been freeze thawed, performed at $22^{0}C$ gave a Kd of 75-300nM (Yang and Olsen 1987). ^{3}H muscimol binding studies performed on membranes isolated from HEK cells expressing α1 and β2 subunits gave Kd's of 20nM, (Moss et al 1991) and 13nM (Pritchett et al 1988). Using a Baculovirus expression system SF 9 cells were infected by α1 and β2 virions. Membranes were isolated from these cells and ^{3}H muscimol binding studies performed. The Kd for ^{3}H muscimol as estimated by this study ranged from 12 to 19nM (Pregenzer at al 1993). Saturation studies performed on benzodiazepine affinity purified $GABA_A$ receptors generate Kd' s for ^{3}H muscimol which range from 14nM (Kirkness and Turner 1986) to 33nM with a lower affinity site (Kd 150nM) observed when the detergent CHAPS was substituted for Triton X 100 (Sigel and Barnard 1984).

A.



B.

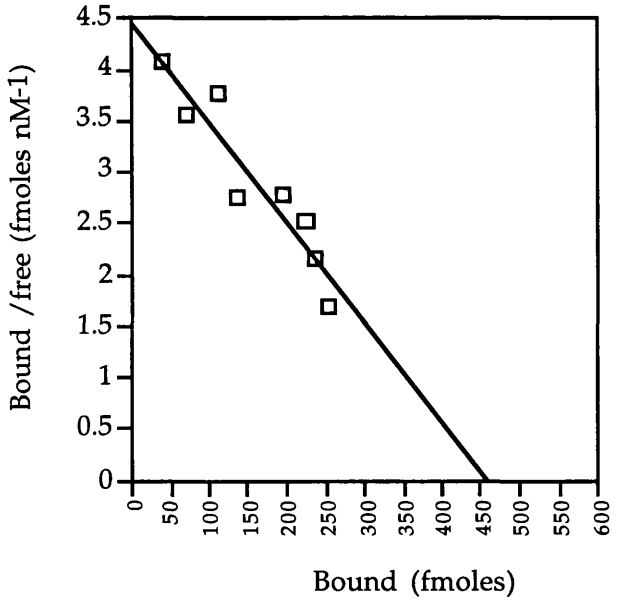


Fig 20. Specific binding of ^{3}H muscimol to BHK cells co-infected by α1 (9E10) and β2 (9E10) virus particles (not to saturation) Panel A. BHK cells were co-infected with α1 (9E10) and β2 (9E10) virus particles. After 20 hours the cells were isolated and used to perform a whole cell ligand binding assay. The points are means +/- two standard deviations. Panel B. Illustrates a Scatchard plot of the data.

3.1.11 Discussion.

In conclusion this chapter describes the characterisation of recombinant Semliki forest virus particles engineered to express the $GABA_A$ subunits α1 (9E10), β2 (9E10), γ2 S (9E10) and γ2 L Flag. The production of the epitope tagged proteins by this expression system has been demonstrated both biochemically and immunocytochemically. The marked suppression of host protein synthesis occurring after only three hours after infection by this expression system has been illustrated. The capability of this system to support double and triple infection has also been illustrated as has the capacity for a viral construct containing two subgeonomic promoters to produce two different proteins. The ability of this viral system of expression to produce recombinant protein in primary cultures of neurones has been clearly shown and the limited toxicity upon such neurones at 24 hours post infection defined by electrophysiological and immunocytochemical assays.

The novel application of the Semliki expression system to the expression of heteroligomeric proteins required the **repetition** of previous observations made using the transient expression of eukaryotic expression plasmids. The viral expression system reproduced the cell biology defined by these earlier experiments.

It is remarkable that even when the entire biosynthetic machinery of a BHK cell has been dedicated to the production of an α1 (9E10) or β2 (9E10) subunits there is no leakage of these subunits on to the plasmamembrane. These results illustrate the significant retention capacity and quality control capability of the endoplasmic reticulum. The observation that the α1 (9E10) and β2 (9E10) subunits bind chaperone proteins such as BIP and Calnexin, (Connolly et al 1996a) the latter actively being retained within the ER, (Rajagopalan et al 1994) may explain why there is little leakage of unassembled subunits into the

secretory pathway. The chaperone protein Calnexin has been demonstrated to be associated with individual unassembled α ,β and δ subunits of the nACh R but not associated with dimers of α and δ subunits (Keller et al 1996).

Finally the ability of double infections of α1 (9E10) and β2 (9E10) virus to produce a $GABA_A$ ligand gated ion channel in BHK cells has been demonstrated electophysiologiacally and pharmacologically. GABA gated whole cell currents were only produced when both α1 (9E10) and β2 (9E10) virus particles were used to co-infect BHK cells, no currents were detectable on single infection. This was consistent with the cell biology, no 9E10 antigenicity was observed on the plasma membrane of BHK cells when single infections of α1 (9E10) or β2 (9E10) virus were performed. The large size of the whole cell recordings, 3nA, ten times that of transient transfections by the PRK 5 eukaryotic expression vector (Connolly et al 1996a) reflected the high B max of ^3H muscimol binding, 16pMoles/mg protein, in co-infected cells. The whole cell binding data and electrophysiology support the conclusion that co-infection of cells with α1 (9E10) and β2 (9E10) virus particles gives rise to functional receptors at the cell surface. The whole cell binding studies gave a Kd of 100nM for ^3H muscimol. This contrasted with previous studies which reported the Kd to range from 2-33nM (Kirkness and Turner 1986, Pritchett et al 1988 , Moss et al 1991, Pregenzer at al 1993). Interestingly these studies were performed often using freeze thawed membranes or membranes which had been exposed to detergents rather than whole cell binding. Such unphysiological conditions may have changed the membrane lipid environment and altered the interactions between ion channel subunits. Interestingly these high affinity binding sites do not agree with the median effective doses of GABA measured on whole cells which are in the μM range (Yang and Olsen 1987, Edgar and Schwartz

1993, Rabow et al 1995). The higher Kd for ^{3}H muscimol identified in this study may therefore be more representative of the native receptor.

The inability to demonstrate the appropriate trafficking of the γ2 L FLAG subunit in BHK cells exposed to all three α1 (9E10), β2 (9E10) and γ2 L FLAG virus particles precluded ligand binding and electrophysiological analysis. The inability for the γ2 L FLAG subunit to be appropriately targeted in such triple infections was concluded to be due to an inability to produce efficient triple expression using the Semliki viral expression system. The lack of a specific antibody to the γ2 subunit also made the optimisation of the triple infection difficult to perform.

Chapter 3.2

Chapter 3.2. The assembly of $GABA_A$ receptors composed of α1 and β2 subunits in both cultured neurones and fibroblasts.

Introduction.

The nature of the assembly process which gives rise to a ligand gated ion channel has been most extensively studied for the nAChR of the vertebrate neuromuscular junction and the analogous nAChR present in the electric organ of the marine ray *Torpedo*. The development of stable cell lines expressing *Torpedo* and mouse AChR subunits promoted an increased understanding of the mechanism of assembly. The efficiency of receptor assembly was demonstrated to be temperature dependant. Lowering the temperature from 37°C to 20°C increased the assembly efficiencies for both the *Torpedo* and mouse nAChR. At 37°C the mouse nAChR has been demonstrated to assemble with an efficiency of approximately 30% in muscle cells (Ross et al 1991). This efficiency was consistent with the observation that only 30% of α subunits assemble into functional receptors in BC3H-1 muscle cells (Merlie and Lindstrom 1983).

In comparison the *Torpedo* nAChR assembles with an efficiency of 2-3 % at 26°C which rises to 20-30 % at 20°C. This was also consistent with observations on the low levels of assembly competent α subunits (3% of the total) as judged by high affinity α bungarotoxin binding. The levels of the assembly competent α subunit could be raised nearly 2 fold by incubating the stable α expressing cell line at 20°C. This increase in assembly competent material in combination with the other four subunits of the receptor was argued to be sufficient to raise the efficiency tenfold (Paulson et al 1991). The reason for the assembly process to be so temperature dependant was not commented on. The lower temperature

could promote the correct folding of the protein and or reduce the rate of intermediate degradation.

It was unknown whether the inefficient nature of the nAChR is a characteristic of the assembly of all ligand ion channels. In order to address this, the assembly of a $GABA_A$ receptor composed of α1 (9E10) and β2 (9E10) subunits was analysed using the Semliki virus expression system.

The choice of a heteroligomer composed of α1 (9E10) and β2 (9E10) subunits was made because neither of these subunits is capable of forming homomeric channels (Connolly et al 1996a Krishek et al 1994, Angelotti and Macdonald 1993, Sigel et al 1990). Secondly a receptor composed from α and β subunits is the most simple generally recognised $GABA_A$ receptor (Macdonald and Olsen 1994).

The Semliki expression system has been demonstrated to form functional $GABA_A$ receptors when BHK cells are co-infected with α1 (9E10) and β2 (9E10) virus particles. In order to analyse subunit oligomerisation a combination of sucrose gradient sedimentation and subunit immunoprecipitaion of radioactively labelled proteins was performed. Sucrose gradient sedimentation of virally expressed subunits was used to demonstrate subunit oligomerisation. This was then combined with pulse chase labelling and immunoprecipitaion across the sucrose gradients. The labelled receptor subunits at different locations within the gradients were then quantified and the efficiency of assembly determined. The fate of unassembled subunits was also examined using such pulse chase techniques.

The quaternary structure of $GABA_A$ receptors composed of α, β and γ subunits has been investigated previously and such studies have supported a pentameric organisation to the heteroligomer (Chang et al , Tretter et al 1997,Hadingham et al 1992, Mamalaki et al 1989 and Nayeem

et al 1994). The stoichiometry of receptors composed only of α1 and β2 subunits was unknown. The Semliki expression system in combination with ^{35}S methionine labelling, sucrose gradient centrifugation and immunoprecipitation techniques were used to determine the likely stoichiometry of such a receptor.

The physiological importance of receptors composed of α1 and β2 subunits is unknown. The existence of Zn^{2+} sensitive GABA gated chloride channels has been well characterised in neuronal preparations isolated form the central nervous system (Draghun et al 1990, Smart et al 1991). $GABA_A$ receptors composed only of α1 and β2 subunits are sensitive to Zn^{2+} (Verdoon et al 1990, Draguhn et al 1990, Smart et al 1991, and Angelotti et al 1993). The low single channel conductance of the α1β2 receptors, (Moss et al 1991 and Angelotti et al 1993) is also similar to some populations of neuronal $GABA_A$ receptors (Smart et al 1992 and Kaneda et al 1995).

It was unknown whether the assembly rules for $GABA_A$ receptor subunits defined in HEK transformed cell lines (Connolly et al 1996a) were applicable to the assembly of such subunits within primary neurones. In order to address this question single and double infections were performed on dissociated cultures of SCG neurones. Immunohistochemical analysis of such experiments allowed one to determine if there were similarities to the assembly of the α1 (9E10) and β2 (9E10) subunits in HEK and BHK cells.

3.2 Oligomerisation of the $GABA_A$ ligand gated ion channel studied using the Semliki expression system in tissue culture and primary neurones.

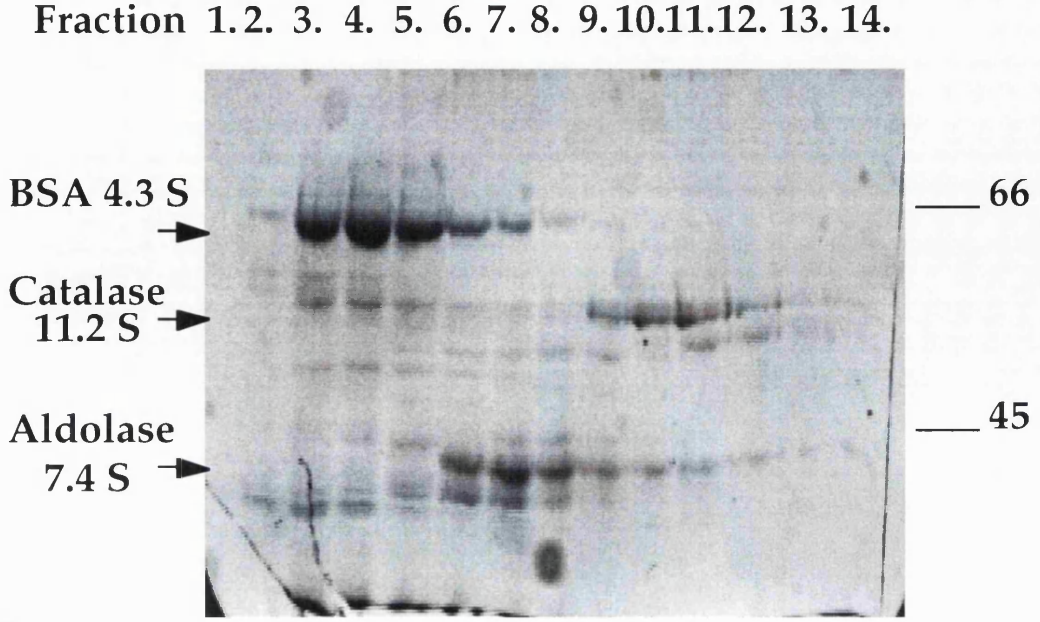
Little biochemical evidence exists illustrating the oligomerisation of $GABA_A$ subunits and the efficiency with which they are formed. The majority of this information is dependant on co-immunoprecipitation

studies which show a biochemical association between subunits (Connolly et al 1996a). The assembly of subunits as demonstrated by changes in the sedimentation coefficient of the $GABA_A$ subunits would be strong bio-physical evidence that heteroligomerisation is occurring, This has been elegantly demonstrated for the nAChR but not for the $GABA_A$ receptor. Changes in the sedimentation coefficients of $GABA_A$ receptor subunits would be expected to occur on the co-expression of α and β subunits.

3.2.1 Calibration of sucrose gradients.

The sucrose gradients were calibrated by the sedimentation of a mixture of proteins which not only had defined sedimentaion values but also could be resolved by SDS PAGE. This necessitated each protein to made of oligomers which had different molecular weights. The proteins chosen, were bovine serum albumen (4.3S, 68 KDa), aldolase (160KDa 7.4S, monomer 40KDa) and catalase (230KDa 11.2S, monomer 58KDa). A 1mg/ml solution of these proteins was sedimented on a 5-20% sucrose gradient using identical settings and solutions as were used to sediment the receptor subunits. The sucrose gradient was fractionated into fourteen 350µl fractions. A portion of each fraction was then run on an 8% PAGE gel. The gel was fixed and stained with Comassie blue. The fractions containing the highest intensity of staining for each protein were then determined and a calibration curve graphed. Fig 21 illustrates a typical Comassie stained gel and the calibration curve. The standard gradient was run three times.

A.



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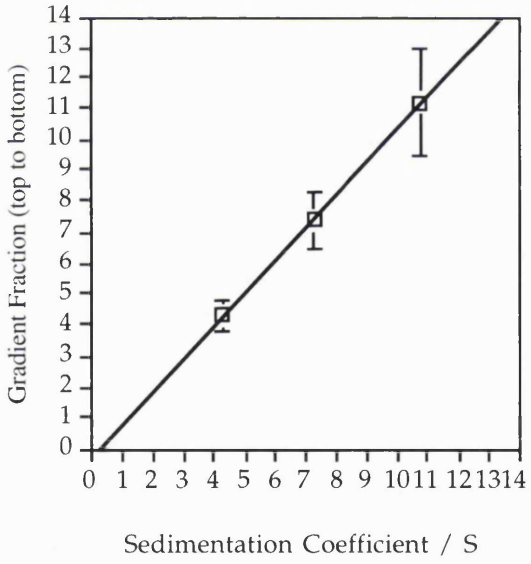


Fig 21. Panel A illustrates a 5-20% sucrose gradient analysis of a 1mg/ml solution of a mixture of protein standards, BSA, Aldolase and Catalase. Each protein has a known sedimentation, BSA 4.3S, Aldolase 7.4S, and Catalse 11.2S. The gradient was fractionated and each fraction resolved by SDS PAGE. The gel was fixed and then stained with comassie blue. The location of the proteins was then noted by the migration of their subunits. BSA a monomer runs at 68KDa, Aldolase a tetramer has subunits which are 40KDa in size and Catalase a tetramer has subunits which are 58KDa in size. Molecular weight standards are KDa. Panel B. is a calibration curve produced from the results of three standard gradients, points are means +/-2SD.

3.2.2 Oligomerisation of α1 and β2 subunits as defined by changes in sedimentation coefficients.

BHK cells were infected either with the α1 (9E10) or co- infected with the α1 (9E10) and β2 (9E10) virus particles. The cells were lysed 16 hours after being infected and the lysate layered onto a 5-20% linear sucrose gradient.The subunits were sedimented on the gradient by centrifugation and the position of the α1 (9E10) subunit determined by western blotting across the gradients. Two blots were performed one using the (9E10) monoclonal Fig 22 (A) for the single infection and a second using a polyclonal antibody to the α1 subunit for the double infection , Fig 22 (B). It is clear that there is a marked shift of the α1 (9E10) subunit from 5S to 9S on co-expression of the β2 (9E10) subunit. This shift in oligomerisation is indicative of subunit oligomerisation. A reciprocal experiment, western blotting with a β2 specific antibody failed to produce a clear signal. To demonstrate that the β2 (9E10) subunit also changed its sedimentation pattern in an α1 (9E10) dependant fashion (9E10) immunoprecipitations were performed across sucrose gradients. The gradients had been loaded with lysates from BHK cells that had either been infected by β2 (9E10) virus alone or in combination with α1 (9E10) virus. The incorporation of radioactive methionine also enabled the quantification of immunoprecipitated proteins using a Biorad phosophorimager.

BHK cells were infected with either the α1 (9E10) or β2 (9E10) virus particles or co-infected with both virus particles. The cells were radiolabelled for one hour, four hours after the infection and subsequently lysed in non denaturing lysis buffer. The proteins within the lysates were separated by sucrose gradient sedimentation and their positions within the gradients determined by immunoprecipitation using the (9E10) monoclonal antibody. The gradients were fractionated

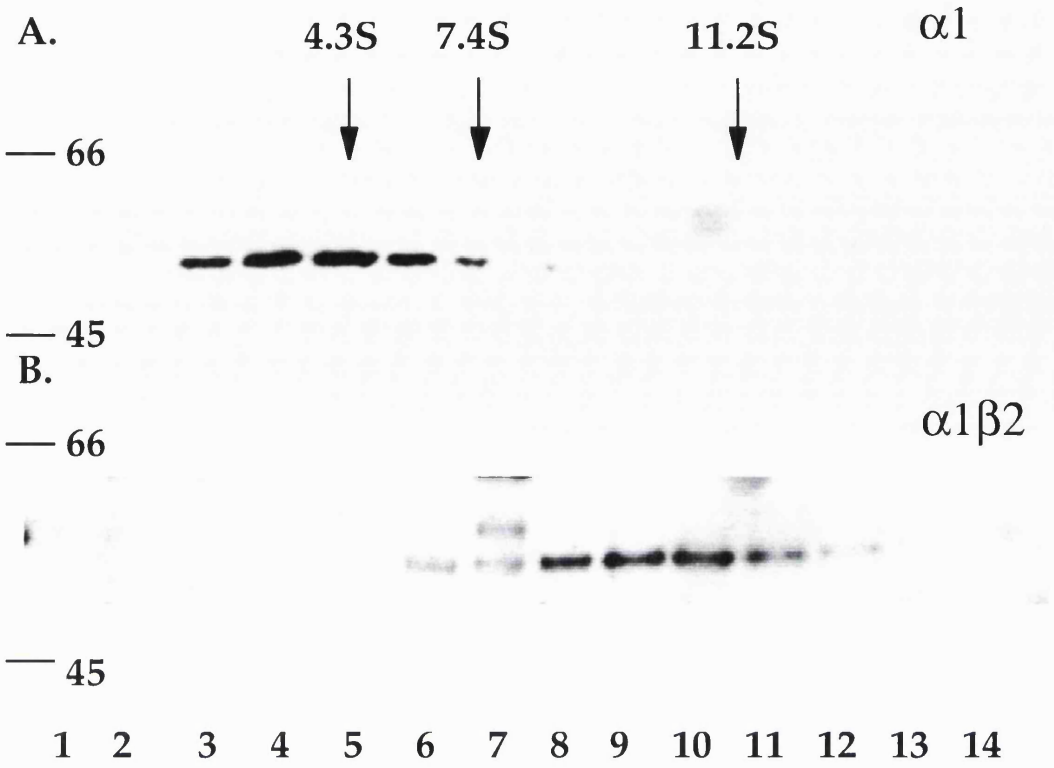


Fig 22. Differential sedimentation of the α1 (9E10) subunit dependant upon coexpression with the β2 (9E10) subunit. BHK cells were infected either with the α1 (9E10) virus al one (A.) or in combination with the β2 (9E10) (B.). The cells were lysed after being infected for 16 hours and the lysate subjected to sucrose gradient centrifugation. The gradient fractions were seperated by SDS PAGE and the location of the α1 subunit in the gradient identified by western blotting using the 9E10 monoclonal for the single infection, (A.) and a polyclonal antibody against the α1 subunit for the double infection (B.). Sedimentation coefficients for the alpha 1 myc subunit were determined with reference to proteins with known sedimentation characteristics. BSA, 4.3 S, Aldolase, 7.4 S and Catalase, 11.2S.

and each fraction immunoprecipitated with 10μg of 9E10 monoclonal antibody. The precipitates were subjected to SDS PAGE. The gel was fixed, dried and then exposed to a Biorad phosphorimager screen. The phosphorimager screen was then read and the signals for the individual subunits plotted as a function of gradient fraction. It is clear from Fig 23 that the α1 (9E10) and β2 (9E10) proteins have relatively broad sedimentation profiles, peaking at 5S when they are expressed

independently. The 5S sedimentation value for the α1 (9E10) subunit is consistent with results of the western blot illustrated in Fig 22. The raw data for each subunit is shown in Fig 23 (A) for comparison. In order to demonstrate a clear shift in the sedimentation coefficients of both the α1 (9E10) and β2 (9E10) proteins on co-infection a pulse chase experiment was performed. The results of such an experiment are illustrated in Figs 24 and 25, and are described in detail in the next section. It is clear that in the 6 hour and 20 hour chase points both the α1 (9E10) and β2 (9E10) subunits co-migrate in the gradient peaking with a sedimentation coefficient of 9S. Therefore, the β2 (9E10) subunit also shifts to a 9S location when co-expressed with an α1 (9E10) subunit.

3.2.3 The assembly of the $GABA_A$ receptor is an inefficient process but once formed the assembled receptor is stable compared with unassembled subunits.

The production and stability of the 9S complex formed on co-expression of α1 (9E10) and β2 (9E10) subunits was studied using a pulse chase protocol. BHK cells were co-infected with α1 (9E10) and β2 (9E10) virus particles with a multiplicity of infection of 10. The infected cells were labelled with ^{35}S methionine for 1 hour and chased for defined periods with excess cold methionine. Cell lysates were then prepared and subjected to sucrose density gradient fractionation. The α1 (9E10) and β2 (9E10) subunits were immunoprecipitated from the gradient fractions using 10 μg of affinity purified (9E10) monoclonal antibody and subjected to SDS-PAGE. The gel was fixed, dried and then exposed to a Biorad phosphorimager screen. The bands corresponding to the α1 (9E10) (52KDa) and β2 (9E10) (56KDa) were then quantified from each fraction. Fig 23 illustrates the results of the pulse chase analysis.

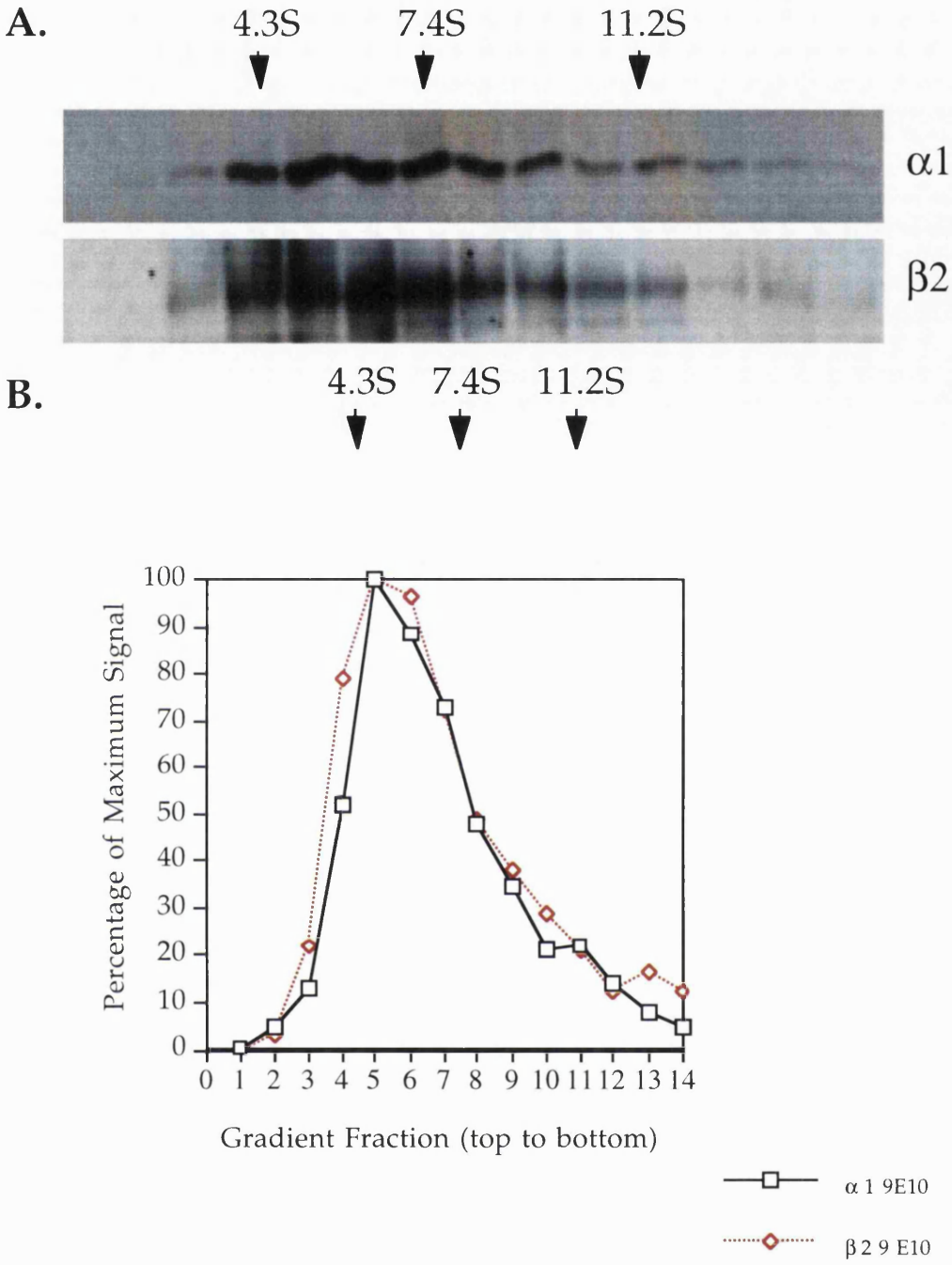


Fig 23. Immunoprecipitation of ^{35}S lab elled $GABA_A$ α1 (9E10) and β2 (9E10) subunits from sucrose gradients. BHK cells were infected with either the α1 (9E10) or the β2 (9E10) virus. The cells were labelled with 250μCi/ml of ^{35}S methionine for one hour at four hours post infection. The cells were then lysed in non denaturing lysis buffer and subjected to sucrose gradient centrifugation. The gradients were fractionated and each fraction immunoprecipitated with the monoclonal antibody 9E10. The precipitates were resolved by SDS PAGE and quantified by a Biorad phosphorimager. The signals were then graphed as a percentage of the maximum signal.

After 0 hours of chase the peak of activity for each protein sedimented at 5S. However as can be seen in Fig 24 and 25, a shoulder of activity can be seen extending to 9S . This was not seen when cells were infected with either the α1 (9E10) or β2 (9E10) virus particles, see Fig 23. In contrast after 6 hours of chase the majority of labelled subunits migrated at 9S Fig 24 and 25. A marked reduction in the total amount of receptor subunits that were initially labelled was also clearly evident. Approximately 33% of the initial counts present at 0 hours of chase remained after 6 hours of chase . After 20 hours of chase the 9S peak of activity was still clearly evident with 31% of the starting counts remaining see Fig 25 and Table 1. The 9S peak presumably forms quickly with a time scale less than that of the pulse (1 hour) but is difficult to resolve from unassembled receptors at this time. The 9S pool is stable compared with the unassembled receptors, with only a 10% loss in fourteen hours, see Fig 25 and Table 1. These results suggest that the assembly of subunits occurs quickly but inefficiently and that once formed the heteroligomer is stable in contrast to unassembled subunits which are degraded.

Table. 1. Assembly of a GABA A receptor composed from α1 (9E10) and β2 (9E10) subunits.

Chase	0 Hours	6 Hours	20 Hours
Subunit α1+ β2			
Fraction Counts 5	146393	21912	8481
Fraction Counts 8	59215	52487	47661

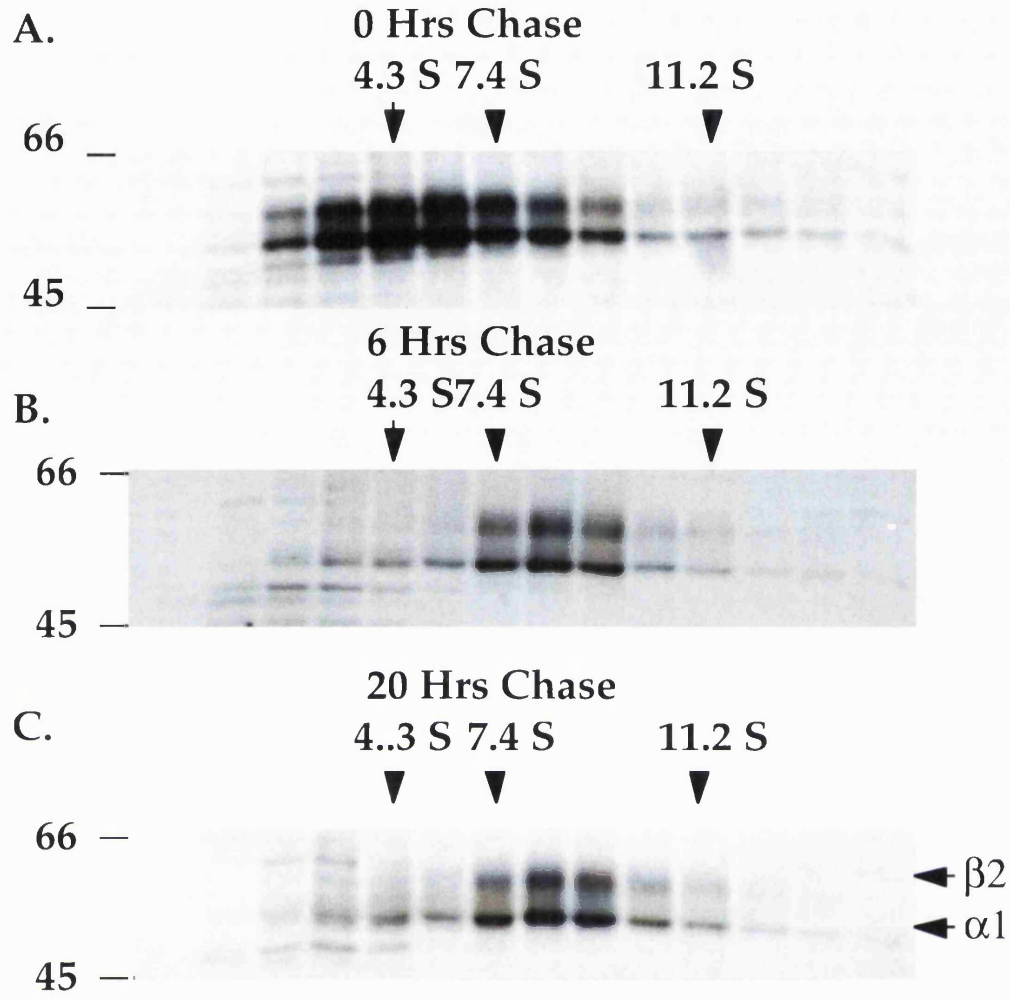


Fig 24. Pulse Chase analysis of $GABA_A$ receptor assembly. BHK cells were coinfected with the α1 (9E10) and β2 (9E10) virus particles. The cells were labelled with 150μCi/ml of ^{35}S methionine for one hour at 4 hours post infection. The cells were chased for 0Hrs, (A), 6 Hrs (B) and 20 Hrs (C) with excess cold methionine in BHK medium. The cells were lysed in non denaturing lysis buffer and the lysate subjected to sucrose density centrifugation. The gradient was fractionated and each fraction immunoprecipitated with the monoclonal antibody 9E10. The α1 (9E10) and β2 (9E10) subunits were resolved by SDS PAGE and the signals for each protein quantified by a Biorad phosphorimager. Location of the standard proteins BSA (4.3 S) , Aldolase (7.4 S) and Catalase (11.2 S) are shown for reference.

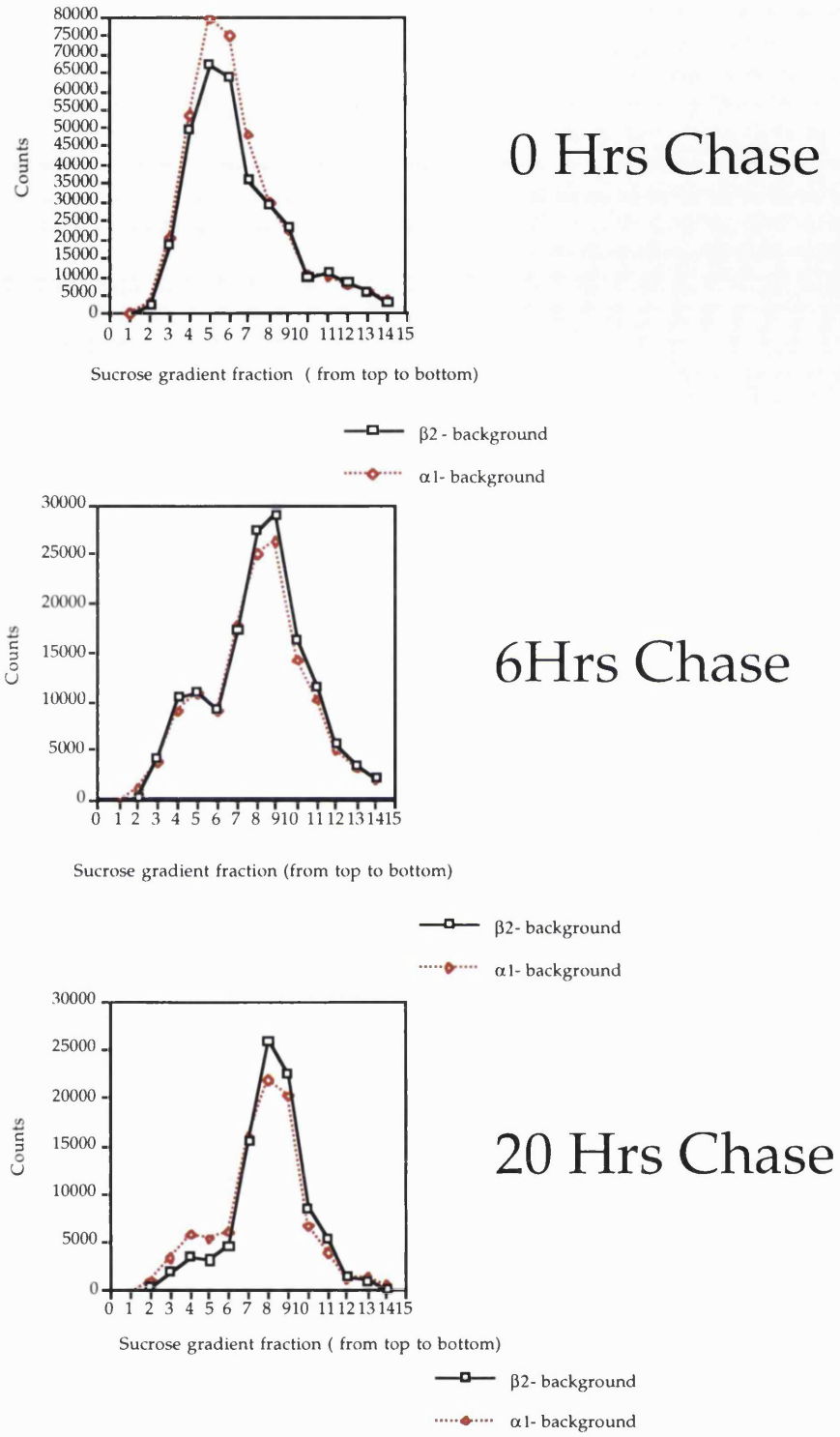


Fig 25. Quantification of receptor assembly. The levels of incorporated methionine in the α1 (9E10) (open circles) and β2 (9E10) (closed circles) subunits were quantified in gradient fractions for cells chased for 0 Hrs (A), 6Hrs (B) and 20 Hrs (C) using a Biorad phosphorimager. Background was subtracted using the same volume that was used to integrate the subunit signals.

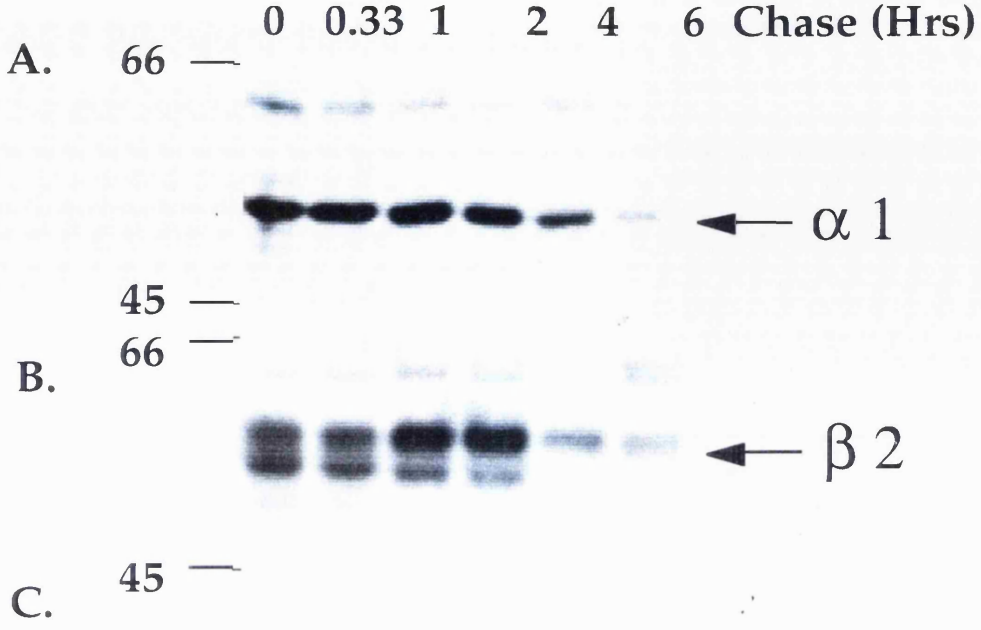
3.2.4 Unassembled receptor subunits are degraded.

Pulse chase and immunoprecipitation studies were performed on single infections of BHK cells with either the α1 (9E10) or β2 (9E10) virus particles. These experiments were utilised to define the half lives of these two subunits when expressed independently. The results of these experiments are shown in Figs 26 (A) , (B) and (C). Half lives of between 2 and 4 hours were determined for the α1 (9E10) and β2 (9E10) subunits. The half lives of homomeric subunits are consistent with the dramatic loss of receptor subunits seen during the first 6 hours of the pulse chase experiment performed on cells co-infected with both the α1 (9E10) and β2 (9E10) virus particles (Figs 24 and 25).

3.2.5 The time dependence of receptor surface expression.

In order to investigate the rate at which receptors appear on the cell surface of BHK cells, cells were co-infected with α1 (9E10) and β2 (9E10) viruses. The infected cells were then fixed after differing time periods and the level of surface expression was determined by fluorescence immunocytochemistry upon intact cells. The specimens for each time point were prepared simultaneously to eliminate variation in solutions or antibody concentrations. Surface receptor levels were quantified using confocal microscopy as shown in Fig 27. The settings of gain and iris diameter were chosen so that the maximal obtainable signal was not saturating the detection apparatus. The linear nature of the fluorescence detection system of the confocal microscope is checked regularly using fluorescently labelled microspheres. The corresponding fluorescent images are shown in Fig 28. It was not possible to detect surface expression of the (9E10) epitope at 6 hours post infection and only at eight hours post infection was reliable surface expression detectable, see Fig 28 (D). It is interesting to note that an apparent lag exists between

oligomerisation evident within one hour, and cell surface receptors at eight hours. When a similar time course experiment is performed on



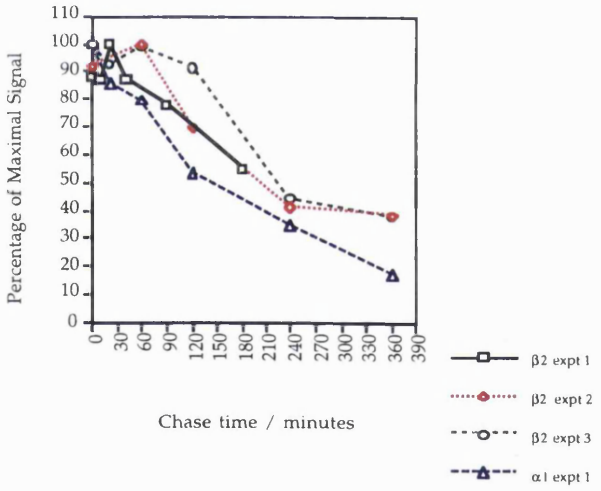


Fig 26. A. , B. and C. Degradation of single $GABA_A$ receptor subunits expressed in BHK cells. BHK cells were infected with α1 (9E10) or β2 (9E10) virus particles. The cells were labelled with ^{35}S Methionine 400μCi/ml for 20 minutes , four hours after being infected by virus. The cells were then chased for differing periods of time. The cells were lysed and receptor subunits isolated by immunoprecipitation with the monoclonal antibody 9E10. Precipitated material was resolved by SDS-PAGE and the subunit levels quantified using a Biorad phosphorimager. Figure A. and B, illustrates the decrease in signal of the α1 (9E10) and β2 (9E10) subunits respectively with increasing times of chase (Hours). Figure 26 C. is a graphical representation of a collection of the pulse chase experiments.

BHK cells infected with a single viral species it is possible to detect (9E10) signal as early as 4 hours post infection in permeabilised cells.

Table 2. Raw data for time course of surface expression.

Time post infection.	Mean pixel density
0	0
2	0
6	0
8	37.0, 38.46, 38, 34.81, 27.66, 32.64, 38.7 n=7
10	84.3, 92.4, 93.7, 85.53, 89.98, 85.17 n=6
24	125.24, 122.43, 128.82, 130.94, 131.66, 138.49, 131.11, 140.00, 138.00 n=9

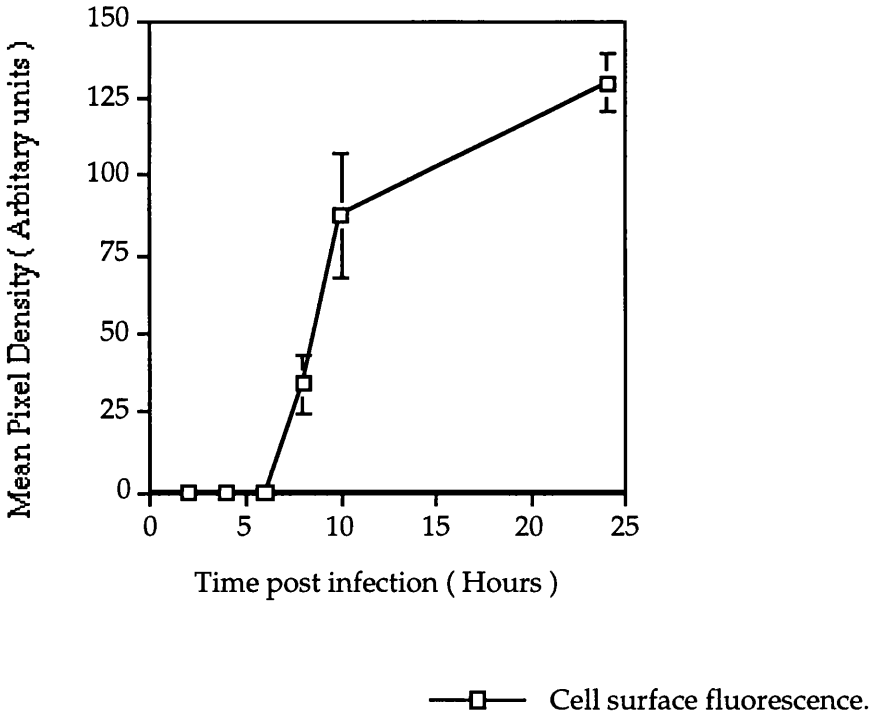


Fig 27. Time dependence of surface expression of receptors composed of α1 (9E10) and β2 (9E10) subunits. Co-infected cells were fixed at differing time points after infection and processed simultaneously for fluorescence with the 9E10 monoclonal antibody without permeabilisation. Images were collected and quantified from cells using a confocal microscope using identical iris, gain and power settings. Data was collected from at least 5 cells for each time point.

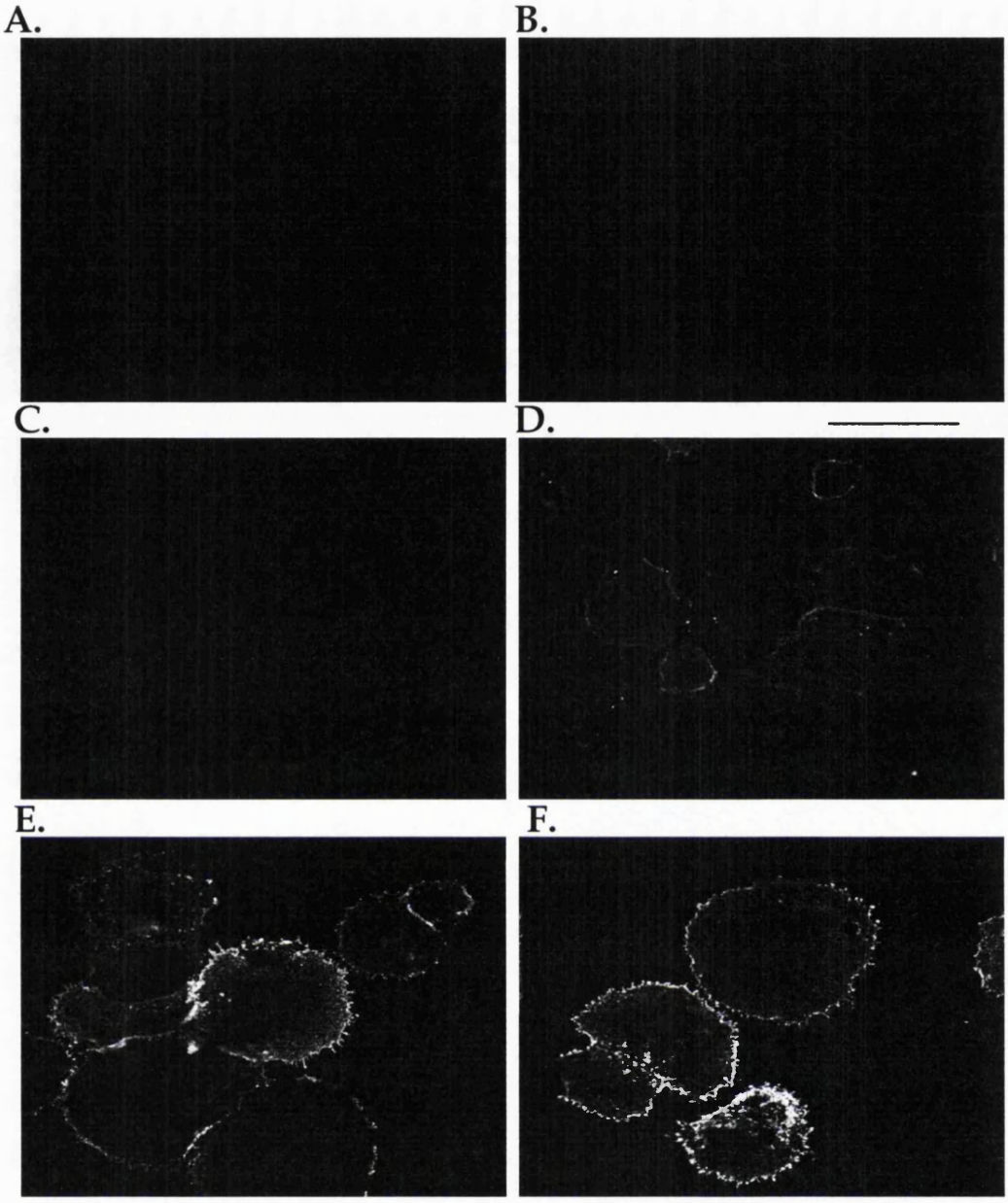


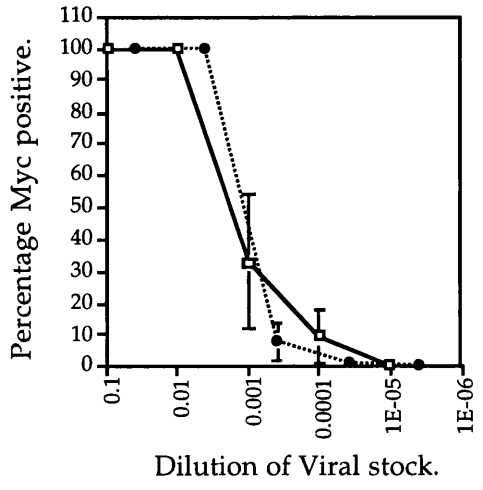
Fig 28. Images illustrating the time dependent expression of receptors composed of α1 (9E10) and β2 (9E10) at the cell surface of BHK cells. BHK cells were co-infected with α1 (9E10) and β2 (9E10) virus particles and at time points after infection were fixed. The specimens were then processes simultaneously for fluorescence using the 9E10 monoclonal antibody. Panel A is a control, non infected cells, B 4 hours post infection, C 6 hours post infection, D 8 hours post infection, E 10 hours post infection, F 24 hours post infection. Scale represents 50μm.

3.2.6 The ratio of α1 to β 2 virus particles is critical for the production of surface receptors.

In order to optimise the expression of α1 (9E10) and β2 (9E10) at the cell surface differing ratios of α1 (9E10) to β2 (9E10) virus were used to co-infect BHK cells. The β2 (9E10) and α1 (9E10) virus preparations had similar titres of 2 x10^8 virus particles per ml, as determined by serial dilutions, see Fig 29 (A). It was clear that when a 10 to 1 ratio of β2 (9E10) to α1 (9E10) was used, very low levels of surface expression was detected, Fig 29 (B) and (E). In contrast reversal of the ratio this time with ten times as many α1 (9E10) virus to β2 (9E10) produced high levels of surface expression, see Fig 29 (B) and (C) . Excess β2 (9E10) subunits must therefore inhibit the oligomerisation and or transport of the heteroligomer to the cell surface.

The inhibition of transport to the cell surface by excess β 2 suggests that the intermediate which is formed during the heteroligomerisation of α1 (9E10)and β2 (9E10) is inhibited by excess β2 (9E10). In contrast excess α1 (9E10) does not inhibit the transport of heteroligomers to the cell surface.

A.



B.

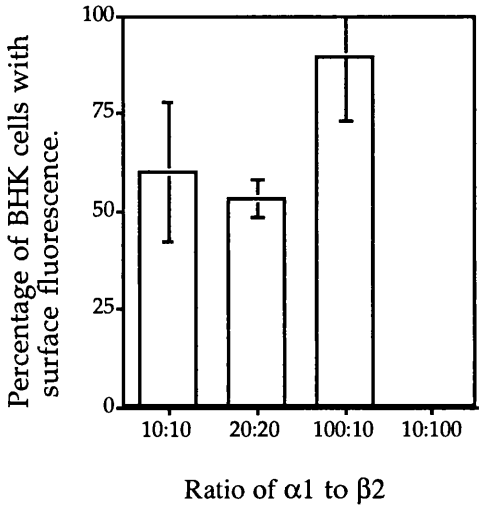


Fig 29 (A and B).

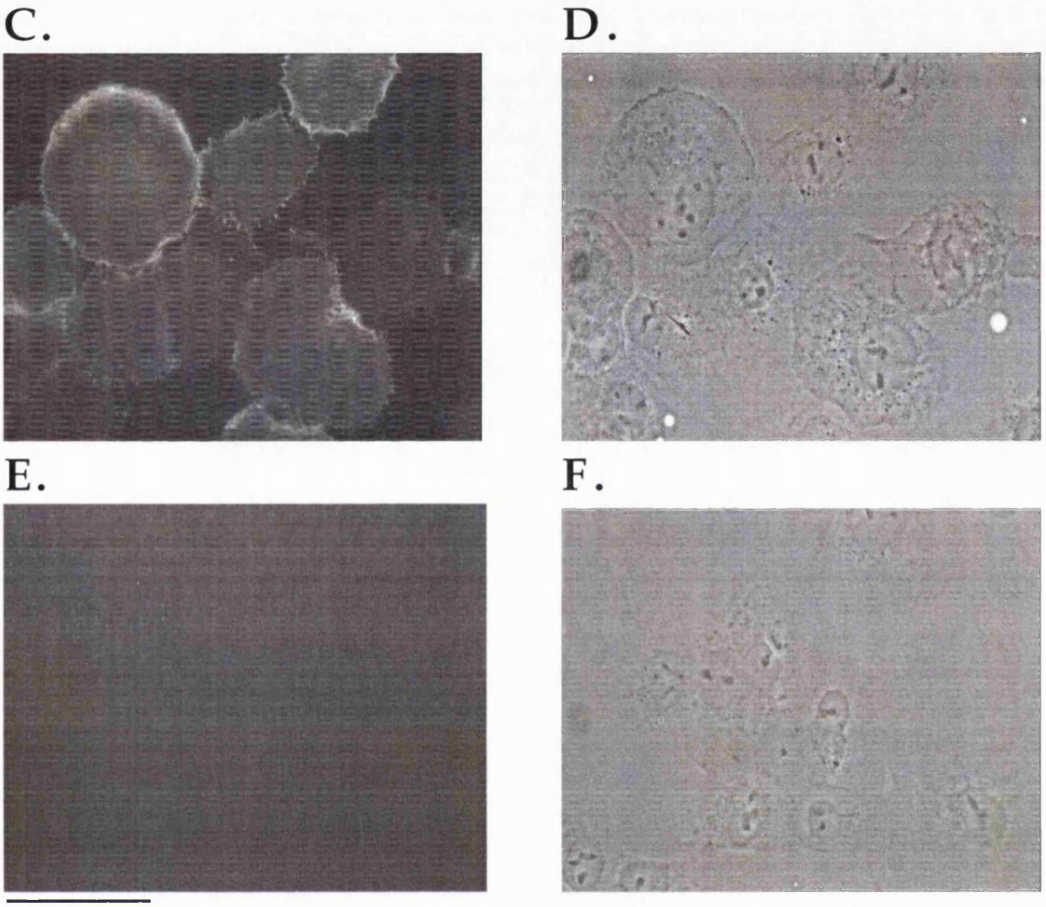


Fig 29. Excess β2 (9E10) inhibits the cell surface expression of receptors composed from α1 (9E10) and β2 (9E10) subunits. Differing ratios of α1 (9E10) to β2 (9E10) virus was used to infect BHK cells. The percentage of BHK cells exhibiting cell surface fluorescence, using the 9E10 monoclonal, was evaluated at 24 hours post infection. Panel A. illustrates the similar titres of the two virus preparations. Panel B. is a histogram depicting the results of the ratio experiments. Panel C. illustrates the high frequency of cell surface fluorescence using the 100:10 ratio of alpha 1 : to beta 2. The phase image of the same field is shown in D. The results of the inverse ratio of 10: 100 are shown in E. with the phase image of the same field in F.

3.2.7 The subunit ratio of $GABA_A$ receptors composed of α 1 (9E10) and β 2 (9E10) is 1 to 1.

It is possible using the Semliki expression system to consistently produce ^{35}S methionine radiolabelled heteroligomers of α1 (9E10) and β2 (9E10). Both receptor subunits were tagged at the N terminus with the same epitope tag to which the monoclonal antibody (9E10) could bind. The two subunits could be precipitated with similar efficiencies given that the epitope at the N terminus of the subunits was equally accessible to the antibody. The ratio of α1 (9E10) to β2 (9E10) within the 9S sedimenting population could then be estimated by immunoprecipitating the (9E10) tagged subunits from the fractions of a sucrose gradient containing 9S heteroligomers. The immunoprecipitated proteins were resolved by SDS-PAGE and quantified by phosphorimager analysis. The number of counts from the α1 (9E10) and β2 (9E10) proteins was normalised for the differing methionine content of the two proteins. The results of one such experiment is shown in Fig 30 (A). The ratio of α1 (9E10) to β2 (9E10) in this particular experiment was 0.9. The results from three of such experiments for the ratio of α1 (9E10) to β2 (9E10) were: 0.8, 0.8, and 0.9. It was possible that unassembled subunits were skewing the results. This was addressed by analysing the results of pulse chase experiments where the cells were pulsed for one hour and chased for 20 hours. During the 20 hours of chase all the unassembled subunits would be degraded. The ratio of α1 (9E10) to β2 (9E10) was calculated from such a pulse chase experiments to be 1.1 to 1

3.2.8 Cell Surface Immunoprecipitation of $GABA_A$ receptors.

Pulse chase studies in combination with immunoprecipitation across sucrose gradients has provided evidence that the ratio of α1 (9E10) to β2 (9E10) in an assembled $GABA_A$ receptor is between 0.8 and 1.25. To further characterise assembled receptors immunoprecipitation was

performed on the population of subunits that had been transported to the cell surface. BHK cells were co-infected with α1 (9E10) to β2 (9E10)

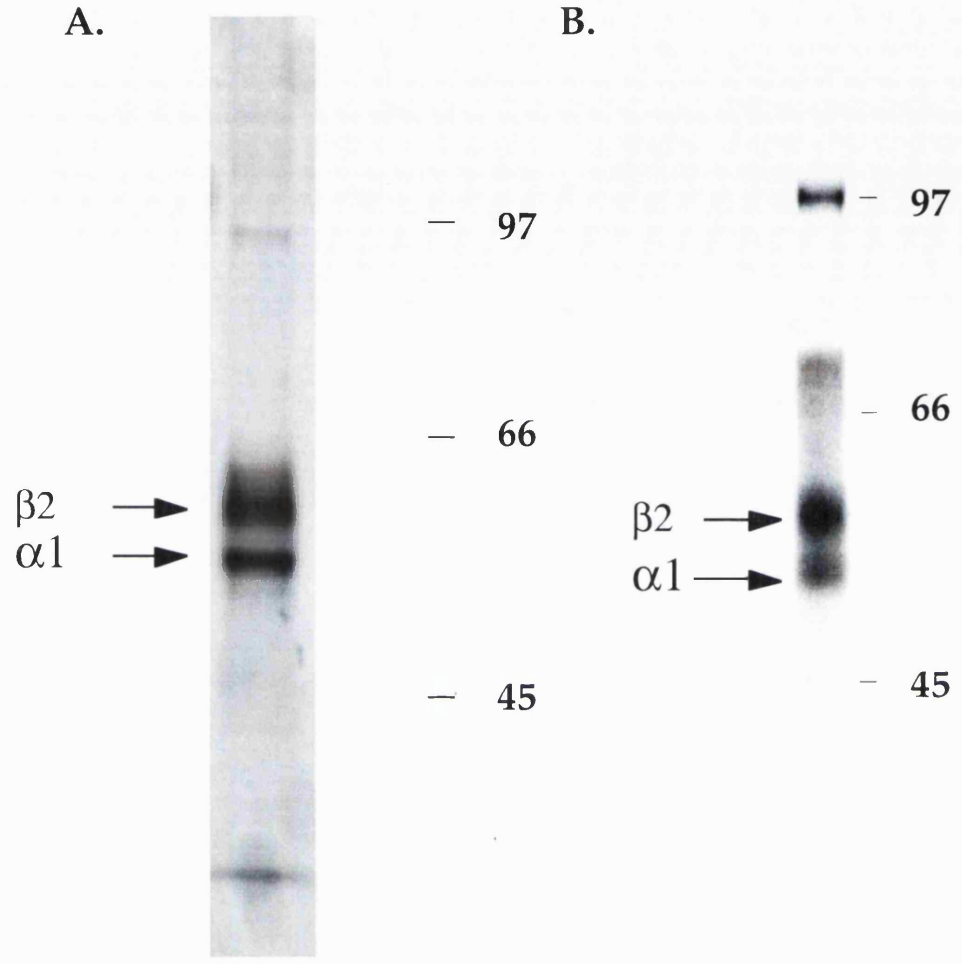


Fig 30. Subunit ratio of $GABA_A$ receptors composed of α1 (9E10) and β2 (9E10). Panel A. BHK cells expressing both receptor subunits were pulse labelled with ^{35}S methionine (200 μCi/ml) for 2 hours at three hours after infection. The cells were then chased for 12 hours with excess cold methionine. Cell lysates were then subjected to sucrose density centrifugation and the fractions coresponding to the 9S peak pooled and immunoprecipitated with the monoclonal antibody 9E10. The subunits were then resolved by SDS-PAGE and quantified using a phosphorimager. After correction for methionine content (α1 (9E10) = 9, β2 (9E10) = 15) a ratio of 0.9 (α1 (9E10): β2 (9E10)) was found for the experiment shown.

B. Labelled cells were exposed to the monoclonal antibody 9E10 at 4°C for one hour. The cell surface receptor population was then isolated via immunoprecipitaion with protein G in the presence of excess 9E10 peptide. The precipitate was resolved by SDS-PAGE and qunatified using a Biorad phosphorimager. A ratio of 1.1:1 was found for the α1 (9E10) to β2 (9E10) ratio in the experiment shown.

virus particles with a multiplicity of 10. The cells were then incubated in the presence of ^{35}S methionine translabel . After being incubated in the presence of the radiolabel for 14 hours the cells were incubated in saturating amounts of (9E10) monoclonal antibody (10μg/ml) at 4°C for one hour. The cells were then lysed in non denaturing lysis buffer in the presence of excess (9E10) peptide (1μg/ml), and the (9E10) monoclonal immunoprecipitated using protein G sepharose which had been preabsorbed with non infected, non radioactive BHK cell lysate. The precipitates were resolved by SDS-PAGE and quantified by phosphorimager analysis. The results of such an experiment is shown in Fig 30 (B). The ratio of the α1 (9E10) to β2 (9E10) in this experiment, after correcting for relative methionine contents was 1.1:1

This result in combination with the previous results are consistent with a 1:1 ratio of α1 (9E10) to β2 (9E10) subunits suggesting that the $GABA_A$ receptor formed from these subunits is composed of equal numbers of α1 (9E10) and β2 (9E10) subunits. This would indicate a tetrameric or hexameric but interestingly not a pentameric tertiary structure.

Table 3. Raw data for the stoichiometry studies.

Expt	α1 counts	β2 counts
1	12791	26270
2	37086	74243
3	10506	19130
4 (20 hr chase)	6934	10932
6 ((9E10) incubation)	29996	45509

3.2.9 Assembly of $GABA_A$ receptors in SCG neurones.

Heterologous expression systems have been used to examine the assembly of ion channels including the $GABA_A$ receptor (Blair et al 1988, Pritchett et al 1988, Sigel et al1989, 1990,Verdoorn et al 1990, Draguhn et al 1990 Angelotti and Macdonald 1993, Connolly et al 1996a). However it is not known whether the assembly of ion channels within transformed cell lines or *Xenopus* oocytes is representative of neuronal assembly mechanisms. Recently the existence of neuronal specific chaperone molecules has been suggested (Ferreria et al 1997). In order to try and address this question SCG neurones have been microinjected with expression plasmids. However, such experiments require access to expensive equipment and the act of microinjection is traumatic to the neurones. It is also difficult identifying the neurones once they have been microinjected. For these reasons such experiments have not been widely successful. In order to address the question of neuronal assembly of $GABA_A$ receptors a viral expression system which was capable of infecting neurones and expressing recombinant protein was put to use. The viral expression system which was chosen was the Semliki expression system. The results in this chapter and the previous chapter have characterised this expression system in BHK cells. The neuronal oligomersiation of α1 (9E10) and β2 (9E10) was tested since this is the most simple combination that can give rise to a $GABA_A$ receptor. The capability of the expression systems to support heteroligomerisation of α1 (9E10) and β2 (9E10) proteins has been demonstrated. The transport of either the α1 (9E10) or the β2 (9E10) proteins to the plasmamembrane was shown to be dependant on both proteins being co-expressed in the same cell. This had been demonstrated before in HEK cells (Connolly et al 1996a). This work was also performed in a fibroblast cell line. In order test the hypothesis that the same assembly "rules" were applicable in

neurones, SCG neurones were co-infected or individually infected with α1 (9E10) and β2 (9E10) viral particles .

As can be seen in Fig 31 (D) marked cell body and process staining was produced when the neurones expressed both subunits. In contrast when the α1 (9E10) or β2 (9E10) virus particles were used to infect neurones independently no cell surface fluorescence was observed and staining was confined to the cell body , see Fig 31 (A) and (B). The cell body staining was only detectable in permeabilised neurones, intact neurones gave only background signal, see Fig 31 (C) .

The inability to observe surface fluorescence in SCG neurones can be explained by the rapid inhibition of host protein synthesis by this viral expression system. Viral message would quickly out compete host messages, including those for $GABA_A$ subunits. The competition would be especially great if the levels of the endogenous message were low, such as that associated with proteins with a low rate of turnover.

The retention of α1 (9E10) and β2 (9E10) within the neuronal cell body when they are separately expressed implicates the existence of chaperone molecules. Calnexin and Bip have been detected at high levels within neurones (Villa et al 1992). The perinuclear observed when the subunits are separately expressed is consistent with the localisation of these subunits to the rough ER of the neurones (Krijnse-Locker et al 1995). This contrasts with cell surface pattern of staining found when both subunits are co-expressed. (9E10) staining could be observed on the cell bodies and the neuronal processes. It was also possible to detect regions of higher intensity staining along the processes. These may represent clustering of the virally produced receptor.

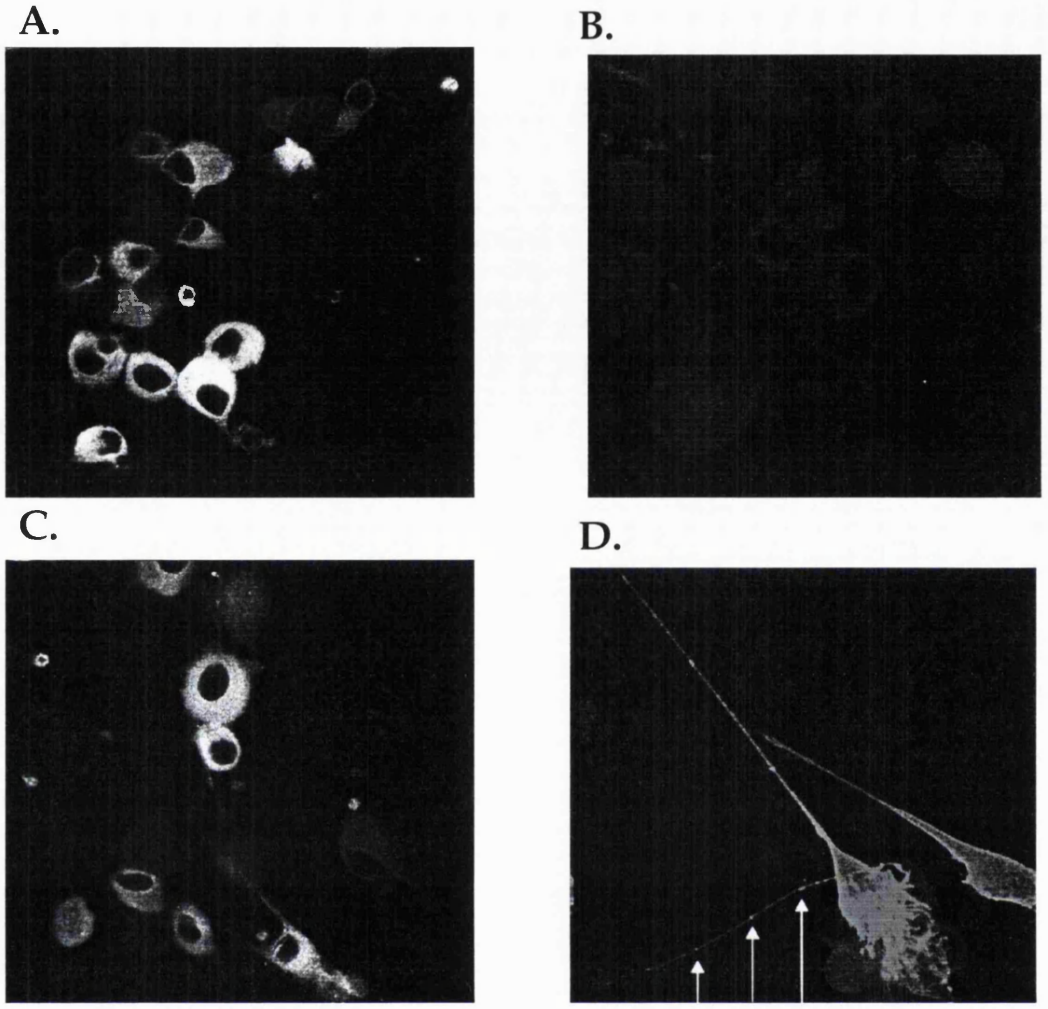


Fig 31. Infection of cultured SCG neurones with Semliki Forest virus expressing $GABA_A$ receptor subunits. Cultured SCG neurones were infected after three days in culture and receptor expression determined by immunofluorescence using the monoclonal antibody 9E10. Immunofluorescence was conducted 12 hours after infection in the presence (+) or absence (-) of detergent. Images were collected using a confocal microscope from cells infected with : A. α1 (9E10) (+) B, β2 (9E10) (+) C, α1 (9E10) (-) and D, α1 (9E10) and β2 (9E10) (-).

3.2.10 Discussion.

When α1 (9E10) and β2 (9E10) virus particles co-infect BHK cells it is possible to demonstrate the formation of an α1 (9E10) β2 (9E10) heteroligomer which has a sedimentation coefficient of 9S. In contrast expression of either the α1 (9E10) or β2 (9E10) subunit produces a 5S complex. Heteroligomerisation of α1 (9E10) and β2 (9E10) subunits has also been demonstrated by co-immunoprecipitation studies in HEK cells using conventional transient transfection methods (Connolly et al 1996a). The generation of this heteroligomer is an inefficient process with approximately 36% of available α1 (9E10) and β2 (9E10) subunits attaining the final 9S receptor conformation. However once formed this 9S is stable in contrast to the unassembled receptor subunits which are quickly degraded with a half life of 2 to 3 hours. Much shorter half lives, approximately 40 minutes, have been estimated for the nAChR subunits (Claudio et al 1989). It should be noted that the viral expression system inhibits host protein synthesis. This would result in a drop in the levels of proteins which have a high turnover. This could be expected in degradative enzymes for example. The half lives determined using the viral expression system may therefore be underestimates.

An apparent lag phase between the assembly of a 9S complex and the detection of subunits at the cell surface was observed. Cell surface subunits were first detected eight hours post infection where as assembly of a 9S complex was clearly visible after six hours of chase and was probably in existence at the end of the hour long pulse. The significance of this lag between assembly and translocation to the surface is unknown. Studies performed using a mouse muscle cell line also identified a lag phase between nAChR subunit synthesis and the formation of nAChR subunits at the cell surface which could bind α–

bungarotoxin (Merlie and Lindstrom 1983). This period was estimated to be one hour.

The 1: 1 ratio of α1 (9E10) to β2 (9E10) subunits in heteroligomeric receptors produced by this expression system as determined by the quantification of sucrose gradient fractions corresponding to mature receptor and by surface immunoprecipitation argues for either a dimer, tetramer , hexamer or an equally distributed population of 3:2 and 2:3 pentamers. The latter suggestion is less likely in light of the results of the experiment where β2 (9E10) subunits are over produced. This reduces surface expression. If a mixed population of pentamers were being produced this would be expected to drive the formation of receptors with the 2:3 ratio of α1 (9E10) to β2 (9E10). Given the unitary dose response curves for α/β receptors, single IC 50 value for Zn^{2+} inhibition and unitary single channel properties this appears less likely (Macdonald and Olsen 1994). The sedimentation characteristics of the receptor would favour a tetrameric model rather than a hexameric model. This result is in contrast to previous studies performed upon $GABA_A$ receptors purified by benzodiazepine affinity chromatography. Such studies are by their nature analysing receptors containing a γ subunit. Two biochemical studies have been performed on $GABA_A$ receptors composed of the α1 and β3 subunits. One group selectively immunoprecipitated receptors from the surface of *Xenopus* oocytes which had been microinjected with the cRNA of α1 and β3 receptors. The results of this study support a tetrameric model (Kellenberger et al 1996). In contrast one other study involving the transient transfection of HEK cells by α1 and β3 expression plasmids support a pentameric model (Tretter et al 1997). The analysis of this experiment is complicated by the ability of the β3 subunit to homoligomerise and give rise to barbiturate sensitive anion channels (Connolly et al 1996b). In an transient transfection one would therefore

have a population of HEK cells which would express the β3 subunit on their surface without the α1 subunit. Such a study would over estimate the proportion of β3 contributing to heteroligomers of α1 and β3 subunits. In comparison β2 homomers are not detectable on the cell surface and would not skew the results.

The tetrameric model is in conflict with the pentameric arrangement of the nAChR at the neuromuscular junction, the neuronal nAChR , the glycine receptor and the 5HT3 receptor. Tetramers of α and γ subunits of the nAChR have been reported to form. The reason given for their formation is the unique position of the γ subunit, which lies between two α subunits. This would give this subunit the capability to interact with both the + and - sides of the α subunit (Kreienkamp et al 1995). The β subunit is also believed to lie between two α subunits. This is supported by the existence of GABA binding sites which are dependant on amino acid residues in the N terminus of the α and β subunits (Sigel et al 1992, Amin and Weiss 1993, Olsen and Smith 1994). The location of the β between two α subunits would also allow it to interact with the + and - sides of the α subunit . The formation of a tetramer like that seen when the α and γ subunits of the nAChR are co-expressed would therefore be expected.

In contrast to $GABA_A$ channels composed only of α1 and β2 subunits the addition of a γ2 subunit produces a channel which is capable of being allosterically modulated by benzodiazepines (Pritchett et al 1988, Pritchett et al 1989, Luddens and Wisden 1991, Mckernan et al 1995). Mutagenic strategies have defined particular amino acids on the α and γ subunits that are critical for the construction of binding domains of benzodiazepines. Mutation of histidine residue 101 in the α1 subunit produced benzodiazepine insensitive $GABA_A$ receptors (Wieland et al 1992), whilst mutation of threonine 142 of the γ2 subunit greatly effected

the efficacy of benzodiazepines (Mihic et al 1994). Photaffinity labelling studies determined the likely benzodiazepine binding site to lie between amino acids 59-148 of the bovine α1 subunit, (Stephenson and Duggan 1989) with histidine 101 being the site with the greatest labelling (Olsen and Smith 1994). It is proposed that the likely benzodiazepine binding site is located between the α and γ subunit (Smith and Olsen 1995).

Mutagenic strategies have defined residues on the α and β subunits that are important for GABA binding. A single point mutation at phenylalanine 64 of the α1 subunit markedly affected GABA affinity for receptors containing this mutant. (Sigel et al 1992). Interestingly a natural splice variant of the α6 subunit exists which results in the loss of phenylalanine 64. Heterologous expression of this splice variant with β2 and γ2 subunits did not produce functional $GABA_A$ receptors (Korpi et al 1994). Phenylalanine 65 was demonstrated to be photoaffinity labelled by ^{3}H muscimol, (Smith and Olsen 1994). Mutagenic strategies also identified two regions in the β2 subunit that were important for the activation of the $GABA_A$ receptor by GABA. The first region included tyrosine 157 and serine 160 and the second region included tyrosine 202 and serine 205 (Amin and Weiss 1993). The location of the GABA binding site was suggested to involve these subunits and lie between the α and β subunits (Smith and Olsen 1995).

Thus a pentameric arrangement may indeed occur in receptors produced from α1, β 2 and γ2 subunits, with an order α1β2γ2α1β2. However in the absence of the γ subunit it is proposed that a tetramer α1β2α1β2 forms. The suppression of surface expression by the production of excess β over α may suggests that the intermediate is an α1β2α1 trimer rather than β2α1β2. A model for assembly of pentameric and tetrameric $GABA_A$ receptors is shown in Fig 32.

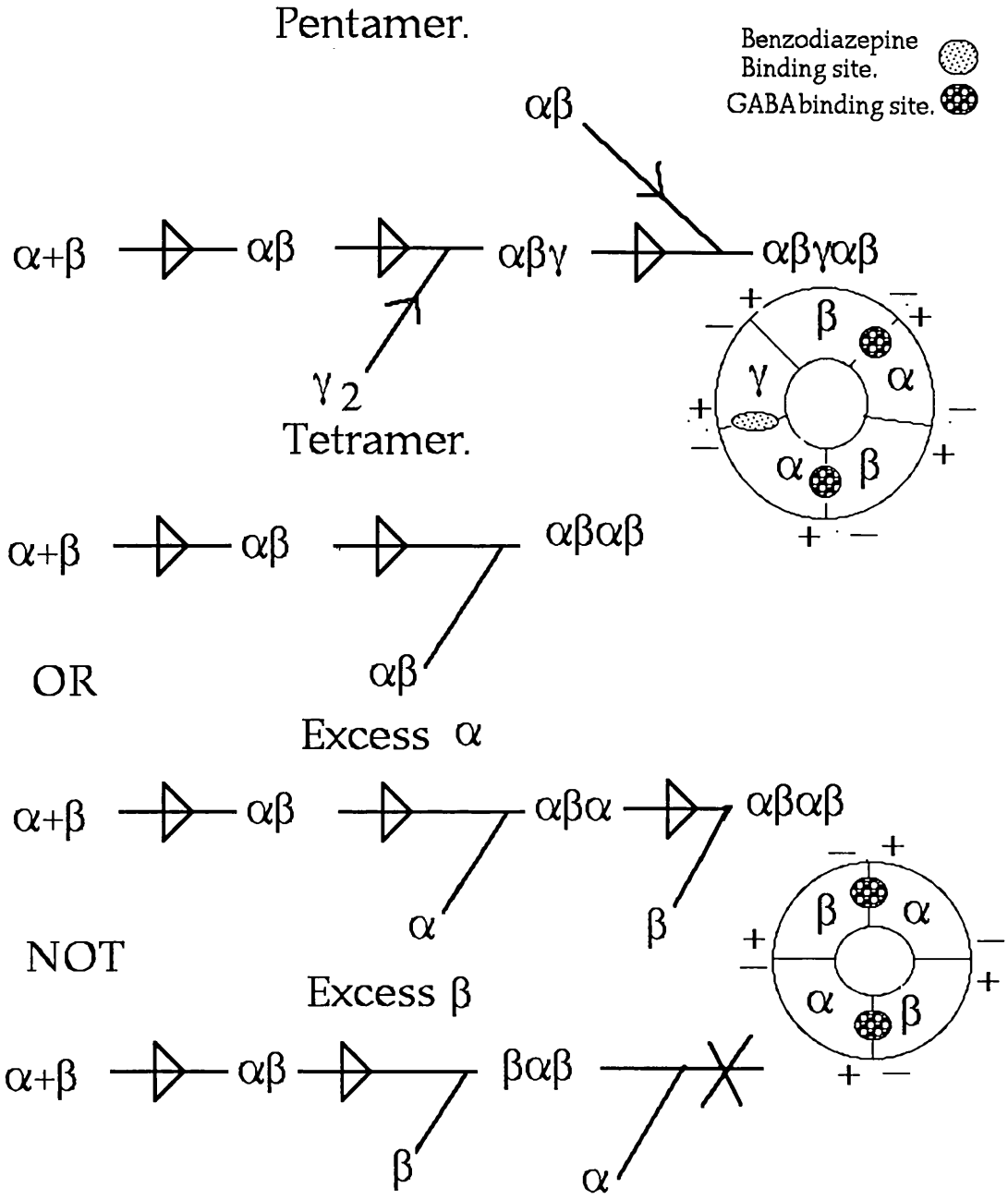


Fig 32 Model of oligomerisation. - an + refer to the sidedness of the subunit.

The production of a pentameric channel from an α1 and β2 subunits would necessitate the positioning of an α1 or a β2 subunit adjacent to itself , that is α1β2α1β2α1 or β2α1β2α1β2 for example. It is known that neither the α1 or β 2 homoligomerises , in contrast to glycine receptor subunits and the β3 subunit of the GABA receptor (Sontheimer et al

1989; Connolly et al 1996a, Connolly et al 1996b). Such arrangements would therefore be less likely than a tetramer where α1 and β2 subunits are interspaced.

Evidence to suggest a difference between $GABA_A$ channels composed of α and β subunits and those with an α, β and γ subunit come from differences in the single channel conductance of such receptors. Receptors composed of only α and β subunits have a lower single channel conductance, 15pS compared to the 28pS produced when a γ subunit is also introduced (Angelotti and Macdonald 1993). The two types of receptor differ in their sensitivity to zinc ions, α β channels are inhibited by Zn^{2+} where as α β γ receptors are insensitive to Zn^{2+} (Draguhn et al 1990). The existence of receptors composed of α and β subunits in neurones has not been established. However GABA gated chloride channels which are highly sensitive to Zn^{2+} and have a low single channel conductance have been described in neurones (Kaneda et al 1995).

The ability to perform well controlled experiments on a negative background has been a major advantage of studies expressing cDNA's of subunits in cell lines and *Xenopus* oocytes. However it is not known whether the assembly process which occurs in fibroblasts and oocytes is representative of the assembly events in neurones. Using the broad host range of the Semliki expression system it was for the first time possible to address this criticism. The results of experiments where SCG neurones were infected with the α1 (9E10) and β2 (9E10) virus particles correlated exactly with the results of experiments performed in BHK and HEK cells. In order to observe the trafficking of the (9E10) epitope to the neuronal plasmamembrane the SCG neurones needed to be co-infected by the α1 (9E10) and β2 (9E10) virus particles. It can be concluded that the studies

regarding the assembly of ion channel subunits in transformed cell lines are applicable to the assembly process in neurones.

Virally expressed subunits were not found to assemble with endogenous subunits. The viral expression rapidly inhibits host protein synthesis. The levels of viral message rapidly rise and out compete endogenous messages. The production of endogenous $GABA_A$ subunits would rapidly fall. The very rapid nature with which ion channel assembly takes place suggests that the initial events are co-translational (Green and Claudio 1993). Such a process would require some parity between the levels of the different messages and the insertion of the different nascent polypeptides into adjacent regions of membrane. Evidence to support this suggestion arises from studies performed on the assembly of the HLA-DR histocompatibility antigens (Kvist et al 1982). These authors found that the assembly *in vitro* using pancreas microsomes was only observed when the amount of membranes used became limiting. It would be expected that if one subunit was expressed virally in the SCG neurones any parity in the levels of message would be quickly lost and the virally expressed subunit would flood the endoplasmic reticulum of the neurone making co-translational assembly improbable.

Chapter 3.3 the γ 2 subunit of the $GABA_A$ receptor.

3.3.1 Introduction to the pharmacological and physiological Importance of the γ 2 subunit.

The γ2 protein is the most widely expressed of the $GABA_A$ γ subunits. The γ subunit is crucial in conferring to the $GABA_A$ receptor its capacity to be allosterically modulated by benzodiazepines. The type of benzodiazepine pharmacology is however determined by the α subunit (Pritchett et al 1989b, Luddens and Wisden 1991). The γ subunits contribution to the $GABA_A$ receptor is interesting historically. Initially when the $GABA_A$ receptor was first cloned the γ subunit was not included and it was not until Pritchett and colleagues later cloned this subunit did its pharmacological significance become apparent (Pritchett et al 1988 , Pritchett et al 1989a, Luddens and Wisden 1991, Mckernan et al 1995).

The physiological importance of the γ2 subunit has been illustrated by the production of transgenic mice that had the γ2 subunit knocked out by the homologous recombination of a construct which disrupted exon 8. This exon codes for the second and third transmembrane domains of the γ 2 subunit . The γ2 null animals had a normal development *in utero* but within 2 days of birth the majority of animals had died and all the homozygotes were dead by eighteen days. The precise cause of death was not determined. There were no histological abnormalities of the gastrointestinal and neuroendocrine system. The homozygotes although capable of feeding demonstrated a marked retardation in growth. Interestingly the animals also showed a marked hyperactivity of body and limb movements (Gunther et al 1995).

The important contribution of the γ2 subunit to the function of $GABA_A$ receptors was illustrated by the reduction of the main conductance state of the channel from 28 to 15 pS. (Angelotti and Macdonald 1993) The

importance of the γ2 subunit in generating benzodiazepine sensitivity to the $GABA_A$ channel in vivo was illustrated by the 94% reduction in flumazenil binding sites and the lack of effect of oral diazepam on the γ2 deficient animals. The consequences of loss of the γ2 subunit upon $GABA_A$ receptor assembly and distribution within the central nervous system were also studied. Interestingly no distortion of the normal distribution of α and β $GABA_A$ subunits were reported. This work indicates that the absence of a γ2 subunit is not critical for the assembly of functional $GABA_A$ receptors. However such a complement of receptors is not sufficient to ensure adequate postnatal development and is lethal (Gunther et al 1995).

3.3.2 Studies of the γ2 subunit.

The predicted size of the γ2 protein based upon its cDNA is approximately 48KDa without glycosylation. The native size of the γ2 subunit is controversial. Western blots of brain lysates using γ2 specific antibodies have produced a range of results from 43 KDa (Araki et al 1993), to 49 KDa (Fernando et al 1995 and San Juan et al 1995). The instability of the γ2 subunit has been suggested to contribute to the range of molecular sizes of this subunit (Hadingham et al 1992 and Connolly et al 1996a). The instability of the γ2 subunit is emphasised in the study by Hadingham et al 1992. In this study they could not detect the γ2 subunit by western blotting even in a stable cell line known to produce benzodiazepine sensitive channels.

The cell biology of this subunit is also interesting. There is controversy as to the subunits needed to be co- expressed with the γ2 protein in order to form GABA gated Chloride channels. It has been reported that such channels can be formed when an α1 subunit is co-expressed with a γ2 subunit (Verdoon et al 1990; Draguhn et al 1990) whilst others believe that a β subunit is also required (Sigel et al 1990; Connolly et al 1996a).

When the γ2 subunit was expressed alone in HEK 293 cells GABA gated chloride currents were produced but the currents were of very small amplitude (50pA) (Shivers et al 1989). This contrasts with the 500 pA currents produced when HEK cells expressing α1 (9E10) and β2 (9E10) subunits are exposed to GABA (Connolly et al 1996a). It is possible that low levels of $GABA_A$ receptor subunits exist within some expression systems which would complicate the interpretation of homomeric receptor expression (Ueno et al 1996).

The picture is further complicated by the discovery of an alternative splice variant of the γ2 subunit (Whiting et al 1990, Kofuji et al 1991). The long variant differs by only eight amino acids which lie in the major intracellular loop between transmembrane domains three and four. This insert contains a site for protein kinase C and Calcium / Calmodulin Type II Kinase phosphorylation (Whiting et al 1990, Moss et al 1992, McDonald and Moss 1994), and introduces the eight amino acids between two proline residues. The γ2 subunit also contains a consensus site for tyrosine phosphorylation. Tyrosines 365 and 367 in the short and tyrosines 373 and 375 in the long form.

The biochemical instability of the γ2 protein had made it a difficult subunit to characterise. It was therefore felt appropriate to apply the Semliki expression system to determine the size of the γ2 L and S proteins . The viral expression system was also used to analyse the cell biology of the γ2 subunit in tissue culture cells and primary cultures of neurones expression system.

3.3.3 Tyrosine Phosphorylation of Ligand gated ion channels.

The existence of a consensus site for tyrosine phosphorylation in the γ2 subunit (Songyang et al 1995), suggested that it could be a target for tyrosine kinases. Studies performed on the NMDA and nAChR suggest that this is a possibility. The high levels of tyrosine kinases found within

the central nervous system lead people to believe that they could have a regulatory role in modulating synaptic transmission (Swope et al 1992). The functional consequences of tyrosine phosphorylation of ligand gated ion channels were first determined for the nAChR. Tyrosine phosphorylation of this receptor caused an increase in the rapid rate of desensitisation on exposure to cholinergic ligands (Hopefield et al 1988). Tyrosine phosphorylation of this particular receptor is also thought to modulate its cell biology. Evidence exists which supports a central role of tyrosine phosphorylation in the clustering of muscle nAChR via the actions of Agrin and subsequently MuSK (DeChiara et al 1996; Glass et al 1996; Gillespie et al 1996). The existence of a physical link between acetylcholine receptors and tyrosine kinases has recently been established by immunoprecipitation, for Src and Fyn (Fuhrer and Hall 1996).

The demonstration that the NMDA receptor can also be regulated by tyrosine kinases and tyrosine phosphatases indicated that ligand gated ion channels within the central nervous system were open to this method of modulation. Tyrosine kinase inhibitors reduced NMDA mediated whole cell currents whilst intracellular application of c Src potentiate the currents (Wang and Salter 1994). Recent studies of the NMDA receptor have demonstrated a physical association by immunoprecipitation of this receptor and Src. The study also demonstrated that on activation of the Src by the addition of an activating peptide, an increase in the mean open time and open probability could be observed. Inhibitory antibodies to Src caused a decrease in the probability of opening and mean open time (Yu et al 1997).

The existence of a consensus site of tyrosine phosphorylation in the $\gamma 2$ subunit suggested that the $GABA_A$ receptor was also a potential target of tryosine kinase activity. In order to test this transient transfections of $\alpha 1$,

β1 , γ2 L (9E10) or γ2 S (9E10) and v src were performed. Immunoprecipitations from such transfections followed by antiphosphotyrosine western blotting were used to establish whether the γ2 subunit was tyrosine phosphorylated. The functional consequences of tyrosine phosphorylation were investigated electrophysiologically by Professor Trevor Smart.

3.3.4 The γ2 short protein migrates differently to the long splice variant on SDS Polyacrylamide electrophoresis.

Experiments performed to characterise the γ2 L FLAG and γ2 S (9E10) subunits in Chapter 3.1 indicated that the short splice variant of the γ2 subunit was larger, (50KDa), compared to the γ2 L FLAG subunit (45KDa). To check that it was not an artefact of the viral expression system or of the cells used to produce the protein a transient transfection of HEK 293 cells with a γ2 S (9E10) and a γ2 L (9E10) tagged PRK 5 eukaryotic expression constructs was conducted, followed by a (9E10) immunoprecipitation and a (9E10) western blot of the immunoprecipitate. The result of this experiment can be seen in Fig 33. The transient transfection produced identical results to that produced by viral infection. The long migrating at 45 and the short at 50KDa.

An explanation of this observation could be that the difference in sizes was due to differing N linked glycosylation. To address this question BHK cells were infected by the γ2 L FLAG and γ2 S (9E10) virions. The cells were then labelled with 200uCi / ml of ^{35}S methionine translabel three hours after infection in the presence of 5μg/ml tunicamycin which was added in order to block all N linked glycosylation. Cells were lysed and immunoprecipitation with either FLAG or (9E10) monoclonal antibody. The result of this experiment can be seen in Fig 34.

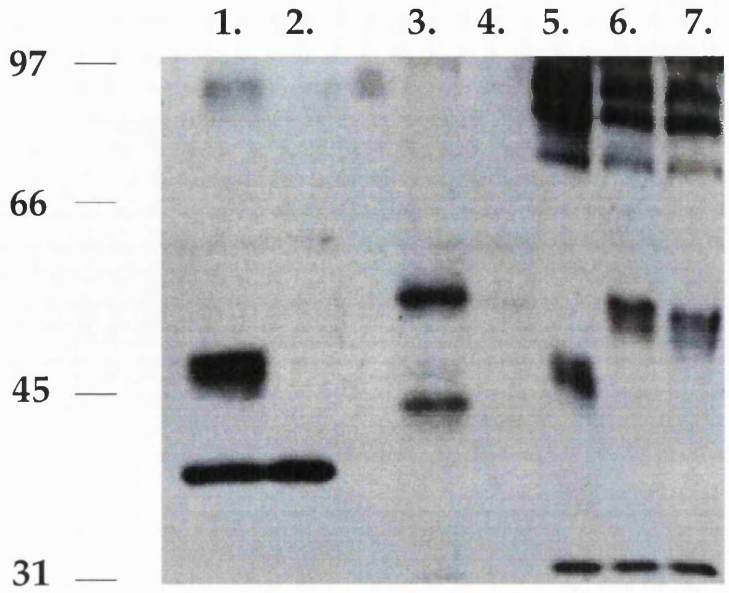


Fig 33. The γ2 S (9E10) subunit migrates differently from the γ2 L FLAG subunit on SDS-PAGE. Lane 1, γ2 L FLAG virally expressed in BHK cells detected by western blotting with the monoclonal antibody FLAG. Lane 2, non infected BHK cells acting as a control for the FLAG western blot. Lane 3, γ2 S (9E10) virally expressed in BHK cells, western blotted with the monoclonal antibody 9E10. Lane 4 non infected BHK cells also western blotted with the 9E10 monoclonal , acting as a control for the 9E10 blot. Lane 5, 9E10 western blot of a 9E10 immunopricipitate from HEK 293 cells exprssing the γ2 L (9E10). Lane 6 9E10 western blot of a 9E10 immunoprecipitate from HEK 293 cells expressing γ2 S (9E10). Lane 7 9E10 western blot of a 9E10 immunoprecipitate from HEK 293 cells expressing the γ2 S (9E10) tyrosine mutant. In lanes 5 to 7 the proteins described were expressed using the PRK5 expression plasmid rather than the viral system.

The unglycosylated γ2 S (9E10) protein could be clearly observed migrating at 45 KDa in size where as the non glycosylated γ2 L FLAG migrated with an apparent size of 31 KDa. Interestingly the 45 KDa size of the non glycosylated γ2 S (9E10) corresponded to a specific band in western blots of whole cell lysates from γ2 S (9E10) expressing cells compare with Fig 10 (B) . The difference in molecular size as determined by SDS PAGE was therfore not due to differing N linked glycosylation of the two proteins.

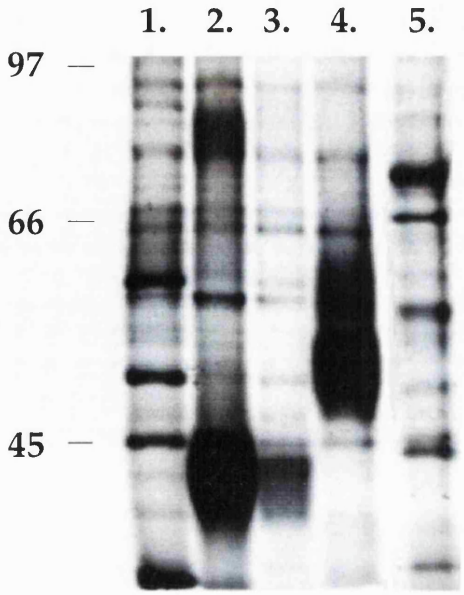
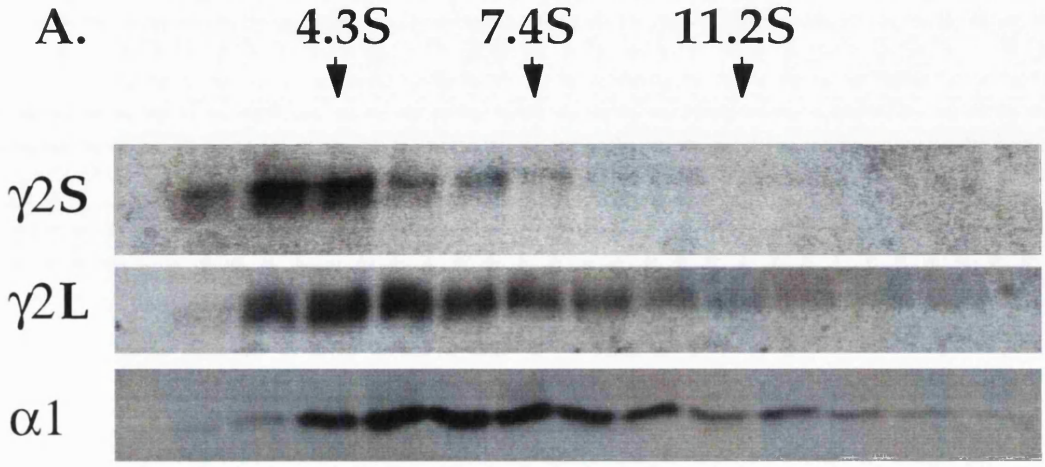


Fig 34. The γ2 S (9E10) and γ2 L FLAG proteins are glycosylated when they are produced using the Semliki expression system. Lane 1 FLAG immunoprecipitation from γ2 L FLAG infected BHK cells . The cells had been treated with tunicamycin (5μg/ml). Lane 2, repeat of FLAG immunoprecipitation on BHK cells infected with the γ2 L FLAG virus, this time in the absence of tunicamycin. Lane 3, 9E10 immunoprecipitation from BHK cells infected with γ2 S (9E10) in the presence of tunicamycin (5μg/ml). Lane 4 Repeat of 9E10 imunoprecipitation on γ2 S (9E10) infected cells in the absence of tunicamycin. Control Ig G immunoprecipitation from BHK cells virally infected with the γ2 S (9E10) virus in the presence of tunicamycin (5μg/ml).

3.3.5 The γ2 S (9E10) protein does not form the 5 S aggregate seen when the α1 (9E10) , β2 (9E10) or γ2 L FLAG proteins are individually expressed.

The α1 (9E10), β2 (9E10), γ2 S (9E10) and γ2 L FLAG subunits were individually virally expressed in BHK after three hours of infection the cells were labelled with 200μCi/ml of ^{35}S methionine for one hour. Cells were lysed and subjected to sucrose gradient ultracentrifugation. The gradient was fractionated and each fraction immunoprecipitated with of affinity purified monoclonal antibody. The precipitates were run on an 8% SDS PAGE gel which was subsequently , fixed, dried down and bands

quantified by a Biorad Phosphorimager. Fig35 (A) and (B) illustrates the profiles of the α1 (9E10), β2 (9E10), γ2 L FLAG and γ2 S (9E10) proteins. In



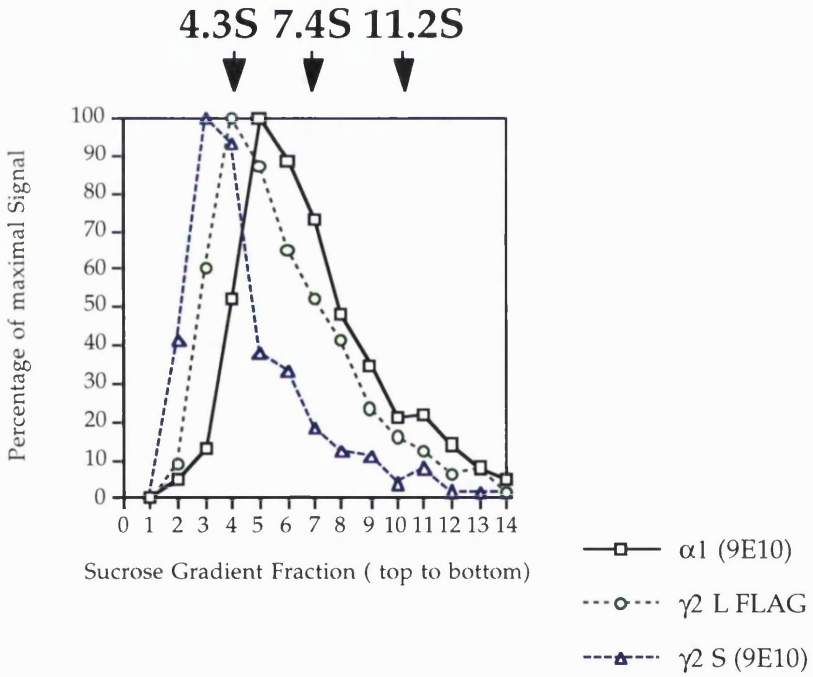


Fig 35 A.The γ2 S (9E10) protein does not form a 5S aggregate like the α1 (9E10) and β2 (9E10) subunits. BHK cells were seperately infected with the γ2 S (9E10), γ2 L FLAG and α1 (9E10) virus particles. Four hours after infection, the cells were labelled for one hour with 250μCi/ml ^{35}S methionine before being lysed. The lysate was then subjected to sucrose gradient centrifugation. The gradient was fractionated and each fraction immunoprecipitated with the monoclonal antibody FLAG or 9E10. The precipitates were resolved by SDS PAGE gel and the signals quantified by a Biorad phosphorimager. Panel B is a graphical representation of the data, signals being expressed as percentages of the maximal signal.

Fig35 (A) it is clear that the profile of the γ2 S (9E10) protein is quite different from that of the α 1 or γ2 L FLAG proteins. The γ2 S (9E10) profile is much sharper and peaks at a lower sedimentation coefficient, 3S as compared to 5S. The γ2 S (9E10) profile is quite different from that of the γ2 L FLAG, see Fig 35 (B). The γ2 L FLAG protein has a much broader profile similar to the α1 (9E10) and β2 (9E10) proteins, it also peaks at a higher sedimentation value of 4S.

3.3.6 The γ2 Short and Long subunits have differing abilities to access the cell surface.

BHK cells were infected individually by the γ2 S (9E10) and γ2 L FLAG virus particles. After an overnight incubation at 37°C the cells were fixed with 3% paraformaldehyde and examined immunocytochemicaly for the presence of the (9E10) and FLAG epitopes. Infected cells were kept intact or permeabilised with 0.1% NP40. The results of this experiment can be seen in Fig 36 (A, B and C). Only in permeabilsed cells could Flag antigenicity be detected, see Fig 36 (A) and (B). The pattern of the FLAG antigenicity was characteristic of an ER morphology. In contrast the γ2 S (9E10) signal could be found on the surface of intact cells, see Fig 36 (C) and in permeabilised specimens, where a punctate intracellular staining morphology was observed , see Fig 8 (D). This pattern of staining is characteristic of endosomal staining (Hopkins et al 1994).

3.3.7 The γ 2 S (9E10) subunit can endocytose from the cell surface.

In order to examine the possible endocytosis of the γ2 S (9E10) subunit BHK cells were infected with the γ2 S (9E10) virus. After an overnight incubation the cells were incubated for one hour in saturating amounts of (9E10) monoclonal antibody. γ2 S (9E10) subunits that passed via the surface of the cell would be exposed to the (9E10) antibody. Endocytosed γ2 S (9E10) protein could then be detected by performing immunocytochemistry on permeabilised cells. The immunofluorescence

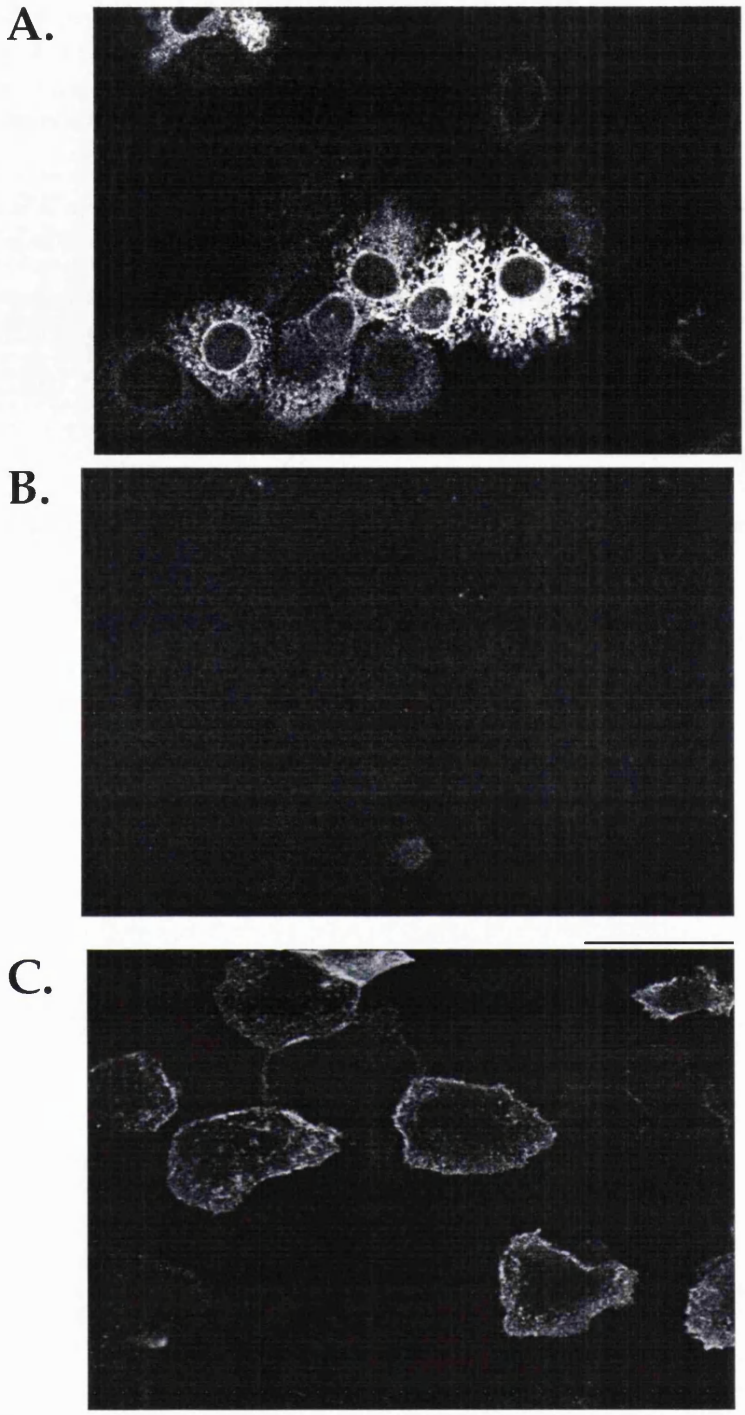


Fig 36. Immunoflurescence of BHK cells infected with the γ2 L FLAG and γ2 S (9E10) virus particles.BHK cells were infected with the γ2 L FLAG virus (A) and (B) or the γ2 S (9E10) virus (C). The cells were fixed and immunoflurescence conducted 16 hrs post infection. The FLAG monoclonal was used to perform immunofluorecence on the γ2 L FLAG infected cells in the presence (A) and absence of detergent (B). The 9E10 monoclonal antibody was used to perform immunofluorescence on the γ2 S (9E10) infected cells in the absence of detergent (C). Scale bar represents 50μm.

was performed using only the fluorescene conjugated secondary anti mouse antibody. This would detect endocytosed (9E10) bound to endocytosed γ2 S (9E10) protein. Fig 37 illustrates the results of this experiment. The punctate staining is characteristic of an endosomal /lysosomal morphology, see Fig 37 (A) and(B).

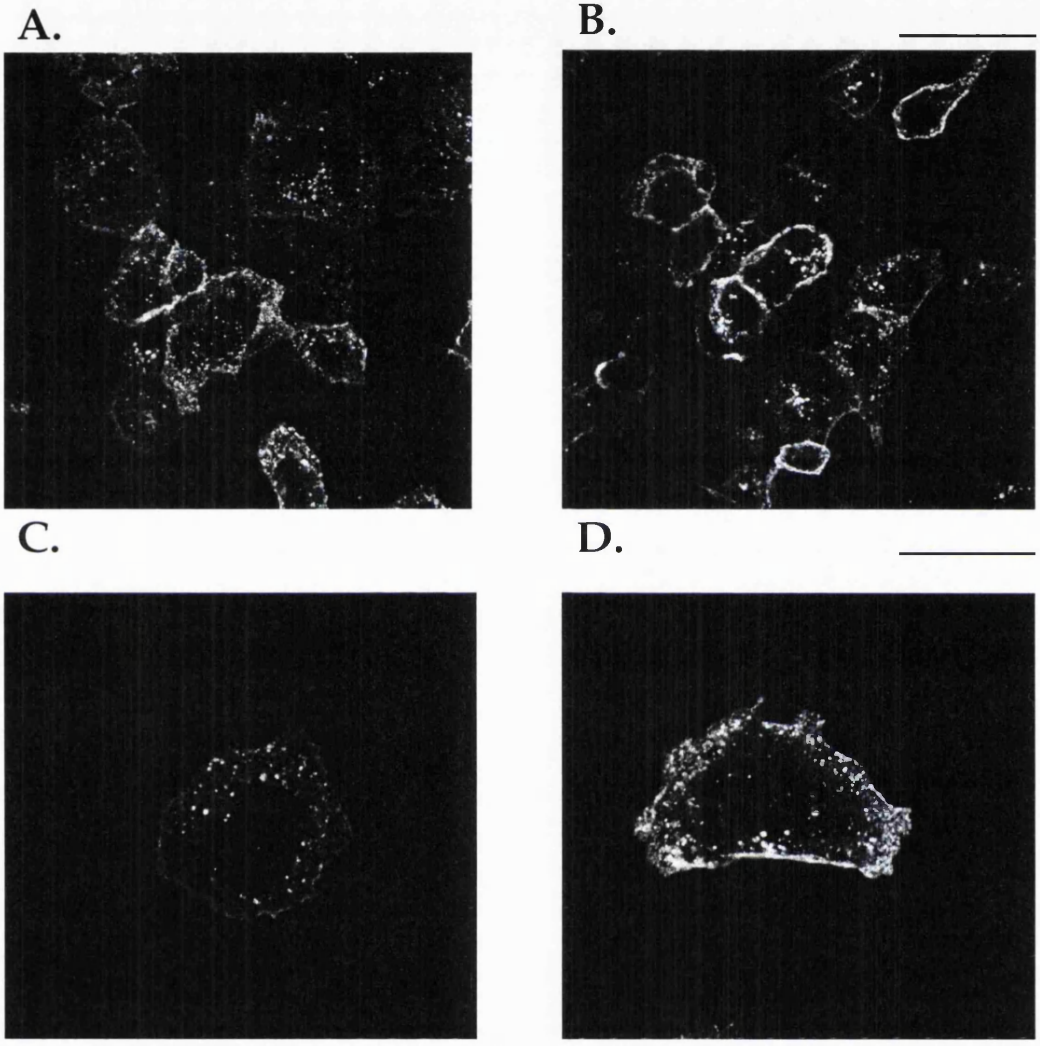


Fig 37. The γ2 S (9E10) subunit can endocytose from the plasmamembrane of BHK cells. BHK cells were infected with the γ2 S (9E10) (A)and (B) or the γ2 S (9E10) double tyrosine mutant virus (C) and (D). Cells were incubated in 9E10 containing culture medium for one hour at 37°C. The cells were then fixed and permeabilised. Immunofluorescence was then performed using a secondary anti mouse antibody. Upper scale bar 50μm, lower scale bar 25μm.

An identical experiment was performed with a virus that produced a mutated form of the γ2 S (9E10) protein which had the two tyrosines at

the concensus site of phosphorylation mutated to phenylalanine. The two tyrosines conformed to a potential endocytosis motif (Trowbridge et al 1993, Marks et al 1997). As can be seen in Fig 37 C and D the tyrosine mutant produced a similar pattern indicating that it too was capable of trafficking to the surface and subsequently being endocytosed. An alternative explanation for these results would be that the divalent antibody was crosslinking receptor subunits and causing them to be endocytosed. The saturating levels of antibody that were used would argue against this alternative explanation. Under such conditions the antibody epitope interactions would be monovalent and crosslinking of receptors would occurr less frequently.

3.3.8 Electron microscopy of BHK cells infected by the γ2 S (9E10) virus and BHK cells which had been co-infected with α1 and β2 virus particles.

The difference in pattern between the pattern of immunofluorescence that was seen for the γ2 S (9E10) and the pattern observed in cells which had been co-infected with α1 (9E10) and β2 (9E10) virus was further studied using whole mount immuno-gold electron microscopy. Using this approach it is possible to compare the differing distributions at a higher resolution and allow further observations to be made.

BHK cells were infected at a multiplicity of 10 with either the γ2 S (9E10) virus or co-infected with α1 (9E10) and β2 (9E10) virus particles. The cells were incubated overnight and then fixed. The presence of the (9E10) epitope was then determined by incubating the intact cells with (9E10) and then localising the (9E10) with anti-mouse conjugated gold. The specimens were then prepared for whole mount electron microscopy. Ms Adele Gibson kindly prepared these specimens and performed the electron microscopy. The results of the experiments are illustrated in

Fig 38 A. BHK cell infected with α1 (9E10) and β2 (9E10) virus particles. Fixed and processed for whole mount, 5nm Gold immunogold microscopy. Scale bar is equivalent to 1μm. Figure printed as a negative.

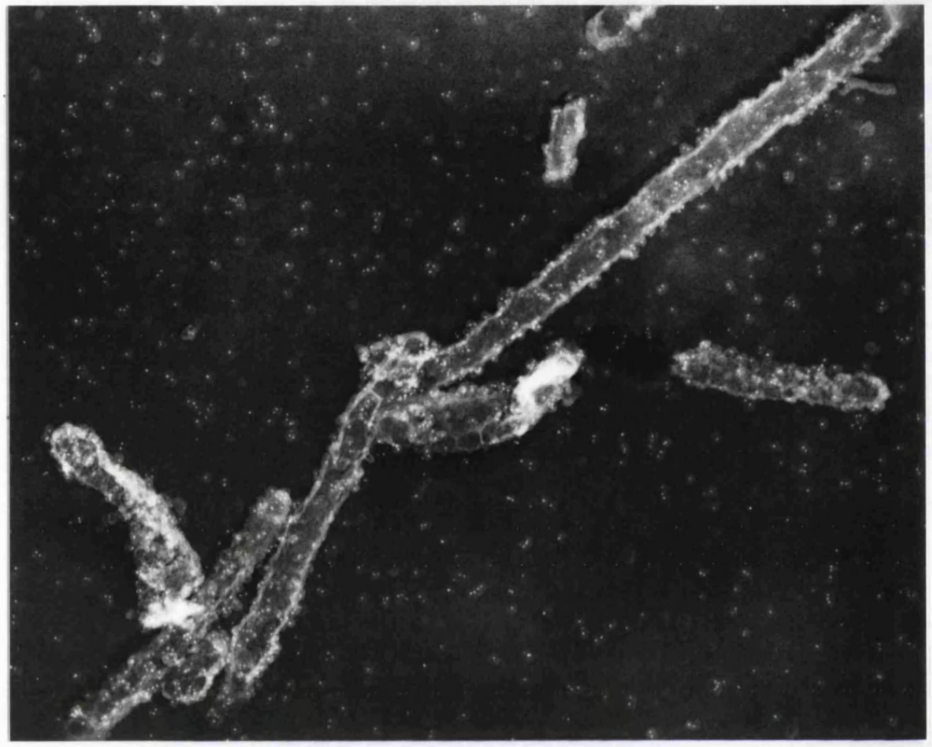


Fig 38 B. BHK cell infected with α1 (9E10) and β2 (9E10) virus particles. Fixed and processed for whole mount, 5nm Gold immunogold microscopy. Scale bar is equivalent to 1μm. Figure printed as a negative.

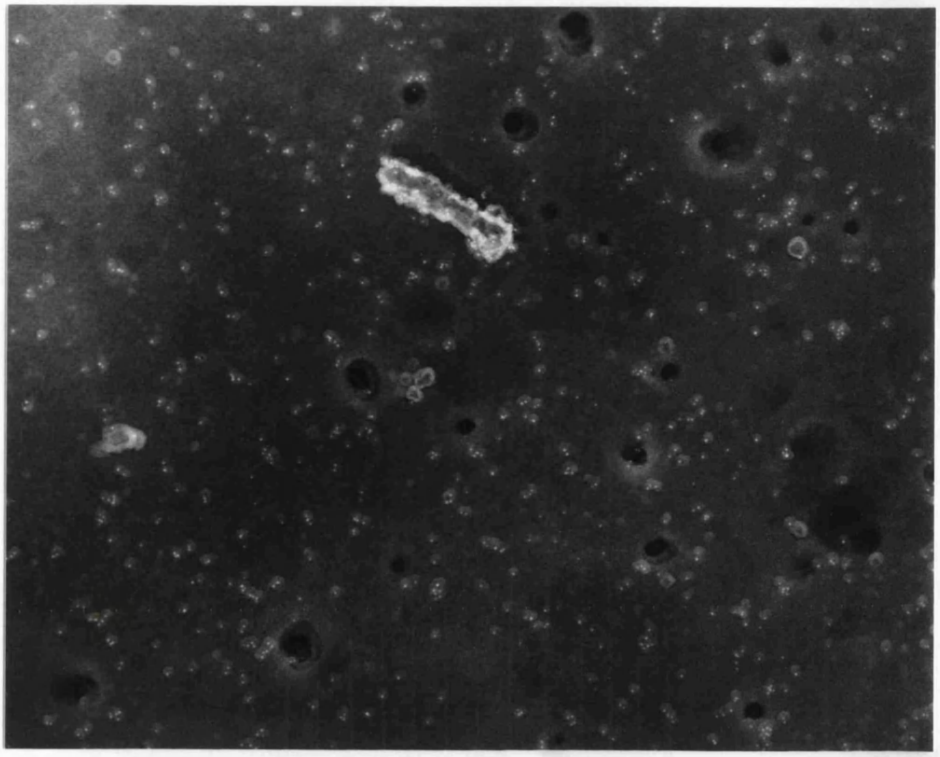


Fig 39 A. BHK cells infected with γ2 S (9E10). Fixed and processed for whole mount, 5nm Gold immunogold microscopy. Scale bar represents 1μm. Figure printed as a negative.

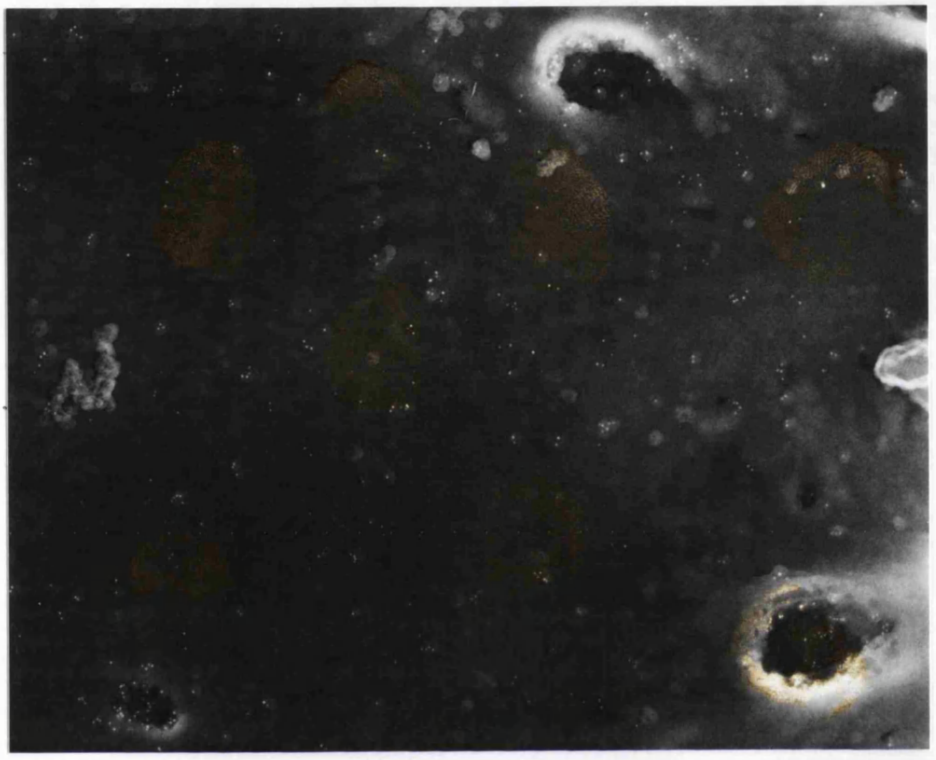
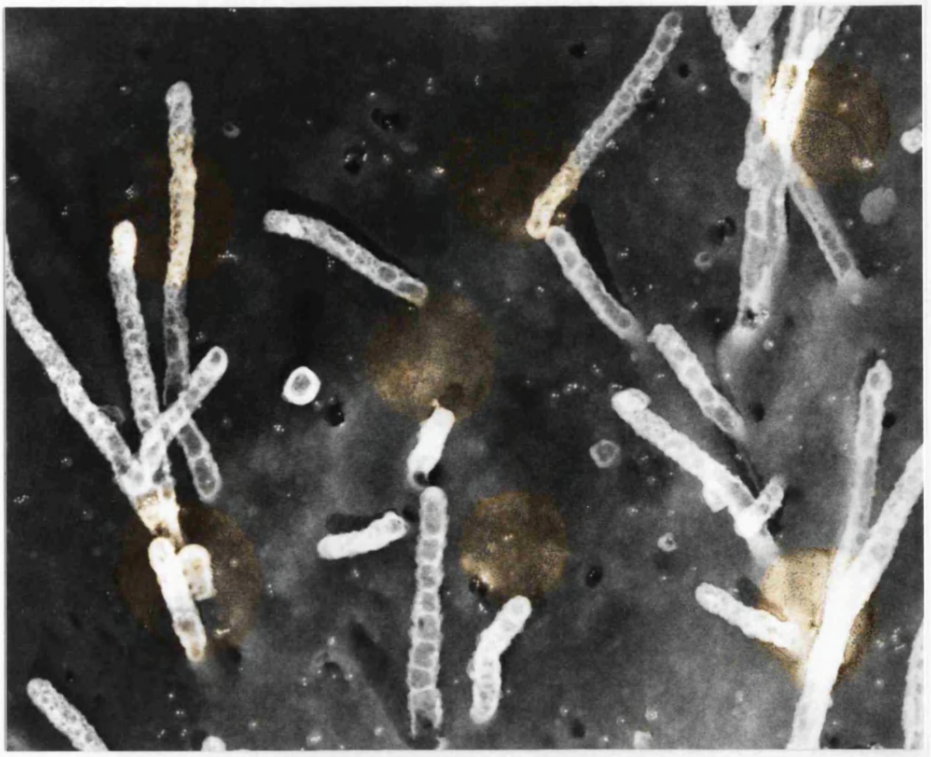


Fig 39 B. BHK cells infected with γ2 S (9E10). Fixed and processed for whole mount, 10nm Gold immunogold microscopy. Scale bar (as above) represents 1μm. Figure printed as a negative.



Figs 38 and 39 . Several observations can be made. It is clear that less (9E10) signal is detectable on the surface of the cells infected by the γ2 S. (9E10) virus, Fig 39, compared to cells co-infected with the α1 (9E10) and β2 (9E10) virus particles, Fig 38 . Secondly the gold particles seen in the cells infected with the γ2 S (9E10) virus are associated with coated pits, marked by arrows. see Fig 39 A and B, whilst no association can be seen in the cells infected with α1 (9E10) and β2 (9E10) virus particles, see Fig 38 B. This pattern is very similar to the distribution of transferrin receptors

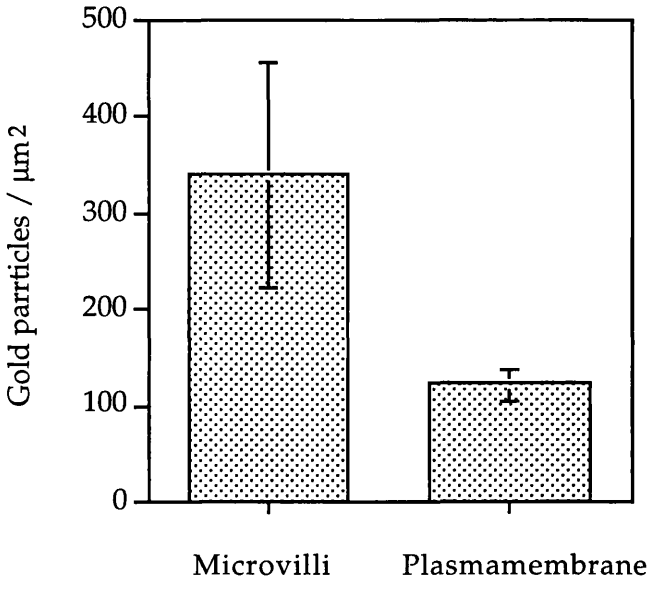


Fig 40. α1 (9E10) β2 (9E10) receptors on the surface of BHK cells are preferentially distributed to the microvilli. BHK cells were co-infected with α1 (9E10) and β2 (9E10) virus particles. The cells were fixed 24 hours post infection and analysed by whole mount 9E10 immunogold electron microscopy. The density of gold particles per μm^2 was determined for the cell surface and microvilli.

which are constitutively endocytosed (Hopkins 1985, Jing et al 1990, Trowbridge et al 1993.). In contrast to the γ2 S (9E10) the distribution of the α1 (9E10) and β2 (9E10) proteins are not associated with coated pits but do have an association with the cells microvilli, see Fig 38 A. This is shown quantatively in Fig 40 , where the density of gold labelling on a

cell's microvilli and plasmamembrane have been compared. The average labelling for the microvilli was 339 gold particles per μm^2 compared with the density of 121 gold particles per μm^2 for the plasma membrane illustrates the different densities of gold labelling. The differing densities are statistically significant $p<0.0005$.

3.3.9 The γ2 short subunit is not limited to the cell body of neurones.

The marked difference in cell biology demonstrated in BHK cells between the long and the short splice variants of the γ2 subunit were pursued in primary cultures of neurones. Superior cervical ganglion neurones (SCG) were infected with the γ2 L FLAG and γ2 S (9E10) virus particles. Fig 41 (A) and (C) illustrates the different distributions of the two proteins. The γ2 S (9E10) protein can be clearly seen to be present in the neuronal cell body and distributed out into the processes, Fig 41 (A). Marked clustering of signal could also be observed, marked by arrows. In contrast the long isoform was confined to the cell body Fig 41 (C). The γ2 L FLAG pattern of distribution was similar to that of the α1 (9E10) and β2 (9E10) subunits when they were individually expressed in SCG neurones, compare with Fig 41 (A) and (B).

The γ2 short (9E10) virus was also used to infect cortical neurones from mice devoid of the γ2 subunit. This was conducted in Hans Moller's laboratory. Fig 41 (B) illustrates an example of the distribution of the γ2 S (9E10) protein when virally expressed in mouse cortical neurones. The fluorescent signal can be detected in the processes of the neurones. The pattern is similar to that observed when the γ2 S (9E10) subunit is expressed in the SCG neurones. Varicosities within the dendrites appeared to accumulate the γ2 S (9E10) subunit. The tapering nature of the main process arising from the cortical neurone illustrated is characteristic of dendrites (Craig and Banker 1994).

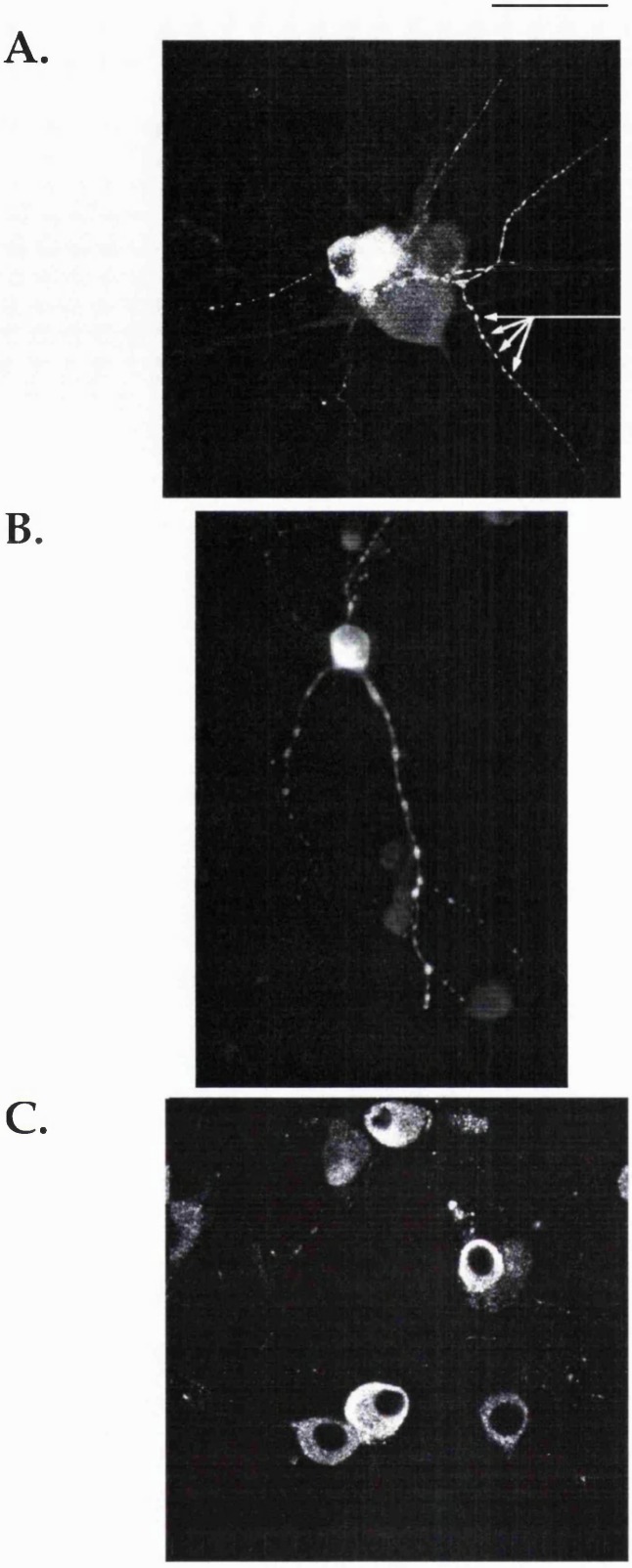


Fig 41.Viral expression of the γ2 S (9E10) and γ2 L FLAG subunits in neurones. Panel A. SCG neurones were infected with the γ2 S (9E10) virus. The neurones were fixed and permeabilsed 20 hours post infection. Immunofluorescence was performed using the 9E10 monoclonal. Panel B. Cortical neurones isolated from mice which had

their γ2 gene disrupted (Gunther et al 1995) were infected with the γ2 S (9E10) virus. Immunofluorescence was performed at 20 hours post infection in the presence of detergent with the 9E10 monoclonal. Panel C. SCG neurones were infected with the γ2 L FLAG virus. The cells were fixed and permeabilised 20 hours post infection. Immunofluorescence was performed using the FLAG monoclonal.

3.3.10 Viral expression of the γ2 short subunit in neurones cultured from mice devoid of the γ 2 subunit.

Hans Moller's laboratory had produced a transgenic mouse that is devoid of γ2 subunits. Cortical cultures were taken from these animals by Mr Christian Essrich. The cortical neurones were infected by the γ2 S (9E10) virus and electrophysiological recordings made to ascertain whether the virus was capable of restoring benzodiazepine pharmacology to these neurones. Immunohistochemistry was conducted to check that the neurones were expressing the recombinant subunit. (9E10) antigenicity was observed, see Fig 41 (B) but it was not possible to detect flunitrazepam potentiated GABA gated chloride currents . The inability to restore GABA currents that were potentiated by benzodiazepines can be explained by the observations made when SCG neurones were infected with either α1 (9E10) or β2 (9E10) virus particles. When such infections were performed it was not possible to demonstrate the assembly of endogenous subunits with the viral expressed subunits. The most likely explanation for this result is that the viral expression system was flooding the host cell's biosynthetic machinery with viral RNA encoding the γ2 S (9E10) subunit, inhibiting the production of endogenous subunits and their oligomerisation with the virally expressed subunit. This an important limiting feature of this expression system.

3.3.11 The γ 2 subunit can be tyrosine phosphorylated.

The existence of a consensus site for tyrosine phosphorylation, (Songyang et al 1995), within the intracellular loop of the γ 2 subunit at

tyrosine residues 365 and 367 for the short splice variant and tyrosine residues 373 and 375 for the long splice variant suggested that this protein could be phosphorylated at these residues *in vivo*. In order to determine whether this occurred within cells constructs were engineered which had the tyrosine residues mutated to phenylalanines. Wild type γ 2 subunits, short or long , were co-expressed with the α1 and β 2 receptor subunits in Human Embryonic Kidney cells.(HEK 293) The v-src tyrosine kinase was also co-transfected with these subunits in to the HEK cells . This was necessary since HEK cells have very low levels of tyrosine kinase activity (Herbst et al 1991) see Fig 42 (A).

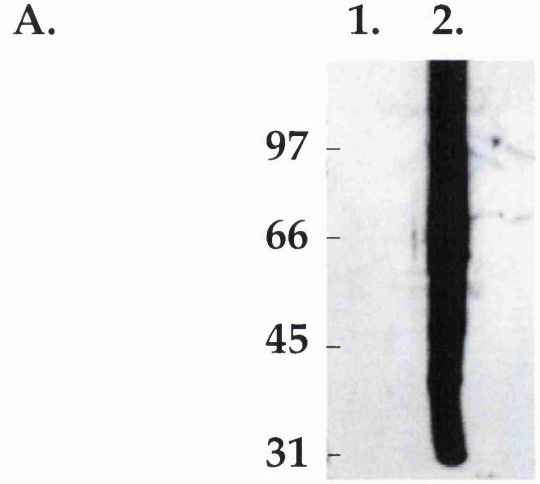


Fig 42 A . HEK 293 cells have low levels of tyrosine kinase activity. HEK 293 cells were transiently transfected with PRK 5 v-src and then lysed in sample buffer. Control untransfected 293 cells were also lysed in sample buffer. The lysates were seperated by SDS-PAGE and then transferred to nitrocellulose. This was then western blotted with a rabbit polclonal antiphosphotyrosine antibody. Lane 1, untransfected, Lane 2, transfected with v-src.

In parallel to the transfection of wild type γ2 subunits the mutated γ 2 subunits, devoid of the tyrosines at the conscensus site of phosphorylation were also co-expressed in 293 cells with α1 β1 and v src. In order to facilitate biochemical and immunohistochemical analysis the γ2 subunits were tagged at the amino terminus with the 9E10 epitope. The cells were allowed to express the tyrosine kinase and $GABA_A$ receptor subunits for 24 hours before being lysed and the γ2 subunits

immunoprecipitated with the monoclonal antibody 9E10. The immunoprecipitates were then subjected to polyacrylamide electrophoresis and subsequently transferred to nitrocellulose. The blot was then assayed for tyrosine phosphorylation using polyclonal anti-phosphotyrosine antibodies. The results of this experiment are illustrated in Fig 42 (B) .

Fig 42 (B) demonstrates that the γ2 L (9E10) subunit can be tyrosine phosphorylated in cells expressing high levels of the tyrosine kinase src. A specific tyrosine phosphorylated band can be seen migrating at 45KDa in lane 3 of Fig 42 B. This corresponds to the size of the γ2 L FLAG protein by FLAG western blotting see Fig 42 (B) lane 5 and by immunoprecipitation, see Fig 33. Expression of the tyrosine mutant

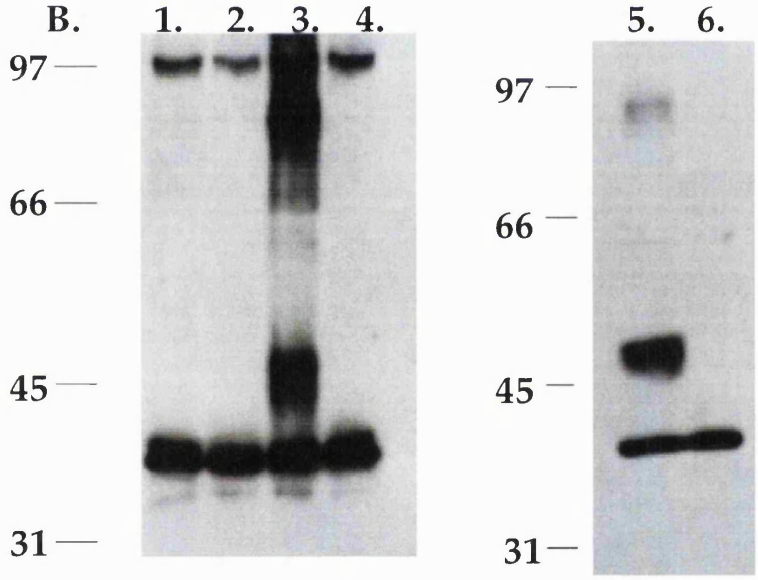


Fig 42 B. The γ2 L (9E10) subunit can be tyrosine phosphorylated. HEK 293 cells were transiently transfected by electroporation with PRK5 v-src alone, Lane 1, PRK5 v-src, α1, β1 and γ2 L (9E10) double tyrosine mutant, lane 3 PRK 5 v-src, alpha1, beta 1 and gamma 2 long my wild type. Lane 4 PRK 5 α1, β1 and γ2 L (9E10) wild type. The cells were lysed 24 hours after transfection and the receptors immunoprecipitated with the monoclonal antibody 9E10. The precipitates were seperated by SDS-PAGE and the gel western blotted with a rabbit polyclonal antiphosphotyrosine antibody. For orientation a FLAG western blot of BHK cells infected with the γ2 L FLAG virus (Lane 5) or not infected (lane 6) is also presented. Migration of protein standards is shown, units are KiloDaltons.

γ2 L (9E10) subunit results in the loss of the tyrosine phosphorylation species in lane 2. Lane 4 correspond to cells in which the wild type γ2 L (9E10) was expressed but no v src co-expressed. This also results in the loss of the phosphotyrosine signal at 45KDa. Finally lane 1 of the phosphotyrosine western blot correspond to cells transfected with v src alone with no $GABA_A$ subunits. This lane also has no phosphotyrosine signal at 45 KDa.

In order to determine if the short splice variant of the γ2 subunit could also be tyrosine phosphorylated in cells a similar experiment was performed as that used to demonstrate tyrosine phosphorylation of the γ2 L (9E10) subunit. The results of this experiment are shown in Fig 43.

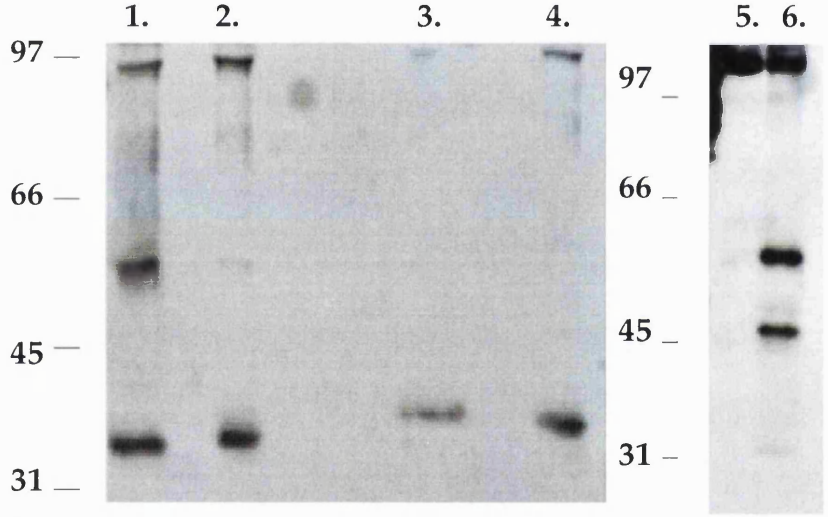


Fig 43. The γ2 S (9E10) subunit can be tyrosine phosphorylated. HEK cells were transiently transfected by electroporation with PRK 5 v-src, α1, β1 and γ2 S (9E10) wild type lane 1, PRK 5 v-src, α1, β1 and γ2 S (9E10) double tyrosine mutant , lane 2, PRK5 α1, β1 and γ2 S (9E10) wild type lane 3 and PRK 5 v-src alone lane 4. The cells were lysed 24 hours after transfection and the receptors immunoprecipitated with the monoclonal antibody 9E10. The precipitates were seperated by SDS-PAGE and the gel western blotted with a rabbit polyclonal antiphosphotyrosine antibody. For orientation a 9E10 western blot of BHK cells infected by the γ2 S (9E10) virus (lane 6) or not infected (lane 5) is also presented. Migration of molecular weight standards is indicated, units are KiloDaltons.

A band can be observed in lane 1 migrating at about 50KDa which corresponds to the size of the γ2 S (9E10) protein, a 9E10 western blot of

cells expressing the γ2 S (9E10) protein is shown in lane 6 for comparison. Lane 2 of the phosphotyrosine blot is an immunoprecipitate from cells which expressed the γ2 S (9E10) tyrosine mutant, v-src, α1 and β1. Expression of the mutant results in the loss of the phosphotyrosine signal at 50KDa. Lanes 3 and 4 are controls, lane 3 corresponds to cells expressing wild type γ2 S (9E10), α1, and β1 but no v-src and lane 4 corresponds to cells expressing v-src alone. No phosphotyrosine signal can be seen in either of these control lanes at 50KDa. It can be concluded that the γ2 S (9E10) protein can also be tyrosine phosphorylated in HEK cells expressing high levels of v-src. Evidence from whole cell orthophosphate labelling experiments performed by Dr Stephen Moss support this data (Moss et al 1995).

3.3.12 Poor specificity of polyclonal anti γ2 phosphotyrosine antibodies.

It would be desirable to determine biochemically whether endogenous $GABA_A$ receptor γ2 subunits are tyrosine phosphorylated. This was addressed by the production of antibodies designed to detect the tyrosine phosphorylated γ2 subunit. Immunohistochemical assays using such phosphospecific antibodies could also be used to study the effect of a physiological stimulus and its consequences upon the tyrosine phosphorylation state of neuronal $GABA_A$ receptors.

Antibodies were raised in guinea pigs to a tyrosine phosphorylated peptide. In order to test the specificity of the antibodies HEK 293 cells were transfected with v src, α1, β2 and γ2 L (9E10) wild type or tyrosine mutants. Unfortunately as can be seen from Fig 44. the antibodies produced lacked specificity. Some cells which contained no red signal, corresponding to the 9E10 tagged wild type γ2 subunit did contain green signal, see Fig44 (A) and (B). When the tyrosine mutant for the γ2 subunit replaced the wild type , Fig 44 (C) and (D), yellow signal was observed indicating that the yellow signal observed in panels A and B

was not purely due to the specific phosphoryation of the γ2 protein. It was interesting to observe that the phosphotyrosine signal was associated with the plasma membrane. Src is known to associate with the plasmamembrane so the membranous pattern may be due to the antiphosphotyrosine antibody detecting src which autophosphorylates itself at tyrosine residues (Bolen 1993). The poor specificity of these antibodies prevented this direct approach of answering whether the γ2 subunit of native neurones is tyrosine phsosphorylated.

It is hoped in the future that this question can be answered by using an antibody directed against the native γ2 subunit and precipitating this from brain lysates and then blotting the precipitate with phosphotyrosine antibodies. Evidence to suggest that the endogenous γ2 protein is tyrosine phosphorylated has recently been produced (Personal communication from Mr Nick Brandon).

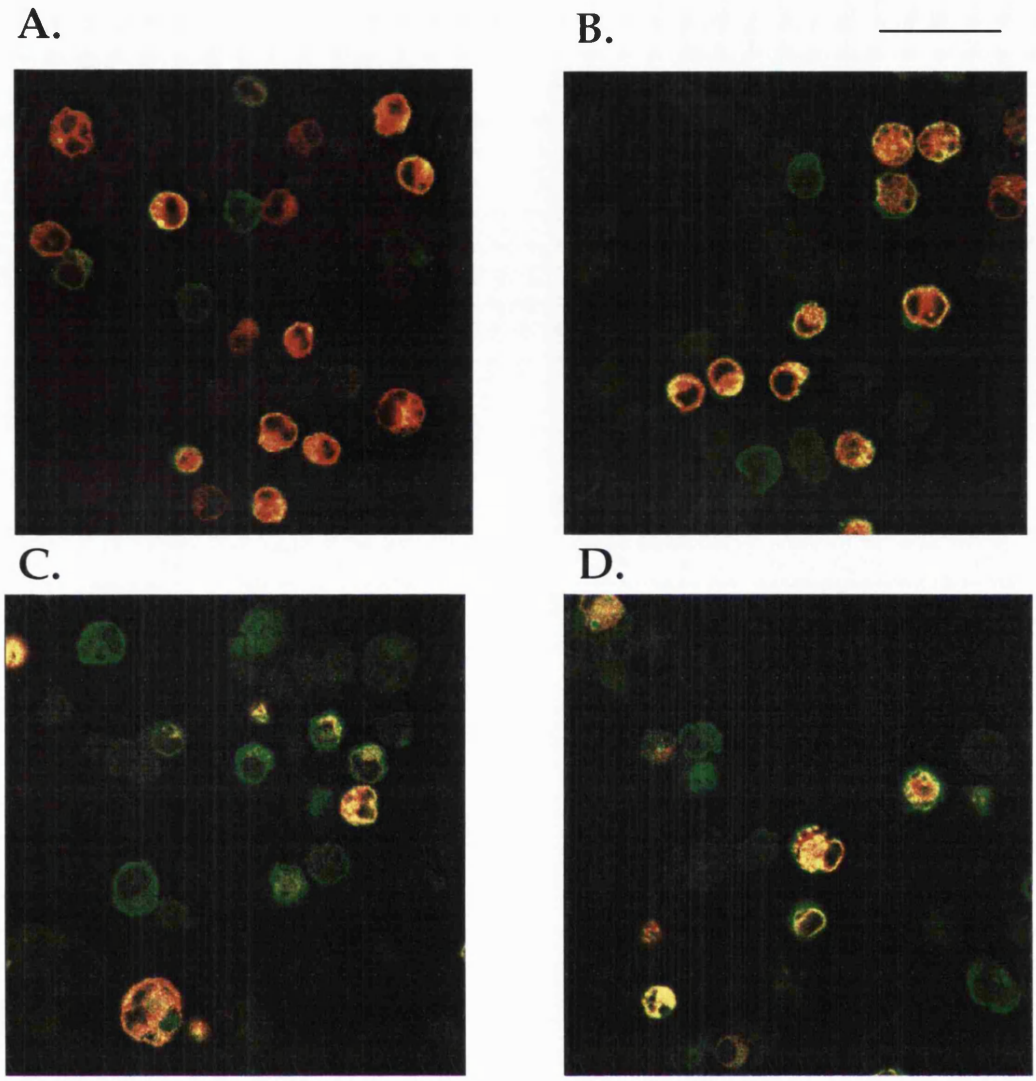


Fig 44. Poor specificity of guinea pig anti γ2 phosphotyrosine antibodies. HEK cell were transiently transfected with PRK5 v-src, α1, β2 and γ2 S (9E10) wild type panels A and B. or PRK 5 v-src, v-src, α1, β2 and γ2 S (9E10) double tyrosine mutant panels C and D. Double immunofluorescence was conducted on the transient transfections. The primary antibody used to detect the γ2 S (9E10) was the mouse monoclonal 9E10. This was detected using a rhodamine anti mouse secondary. The affinity purified guinea pig anti γ2 phosphotyrosine antibody was detected using a fluorescene anti guinea pig secondary. Scale bar equals 50μm.

3.3.13 Discussion.

The behaviour of the γ2 L FLAG and γ2 S (9E10) subunits is quite different , yet the two proteins differ by only eight amino acids. One potential explanation could be that the long isoform misfolds, subsequently binding chaperone proteins. This would result in the retention of the γ2 L FLAG subunit within the endoplasmic reticulum and an increase in the sedimentation coefficient of this isoform of the protein. The chaperone protein, immunoglobulin binding protein (BIP) has been demonstrated to co-precipitate with the γ2 L (9E10) subunit when it is expressed in HEK 293 cell (Connolly et al 1996a). In contrast the γ2 S (9E10) can fold and subsequently exit the endoplasmic reticulum. This may explain the sharper sucrose gradient profile and lower sedimentation coefficient compared with the long splice variant. It is interesting to note a similarity between the sucrose gradient profile of the γ2 S (9E10) $GABA_A$ subunit and that of the assembly competent monomeric α subunits of the nAChR as determined by high affinity (1nM) bungerotoxun binding (Paulson et al 1991).

The γ2 S is not the only $GABA_A$ subunit which does not require the presence of other $GABA_A$ subunits in order for it to exit the endoplasmic reticulum. The β3 subunit also demonstrates this capability but in contrast to the γ2 S protein it can form a chloride ion channel when it alone is expressed in HEK cells (Connolly et al 1996b). The sucrose gradient profile of the β3 protein is also completely different from the γ2 S protein. It is found to migrate in fractions 10 and 11 (Personal communication, Miss Pamela Taylor). This is in keeping with its capacity to form an ion channel. The γ2 S subunit is therefore the only $GABA_A$ subunit so far characterised that can translocate to the cell surface as a monomer.

With this marked difference in the cell biology of the two splice variants one would expect variations in the distribution of the two proteins within the central nervous system. Regions of the rat brain can be identified which have higher levels of the short form compared to the long isoform and vice versa by in situ hybridisation and immunocytochemical techniques . For example the cerebellar Purkinje cells are reported to be enriched in the the γ2 L splice variant whereas the granule cells of the dentate gyrus have more γ2 S (Miralles et al 1994). Other studies have described the distributions of the two isoforms as similar but with varying relative intensities of immunoreactivity (Gutierrez et al 1994). Age dependant variation of the two isoforms of the γ2 subunit has also been observed by immunocytochemistry. Old rats have been reported to have increased levels of the long isoform within the cerebral cortex whilst the levels of the short form have been shown to decrease with age (Khan et al 1996 and Gutierrez et al 1996). The levels of the long isoform of the γ2 subunit have been reported as being negligible at birth but rise to adult levels during the first month after birth as assayed by a reverse transcription polymerase chain reaction (Bovolin et al 1992).

The marked difference in distribution of the γ2 L FLAG and γ2 S (9E10) proteins within superior cervical ganglion neurones is also interesting. The clustered distribution suggests that this subunit is capable of interacting with anchoring molecules. Unlike the Potassium channel (Kim et al 1995) , NMDA (Kim et al 1996), AMPA (Dong et al 1997) or the glycine receptor (Meyer et al 1995) no anchoring molecules have thus far been determined for the $GABA_A$ receptor. The differential distribution of the α2 and α1 subunits also argues for a mechanism of localising subclasses of $GABA_A$ receptor dependant on their subunit composition (Nusser et al 1996). Interestingly whole mount electron microscopy of

BHK cells infected by α1 (9E10) and β2 (9E10) virus particles demonstrated that there was a significantly greater density of subunits localised to the microvilli of the BHK cells. It could be hypothesised that an interaction with the cytoskeleton is occuring at the microvilli. In contrast the γ2 S (9E10) subunit appears to be associated with coated pits. Once at the surface the γ2 S (9E10) subunit is capable of being endocytosed. This was demonstrated by antibody feeding experiments and electron microscopy. The motif that is recognised as a endocytosis signal could be the GYECL . This sequence is found in the intracellular loop of the γ2 subunit and the tyrosine corresponds to tyrosine 367 which is known to be capable of being phosphorylated. The glycine preceding the tyrosine has been shown to be associated with targeting to the lysosome. The state of phosphorylation of the tyrosine is important , active sorting only occurring when the tyrosine is *not* phosphorylated (Marks et al 1997).

Unfortunately when the two tyrosines are mutated to phenylalanines the subunit is capable of undergoing endocytosis. This would tend to indicate that this motif was not important in directing the internalisation of this protein from the cell surface. These results should be interpreted with caution since the mutation of the tyrosine to a phenylalanine may not necessarily remove the endocytosis motif. Work performed on the Insulin like Growth factor II receptor found that replacement of the tyrosine residue by phenylalanine in the endocytosis motif did not prevent endocytosis where as mutation to alanine did (Canfield et al 1991).

The possibility that endocytosis is being potentiated in the absence of tyrosine phosphorylation is interesting since it correlates with functional studies performed on the $GABA_A$ receptors in SCG neurones. Exposing such neurones to genistien a tyrosine kinase inhibitor causes a

time dependant decrease in whole cell GABA chloride currents (Moss et al 1995). It could be proposed that the lack of phosphorylation of tyrosine 367 will result in the down regulation of the $GABA_A$ receptor. The localisation of this putative down regulation domain may be exquisitely sensitive to changes in quaternary structure. Evidence for the sensitivity of such motifs is illustrated by the disruption of lysosomal targeting of Lamp 1 when its endocytosis motif is displaced by one amino acid (Marks et al 1997). One could then hypothesise that changes in the quaternary structure of the $GABA_A$ receptor could alter the capacity for the tyrosine 367 to be phsophorylated and or alter the recognition of this motif as a down regulation signal. Knowing that changes in the quaternary structure of the ligand gated ion channels occur on binding ligand (Unwin 1995) one could postulate that ligand binding could be transduced into down regulation by a change in the quaternary structure of the channel that allows the motif to be recognised as endocytosis signal and or prevents it from being phsophorylated.

The results of this chapter indicate that the tyrosines at 365 and 367 can be phosphorylated *in vivo* in both the γ2 L and S subunits when co-expressed with α1 and β1subunits. The consequences on the function of the receptor were investigated by Professor Smart. His work indicated tyrosine phosphorylation of $GABA_A$ receptors expressed in HEK 293 cells caused an increase in GABA mediated whole cell current. Such modulation could be abolished if the recordings were made from cells in which the wild type γ2 L subunit was substituted with a γ2 L (9E10) subunit which had the tyrosines 365 and 367 mutated to phenylalanines. Studies performed in SCG neurones paralleled the work in 293 cells, with tyrosine phosphorylation causing an increase in whole cell currents whilst tyrosine dephosphorylation mediated by blocking tyrosine kinase activity with genistein caused a decrease in whole cell current. The

mechanism for this change in whole cell current was investigated by single channel analysis of GABA channels in SCG neurones. Tyrosine phosphorylation mediated by the perfusion of c Src and vanadate caused the mean open time and probability of channel opening to rise. No change in channel conductances were observed (Moss et al 1995).

Chapter 4. Final Discussion.

This thesis describes the development and application of a viral vector capable of expressing $GABA_A$ subunits within BHK cells, SCG neurones and organotypic brain slice preparations. The recombinant virus particles dedicated to the production of the α1 (9E10), β2 (9E10), γ2 L FLAG and γ2 S (9E10) were shown to be capable of producing proteins of the appropriate molecular weight. The virally produced proteins were also shown to be glycosylated.

The potential toxicity of the viral expression was illustrated by its rapid inhibition of host biosynthesis, which occurred within 3-4 hours after infection. Apoptosis had also been reported to occur when neurones from new born rats were infected by Sinbis virus (Griffen et al 1994a). The toxicity of the viral expression system was studied by infecting SCG neurones with a recombinant Semliki virus producing Betagalactosidase. The nuclear integrity of the infected neurones was then evaluated using propidium iodide staining (Barres et al 1992 and Coles et al 1993) and found to be intact 18 hours post infection. In order to evaluate the physiological consequences of Semliki viral infection upon neurones a virus was engineered to express a mutated form of tthe Green Fluorescent Protein (Heim et al 1995). Infection by this virus could be detected by exposing the neurones to blue light. The infected neurones were identified by their green fluorescence without the addition of exogenous substrates or fixation. The infected cells had normal resting membrane potentials and retained the ability to discharge action potentials.

The Semliki expression system had previously been directed at the expression of individual proteins (Liljestrom and Garoff 1991, Dehoop et al 1995, Simons et al 1995, Lundstrom et al 1995, Patel et al 1996, Lundstrom et al 1997). There were no reports of the expression system

being used to produce a heteroligomeric protein. Experiments were conducted using mixtures of the α1 (9E10) and β2 (9E10) virus particles which illustrated that such double infections could produce $GABA_A$ receptors at the plasmamembrane as defined by immunohistochemical, pharmacological and electrophysiological methods. The functional and pharmacological characteristics of the virally produced $GABA_A$ receptors were similar to those produced by other transient expression systems (Connolly et al 1996a). Triple infection could be demonstrated using the GFP, α1 and β 2 virus particles. However it was not possible to demonstrate triple infection by α1 (9E10), β2 (9E10) and γ2 L FLAG virus particles. The feasibility of triple of infection is clear as demonstrated by the success of the GFP, α1 (9E10) and β2 (9E10) experiment. This did not test the ability of this system to support the oligomeristion of three separate membrane proteins, since the Green fluorescent protein is a cytoplasmic protein. The titres of the γ2 S (9E10) virus preparations were consistently tenfold less than those produced for the α1 (9E10) and β2 (9E10) subunits which may have been a contributing factor.

Knowing that this system was capable of supporting the assembly of α1 (9E10) and β2 (9E10) subunits it was used to investigate the efficiency with which this process occurs. ^{35}S methionine pulse chase labelling experiments of BHK cells co-infected with α1 (9E10) and β2 (9E10) subunits in combination with sucrose gradient analysis and immunoprecipitation of the epitope tagged subunits allowed the efficiency of oligomerisation in BHK cells to be estimated at approximately 33%. This is in close agreement with the Fig of 30% for the muscle n AChR (Ross et al 1991). Such pulse chase experiments were informative in other aspects. It was apparent that the assembled receptor, as defined by its 9S sedimentation coefficient, was relatively stable with only a 10% drop in signal over 14 hours. This is consistent with

observations using electrophysiological assays which determine the half life of a $GABA_A$ receptor composed of α1 (9E10) and β2 (9E10) subunits to be 50 hours (Personal communication, Professor T.G. Smart). In contrast the unassembled subunits were rapidly degraded with the majority of the signal (80%) being lost in a six hour period. The half life of individually expressed α1 (9E10) and β2 (9E10) subunits were estimated to between 2-3 hours. This was consistent with the pulse chase experiments. The half lives of nAChR subunits were lower, approximately 40 minutes, (Claudio et al 1989), in contrast with the values estimated for the virally expressed $GABA_A$ subunits. This could be a real difference or due to the suppression of host cell biosynthesis by the viral expression system. The time point of four hours post infection, when the half life experiments were conducted, was chosen to try and minimise this effect.

The assembly of the α1 (9E10) and β2 (9E10) subunits was clearly visible after one hour of chase yet the translocation of the receptor to the plasmamembrane could not be demonstrated until eight hours after infection. A lag between the assembly of an ion channel's subunits and the translocation of the receptor to the surface of the cell has been previously demonstrated for the nAChR. This delay was estimated to be one hour in duration (Merlie and Lindstrom 1983).

Mutagenic and biochemical approaches have suggested that stoichiometry of a $GABA_A$ receptor composed of αβ2γ2 or α1β3γ2 are pentameric (Backus et al 1993, Chang et al 1996 and Tretter et al 1997). The structure of receptors composed only from α and β subunits is more controversial. Receptors composed of α1β3 subunits expressed in *Xenopus* oocytes have been reported to be trameric (Kellenberger et al 1996) whilst the same receptors expressed in HEK 293 cells were reported to be tetrameric (Tretter et al 1997). This latter study may have been

complicated by the existence of homomeric receptors composed of β3 subunits. Receptors composed of α1 (9E10) and β2 (9E10) subunits were studied since neither of these subunits can form homomeric channels. In order to determine the stoichiometry of a receptor formed from α1 (9E10) and β2 (9E10) subunits metabolic labelling experiments were combined with sucrose gradient sedimentation and immunoprecipition of receptor subunits using the monoclonal antibody 9E10. It was possible to quantify the amount of signal form the α1 (9E10) and β2 (9E10) proteins in the assembled 9S complex and calculate a ratio of α1 (9E10) to β2 (9E10). When the two signals had been corrected for relative methionine content a ratio of 1:1 was determined. This suggested that the 9S complex was a tetramer or a hexamer. In contrast to the pentameric arrangement of other ligand gated ion channels. One could argue that an equally mixed population of pentamers composed of 3:2 and 2:3 α to β had been produced. The results of the experiment where the relative concentration of β2 (9E10) to α1 (9E10) is increased argue against this mixed population . When the ratio of β2 (9E10) to α1 (9E10) virus particles is increased expression of the α1 (9E10) and β2 (9E10) proteins at the plasmamembrane is decreased. Given the unitary dose response curves for α/β receptors, single IC 50 value for $Zn2^{+}$ and unitary single channel properties a mixed population of receptors appears less likely (Macdonald and Olsen 1994). The tetrameric structure of receptors composed of α1 (9E10) and β2 (9E10) subunits may help to explain the lower single channel conductance of 15-16pS, compared to 28-32pS for receptors composed of α1β2γ2 subunits, which have a pentameric arrangement (Moss et al 1991 and Angelotti and Macdonald 1993, Tretter et al 1997). The analysis of the channel permeability of these two receptor types may resolve this possibility in the future.

The capability of this viral expression system to express recombinant protein within neurones enabled the neuronal assembly of $GABA_A$ receptors to be studied. The viral expression system allowed a comparison of the assembly of $GABA_A$ subunits in BHK and HEK cells with that occurring in neurones. Infections of SCG neurones by recombinant α1 (9E10) or β2 (9E10) virus particles produced identical results to those produced in BHK and HEK cells, namely retention within the endoplasmic reticulum. Given that SCG neurones are known to have endogenous $GABA_A$ receptors it would perhaps have been expected that the individual infections would have produced some cell surface fluorescence. The reason for this not occurring is probably a reflection on the viral expression system which floods the host cell with recombinant RNA quickly out competing endogenous message for the host's translational machinery. Given that the initial assembly process is perhaps thought to be co-translational, it would require some equity in the abundance of the messages for the proteins that were going to heteroligomerise. This is unlikely to occur in this expression system unless one introduces the second message virally. When co-infection of SCG neurones by α1 (9E10) and β2 (9E10) Semliki virus particles was conducted, robust surface expression extending in to the neuronal processes was obtained. Some clustering on the neuronal processes was also observed. The results of these experiments suggested that the assembly rules defined in HEK cells can be applied to neuronal systems.

It was not possible to restore benzodiazepine sensitive GABA currents to cortical neurones prepared from the γ2 knock out mice by infecting them with the γ2 S (9E10) virus. One can explain this using the same argument used to justify the lack of oligomerisation of endogenous GABA receptor subunits in the SCG neurones with virally produced α or β subunits.

The biochemistry, cell and neurobiology of the γ2 S and γ2 L alternate splice variants was also investigated using the Semliki expression system. It was surprising to find that the γ2 short protein appeared to migrate at 50KDa on polyacrylamide electrophoresis whilst the long isoform migrated with an apparent molecular weight of 45KDa. Western blot studies of brain lysates using γ2 specific polyclonal antibodies have shown this protein to have range of molecular weights from 45-50 KDa (Araki et al 1993 Fernando et al 1995 and San Juan et al 1995). This large range of sizes was attributed to the susceptibility of this subunit to proteolysis (Hadingham et al 1992 and Connolly et al 1996a).

The difference in apparent size between the γ2 S (9E10) and γ2 L FLAG was reversed on sucrose gradient sedimentation studies with the short splice variant sedimenting with a lower value of 3S compared to the 4S value for the γ 2 L FLAG protein. The profile of the two gradients were also different the γ 2 S (9E10) having a sharper profile compared to the long splice variant. The differences in sedimentation profiles were hypothesised to be due to the long isoform binding more extensively with chaperone proteins but no evidence to support this exists.

The differences between the two proteins were not solely biochemical in nature. The splice variants had a markedly different cell biology. The long isoform was retained within the endoplasmic reticulum of BHK cells and SCG neurones whilst the short splice variant could traffic to the surface of BHK cells and out into the neuronal processes of SCG neurones. The γ2 S (9E10) protein could also be shown to endocytose from the cell surface of BHK cells. Electron microscopic examination of BHK cells infected by the γ2 S (9E10) virus demonstrated the association of this subunit with coated pits. When BHK cells which had been infected with α1 (9E10) and β2 (9E10) virus were examined electronmicroscopicaly no association of α1 (9E10) and β2 (9E10)

subunits with coated pits could be demonstrated but their was a significant association of these subunits with microvilli.

It could be argued that this is an artificial situation since the γ2 subunits are unlikely to be expressed alone *in vivo* but in combination with other $GABA_A$ subunits. It is interesting that of the subunits which give rise to the $GABA_A$, Glycine and nACh receptors, the γ2 S subunit is the only one that has been demonstrated to migrate to the plasmamembrane as a monomer and then undergo endocytosis.

The potential for the $GABA_A$ receptor to be regulated by tyrosine phosphorylation arose when the sequence of the γ2 subunit was published (Pritchett et al 1988). This subunit contains a consensus site for tyrosine phosphorylation in the intracellular loop between transmembrane domains 3 and 4 of the γ2 subunit. The capability of the γ2 L and S splice variants to be biochemically tyrosine phosphorylated *in vivo* by the archetypal tyrosine kinase src was demonstrated using transient transfection of HEK 293 cells. Tyrosine phosphorylation of the $GABA_A$ receptor by this kinase resulted in an enhancement of GABA gated chloride currents. This enhancement was eliminated when the tyrosines were mutated to phenylalanine residues (Moss et al 1995). The response of the endogenous $GABA_A$ receptors in SCG neurones to tyrosine kinase inhibitors and tyrosine kinase activators was also studied by Moss et al. The results paralleled those determined in the heterologous expression system. The tyrosine kinase inhibitor genistein produced a time dependant decrease in the size of GABA currents recorded from the SCG neurones. In contrast the application of c Src or sodium vanadate produced a time dependent increase in GABA activated currents (Moss et al 1995). This agrees with studies performed on *Xenopus* oocytes expressing $GABA_A$ receptors, where the addition of tyrosine kinase inhibitors caused a decrease in GABA gated currents

(Valenzuela et al 1995). Unfortunately it was not possible to demonstrate biochemically the existence of endogenous tyrosine phosphorylated γ2 protein due to the failure to obtain specific anti γ2 phosphotyrosine antibodies.

The protein Agrin, is released from motor neurones and subsequently associates with the extracellular matrix of the synaptic basal lamina (Magill-Solc and McMahan 1988). This protein has been demonstrated to stimulate the tyrosine phosphorylation of the nAChR (Wallace et al 1991, Ferns et al 1996). In contrast no exogenous factor has been demonstrated to cause the $GABA_A$ receptor to become tyrosine phosphorylated. Evidence exists that the receptor for Agrin includes the protein MuSk (Glass et al 1996). MuSK is a receptor tyrosine kinase which is specifically expressed in skeletal muscle (Valenzuela et al 1995b). In contrast to the $GABA_A$ receptor, the functional consequence of tyrosine phosphorylation of nAChR is desensitisation (Hopfield et al 1988). Tyrosine phosphorylation of the nAChR has also been shown to be important for the clustering of the receptors (Ferns et al 1996). The mechanism by which $GABA_A$ receptors are localised to synapses is presently unknown and one can only speculate that similar clustering molecules may regulate the localisation of the $GABA_A$ receptor and that the clustering of the channels at synapses may be dependant on their phosphorylation.

Future applications of the Semliki expression system with respect to GABA receptors.

It is possible to engineer Semliki viral vectors which make more than one protein using two subgeonomic promoters as shown in this thesis and by others using the Sindbis viral expression system (Raju and Huang 1991). One other approach would be to engineer a vector that contained an internal ribosome entry site, IRES (Jackson et al 1990 and Rees et al

1996). This would enable one promoter to produce two messages. Transgenic studies have used IRES sequences to make targeting constructs which incorporate a reporter and a selection marker (Jones et al 1997). The development of such vectors may allow the viral expression of three integral membrane proteins in a single cell and enable the production of a $GABA_A$ receptor containing α, β and γ subunits.

The Semliki Forest virus system is a powerful biochemical tool but it has one other advantage. It has the ability to infect a broad range of cells including neurones in dissociated cultures and in organotypic cultures. The advantage of organotypic cultures is that the polarity of the neurones are better maintained compared to dissociated cultures. It would be possible to begin to ascertain if for example the transcytosis of the β3 subunit reported in MDCK cells , (Connolly et al 1996b) can occur in polarised neurones. Also double α1 β2 infections of such cultures would allow one to determine whether it is possible to obtain the appropriate trafficking of the $GABA_A$ receptors into dendrites using this system.

Multiple infections are required to produce a functional $GABA_A$ receptor because of its heteroligomeric nature. Virally expressed subunits do not co-assemble with endogenous $GABA_A$ receptor subunits. This is an important limitation of this expression system. It would not be possible, for example, to use this viral expression to introduce a receptor subunit devoid of a particular kinase sight into endogenous $GABA_A$ receptors. However the potential application of this system to the introduction of the $GABA_B$ receptor which is a monomeric protein (Kaupmann et al 1997), would be less problematic and could allow insights into the neurobiology of this particular GABA receptor to be made.

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Assembly of $GABA_A$ Receptors Composed of α1 and β2 Subunits in Both Cultured Neurons and Fibroblasts

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$GABA_A$ receptors are believed to be pentameric hetero-oligomers, which can be constructed from six subunits (α, β, γ, δ, ϵ, and ρ) with multiple members, generating a large potential for receptor heterogeneity. The mechanisms used by neurons to control the assembly of these receptors, however, remain unresolved. Using Semliki Forest virus expression we have analyzed the assembly of 9E10 epitope-tagged receptors comprising α1 and β2 subunits in baby hamster kidney cells and cultured superior cervical ganglia neurons. Homomeric subunits were retained within the endoplasmic reticulum, whereas heteromeric receptors were able to access the cell surface in both cell types. Sucrose density gradient fractionation demonstrated that the homomeric subunits were incapable of oligomerization, exhibiting 5 S sedimentation coefficients. Pulse–chase analysis revealed that homomers were degraded, with half-lives of ~2 hr for both the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits. Oligomerization of the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits was evident, as demonstrated by the formation of a stable 9 S complex, but this process seemed inefficient. Interestingly the appearance of cell surface receptors was slow, lagging up to 6 hr after the formation of the 9 S receptor complex. Using metabolic labeling a ratio of $\alpha1^{(9E10)}$:$\beta2^{(9E10)}$ of 1:1 was found in this 9 S fraction. Together the results suggest that $GABA_A$ receptor assembly occurs by similar mechanisms in both cell types, with retention in the endoplasmic reticulum featuring as a major control mechanism to prevent unassembled receptor subunits accessing the cell surface.

Key words: Semliki Forest virus; $GABA_A$ receptor; density gradients; cultured neurons; assembly; endoplasmic reticulum

$GABA_A$ receptors are the major sites of fast inhibitory neurotransmission within the brain. These receptors are believed to be hetero-oligomers constructed from six classes of homologous subunits: α(1–6), β(1–4), γ(1–3), δ, ϵ, and ρ(1–3) (Macdonald and Olsen, 1994; Davies et al., 1997), generating a considerable potential for receptor heterogeneity. Even within single neurons, there is likely to be heterogeneity of structure, because many neurons often express multiple numbers of receptor subunits (Wisden and Seeburg, 1992; Fritschy and Mohler, 1995). Therefore, to determine the true extent of $GABA_A$ receptor heterogeneity, it is important to understand the mechanisms that govern the assembly of these receptors. Selective oligomerization and/or selective transport of defined subunit combinations to the cell surface (Connolly et al., 1996a) may provide important mechanisms for controlling receptor diversity.

To address these questions, the assembly of recombinant receptors has been analyzed. These studies have focused on the minimal structural requirement for the production of $GABA_A$ receptors displaying the full physiological and pharmacological properties of neuronal receptors. Parallel electrophysiological, biochemical, and morphological studies have demonstrated that homomeric expression does not result in surface expression for the α1, β2 or γ2L subunits in *Xenopus* oocytes, human embryonic kidney (A293) cells, or Madin Darby canine kidney (MDCK) cells (Connolly et al., 1996a,b). In common with the combinations α1γ2L and β2γ2L these homomeric subunits are retained in the endoplasmic reticulum (ER) (Connolly et al., 1996a,b). Expression of receptors composed of α and β subunits produces GABA-gated chloride channels, but the co-expression of the γ2 or γ3 subunits is essential for modulation by benzodiazepines (Macdonald and Olsen 1994).

To explore receptor assembly further, we have developed Semliki Forest viruses (Liljestrom and Garoff, 1991) that express receptor subunits modified with the 9E10 epitope (Connolly et al., 1996a,b). This expression system allows high levels of protein production in a broad range of host cells, including neurons (Liljestrom and Garoff, 1991; deHoop et al., 1995). Here, we use this system to study the assembly of $GABA_A$ receptors composed of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits in both baby hamster kidney (BHK) cells and cultured superior cervical ganglia (SCG) neurons.

MATERIALS AND METHODS

Construction and production of Semliki Forest viruses expressing the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ $GABA_A$ receptor subunits. The murine $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits modified with the 9E10 epitope EDKLISEEDL, between amino acids 4 and 5 of the mature protein, have been described previously (Connolly et al., 1996a,b). Addition of this epitope has been demonstrated to be functionally silent with regard to both receptor pharmacology and physiology (Connolly et al., 1996a,b). These cDNAs were cloned as *Bam*HI fragments into the vector pSFV1 (Liljestrom and Garoff, 1991) using standard recombinant methods. The respective plasmids were linearized with *Nru*1, and cRNA was synthesized using SP6 polymerase. cRNA was synthesized from *Spe*I linearized Helper I plas-

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mid (Liljestrom and Garoff, 1991) also using SP6 polymerase. All manipulations were performed using standard recombinant methods (Sambrook et al., 1989). To produce viral stocks encoding the α1 and β2 subunits, cRNA transcribed from receptor constructs and the helper plasmid were electroporated into BHK cells at a 1:1 ratio. The supernatants from the cells were collected after 2 d and frozen in 100 μl aliquots. Titers for $GABA_A$ receptor subunit-expressing viruses were determined by serial dilution on BHK cells. Expression was determined by immunofluorescence as described below. The $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ viruses had titers of 7×10^7–1×10^8 particles/ml.

Cell culture. BHK cells were maintained in BHK 21 medium (Life Technologies) supplemented with 5% FCS, 20 mM HEPES, pH 7.2, tryptose phosphate broth, penicillin, and streptomycin. Cultured SCG neurons were prepared and maintained as described previously (Krishek et al., 1994).

Infection of BHK cells and cultured SCG neurons. Infection of BHK cells or neurons was conducted by diluting virus into binding medium (RPMI 1640 medium, pH 6.8, 20 mM HEPES, pH 6.8, and 20 mM BSA), and incubating the cells in this solution for 1 hr at 37°C. Normal culture medium was then reapplied after this incubation. The $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ virus particles were used at concentrations of ~10 infectious units per cell.

Immunocytochemistry. Immunofluorescence was performed as described by Connolly et al., (1996a,b). BHK cells and neurons were fixed in 3% parafomaldehyde in PBS for 5 min and then quenched twice with 50 mM glycine for 10 min. Cells were then blocked using 1% BSA and 10% horse serum in PBS. The primary antibodies were applied for 1 hr at the following concentrations: BD17 (anti-β2/β3; Boehringer), 14 μg/ml; 9E10, 5 μg/ml; or a polyclonal antibody raised against a synthetic peptide corresponding to the C terminus of the α1 subunit (amino acids 413–429) (Pollard et al., 1993) at 5 μg/ml. Secondary antibodies (Pierce, Rockford, IL), either fluorescein- or rhodamine-linked anti-mouse and anti-rabbit IgG, were then applied as appropriate for 45 min in blocking solution. Permeabilization of samples was conducted by adding NP-40 to the blocking solution (0.05%). Fluorescence images were analyzed by confocal microscopy (Medical Research Council 1000). Images from SCG neurons were generated by taking an optical *Z* section through the cell at 1 μm intervals and then recombining the images.

Quantification of fluorescence by confocal microscopy. The level of surface membrane 9E10 staining on infected cells was quantified using confocal microscopy as described by Bogler et al. (1993) and Entwistle and Noble (1994). BHK cells were fixed at differing time points after infection. Surface receptor populations were then detected simultaneously via fluorescence using 9E10 antibodies without permeabilization. Individual cells at each time point were selected at random, and the area command was used to collect brightness readings of the plasma membrane, using identical iris and gain settings. The average fluorescence intensities were converted to numerical readings of arbitrary values (pixels). This was performed on at least five cells for each time point.

Sucrose density gradient fractionation. Receptor subunits were subjected to sucrose density gradient fractionation using 5 and 20% linear sucrose density gradients in lysis buffer (Millar et al., 1995). Before loading, solubilized cell extracts were clarified by centrifugation (100,000 × *g* for 10 min). Gradients were calibrated via the inclusion of marker proteins (1 mg/ml) of known sedimentation coefficients: BSA, 4.3 S; aldolase, 7.4 S; and catalase, 11.2 S. Gradients were run in a Beckman SW 55ti rotor at 40,000 rpm for 14.4 hr at 4°C. The gradients were then fractionated (14 350 μl fractions), and receptor localization was analyzed by immunoprecipitation or Western blotting.

Metabolic labeling and immunoprecipitation of $GABA_A$ receptor subunits. For metabolic labeling studies infected BHK cells were starved in methionine-free media for 30 min before labeling with [^{35}S]methionine (ICN/Flow) at 200 μCi/ml for differing periods. Where appropriate, labeled cultures were chased at 37°C with complete growth media. Total protein synthesis was analyzed by SDS-PAGE, after cell lysis in SDS sample buffer. Receptor subunits were isolated from cell extracts or sucrose density gradient fractions via immunoprecipitation as described previously (Moss et al., 1995; Connolly et al., 1996b). Cells were lysed in a buffer containing 2% NP-40, 5 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 50 mM sodium chloride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, and 0.1 mg/ml aprotinin. Receptor subunits were isolated with 9E10 antibody coupled to protein G–Sepharose, followed by SDS-PAGE. In some experiments 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) was used in place of NP-40. For surface immunoprecipitations, expressing cells were chilled to 4°C and incubated in PBS containing 10 μg/ml BSA for 30 min. 9E10 antisera was then applied to the cells (10 μg/ml) in the same buffer for 20 min, followed by 9E10 peptide at 1 μg/ml for 10 min. Surface receptor populations were then isolated using protein G-Sepharose after cell lysis as described above, except that all solutions contained 9E10 peptide at 1 μg/ml.

Western blotting. Receptor subunits were detected in gradient fractions using either 9E10 antibody or a rabbit polyclonal antibody against a peptide corresponding to amino acids of 326–343 of murine α1 subunit (Macdonald and Olsen, 1994). Antibody production in rabbits and Western blotting were performed as described by Krishek et al. (1994) and Connolly et al. (1996a), respectively.

Electrophysiology. Whole-cell recordings from infected BHK cells were conducted at 18 hr after infection as described previously (Krishek et al., 1994).

RESULTS

Production and characterization of Semliki Forest viruses expressing $GABA_A$ receptor subunits

Co-electroporation of BHK cells with cRNA produced form either plasmid $pSFV1\alpha1^{(9E10)}$ or $PSFV1\beta2^{(9E10)}$ with cRNA synthesized from the Helper I plasmid produced high titers of infectious Semliki Forest virus particles (Liljestrom and Garoff, 1991). Typical titers in excess of 7×10^7/ml were obtained on electroporation of 10^6 cells as determined by limiting dilution. To characterize the virally expressed receptor subunits, infected cells were pulse-labeled with [^{35}S]methionine. Subunit expression was then monitored with time by examining total protein synthesis or via immunoprecipitation using 9E10 antibody. Infection of BHK cells with $\alpha1^{(9E10)}$ virus lead to the time-dependent synthesis of a protein with an apparent molecular mass of 52 kDa and a minor band at 50 kDa together with a significant inhibition of host protein synthesis (Fig. 1*A*). In addition to these, weaker bands of 65 and 40 kda were also evident. These bands represent the replicase and capsid protein of the Semliki Forest virus (Liljestrom and Garoff, 1991). Likewise, infection with the $\beta2^{(9E10)}$ virus resulted in the synthesis of a 56 kDa protein (Fig. 1*B*). Semliki Forest virus infection of many cell types has previously been shown to inhibit protein synthesis, via competition of the Semliki Forest virus-encoded RNAs with host cell mRNAs for translation (Liljestrom and Garoff, 1991). To confirm that these proteins represent receptor subunits, immunoprecipitation with 9E10 antibody was performed on labeled cells treated with or without tunicamycin to block N-glycosylation. From cells infected with the $\alpha1^{(9E10)}$ virus a protein of 52 kDa was immunoprecipitated with a minor band of 50 kDa; tunicamycin treatment resulted in the precipitation of a single band of 48 kDa (Fig. 1*C*). In contrast 9E10 antibody precipitated a band of 56 kDa from cells infected with the $\beta2^{(9E10)}$ virus; treatment with tunicamycin reduced the mass of this band to 50 kDa (Fig. 1*D*). The values obtained for the molecular masses of the glycosylated and unglycosylated forms of the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ viruses produced in BHK cells are thus in agreement with those observed on expression in A293 cells (Buller et al., 1994; Connolly et al., 1996a).

Expression of homomeric and heteromeric $GABA_A$ receptors in BHK cells using Semliki Forest virus

Expression of receptor subunits in BHK cells was analyzed by immunofluorescence. Using subunit-specific antibodies, we failed to detect expression of the α1, α6, β1–3, or γ2 subunits in uninfected BHK cells. Infection of cells with single $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ viruses resulted in abundant intracellular staining using 9E10 antibody (Fig. 2*A*,*B*) on permeabilization but no staining in nonpermeabilized cells (Fig. 2*C*). The staining pattern is consis-

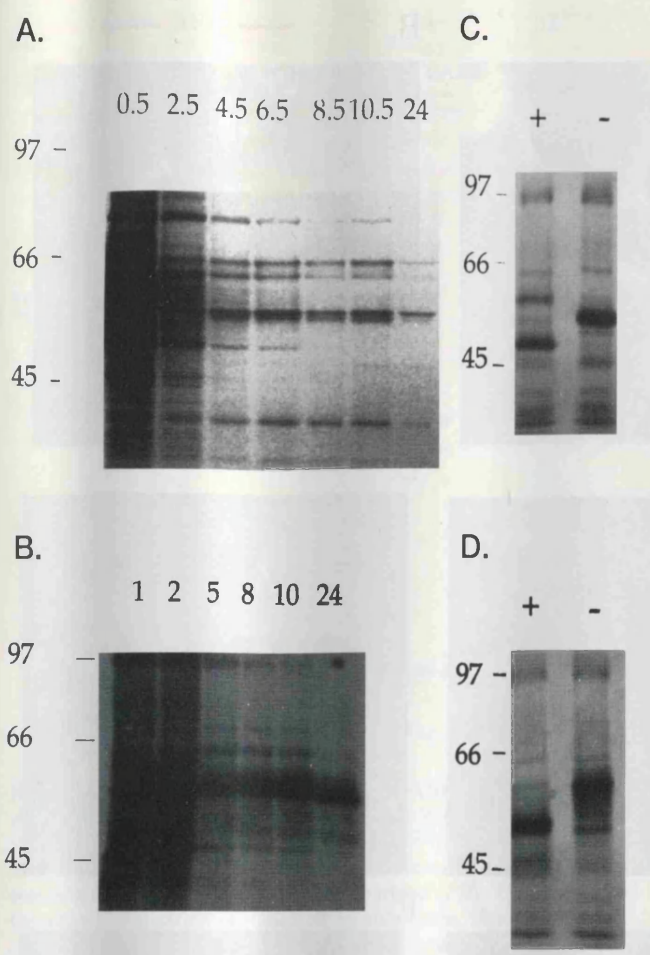

Figure 1. Biochemical characterization of the $GABA_A$ receptor $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits produced on Semliki Forest virus infection of BHK cells. BHK cells were infected with either the $\alpha1^{(9E10)}$ (*A*) or $\beta2^{(9E10)}$ (*B*) subunit virus. At defined periods (between 0.5 and 24 hr as indicated) after infection the cells were labeled for 30 min with 500 μCi/ml [^{35}S]methionine. Infected cells were then lysed, and labeled proteins were resolved by SDS-PAGE followed by fluorography. The subunits were also immunoprecipitated from infected cells labeled with [^{35}S]methionine and treated with (+) or without (−) tunicamycin (5 μg/ml), as shown in *C* and *D* for the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits, respectively. The migration of protein standards (Bio-Rad) is indicated.

tent with ER retention of these subunits on homomeric expression, as demonstrated previously in A293 and MDCK cells (Connolly et al., 1996a,b). In contrast, co-infection with $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ viruses (1:1 ratio) resulted in high levels of surface 9E10 staining (Fig. 2*D*) in ~80% of all cells. The presence of both the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits on the cell surface was also demonstrated using subunit-specific antibodies (Fig. 2*E,F*). To attempt to quantify the level of receptor production, whole-cell binding was performed using tritiated muscimol. High-affinity binding was evident only in co-infected cells, with a B_{max} of 26 pmol/mg protein and K_d values of 4 and 90 nM (results not shown). Similar K_d values for muscimol binding have been reported for the expression of these subunits previously (Pritchett et al., 1988; Moss et al., 1991; Hadingham et al., 1992).

Whole-cell recordings from BHK cells co-infected with $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ viruses exhibited robust GABA-activated membrane currents (Fig. 3). The maximum current density evoked by GABA (100 μM) was 3.5 ± 0.5 nA, significantly higher than expected from conventional calcium phosphate or electroporation studies using these subunits (Connolly et al., 1996a). The membrane currents were sensitive to inhibition by 10 μM Zn^{+2} and were not enhanced by 1 μM flurazepam, and the GABA EC_{50} was 1.5 ± 0.12 μM (n = 8 cells), in agreement with earlier studies (Macdonald and Olsen 1994). In contrast, infection of BHK cells with either single $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ viruses resulted in complete insensitivity to GABA (1 mM) and also pentobarbital (2 mM; n = 5). The latter was used because $\beta1$ and $\beta3$ homomeric ion channels are sensitive to pentobarbital in preference to GABA (Sigel et al., 1989; Connolly et al., 1996b; Krishek et al., 1996). These results suggests that only the $\alpha1^{(9E10)}\beta2^{(9E10)}$ binary combination forms functional ion channels as shown previously (Connolly et al., 1996a).

Sucrose density fractionation of $GABA_A$ receptor subunits

Receptor subunits expressed in BHK cells were fractionated on sucrose density gradients. Receptors were solubilized in a range of differing detergents. Up to 50% of the receptor subunits could be solubilized in 2% NP-40, whereas <20% could be solubilized in 2% CHAPS (data not shown). Therefore, the majority of experiments were performed using solubilization in NP-40. Gradient fractions were then subjected to SDS-PAGE followed by Western blotting with 9E10 antibody. On homomeric expression a major band of 52 kDa corresponding to the $\alpha1^{(9E10)}$ subunit could be detected, which sedimented at 5 S (Fig. 4*A*). In some experiments additional bands of 50 and 48 kDa could also be seen. These bands presumably represent incompletely glycosylated forms of the $\alpha1$ subunit (Fig. 1*C*). The sedimentation of the $\alpha1^{(9E10)}$ subunit was also analyzed in cells co-infected with the $\beta2^{(9E10)}$ virus using an $\alpha1$-specific antibody that recognizes an epitope in the major intracellular domain of this subunit. Co-expression with the $\beta2^{(9E10)}$ subunit resulted in a shift in the sedimentation of the $\alpha1^{(9E10)}$ subunit to 9 S (Fig. 4*B*). This shift is consistent with oligomerization of the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits. An identical shift in sedimentation of the $\alpha1^{(9E10)}$ subunit was seen using CHAPS in place of NP-40. A parallel shift in sedimentation coefficient of the $\beta1^{(9E10)}$ subunit from 5 to 9 S was also seen on co-expression with the $\alpha1^{(9E10)}$ using Western blotting with anti-$\beta2$ subunit antibodies (data not shown). Similar sedimentation coefficients have been previously demonstrated for purified $GABA_A$ receptors and recombinant receptors composed of both α/β and $\alpha\beta\gamma$ subunits (Mamalaki et al., 1987, 1989; Hadingham et al., 1992) and for assembled pentameric muscle nicotinic acetylcholine receptor (AChR) expressed in fibroblasts (Green and Millar 1995). Our failure to detect homo-oligomerization of either the $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ subunit may be attributable to the detergent used. Interestingly, substitution of NP-40 for CHAPS did not result in the detection of slower-sedimenting forms of either homomeric subunit. Furthermore, the use of Lubrol for extraction does not lead to the detection of homo-oligomers for the $\alpha1$ subunit expressed in human embryonic kidney 293 (HEK) cells (Tretter et al., 1997).

To analyze the production and stability of this 9 S complex, co-infected cells were labeled with [^{35}S]methionine for 1 hr and chased for defined periods with excess cold methionine. Cell lysates were then prepared and subjected to sucrose density gradient fractionation. Receptor subunits were precipitated from gradient fractions using 9E10 antibody (Fig. 5), and the levels of incorporated methionine were quantified on a Bio-Rad phosphorimager (Fig. 6). Bands corresponding to 52 and 56 kDa could be detected for the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits, respectively, in gradient fractions (Fig. 5*A–C*). Immediately after a 1 hr labeling

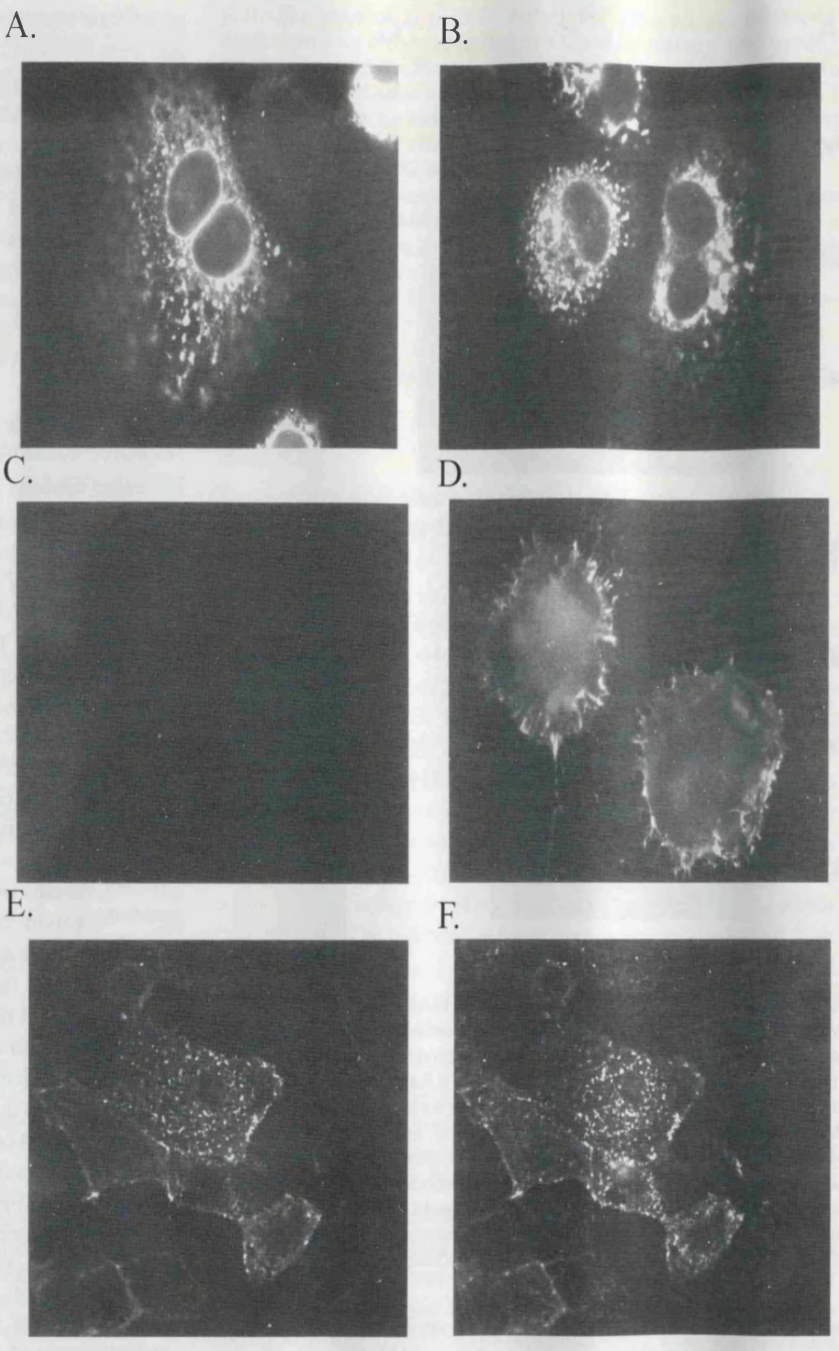

Figure 2. Surface expression of $GABA_A$ receptor subunits in BHK cells. BHK cells were infected with either single $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ virus or co-infected with both, and expression was then determined by immunofluorescence with (+) and without (−) permeabilization 20 hr after infection. Images were then collected by confocal microscopy. *A*, $\alpha1^{(9E10)}$, 9E10 antibody (+); *B*, $\beta2^{(9E10)}$, 9E10 antibody (+); *C*, $\alpha1^{(9E10)}$, 9E10 antibody (−); *D*, $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$, 9E10 antibody (−). Images were collected from cells infected with both subunit viruses and co-stained using a rabbit polyclonal antibody against the $\alpha1$ subunit and a monoclonal antibody (BD17) against the $\beta2$ subunit coupled to fluorescein and rhodamine secondary antibodies, respectively. The images collected for the two channels are shown in *E* and *F* for the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits, respectively.

period, the majority of synthesized subunits sedimented at 5 S. However a shoulder migrating at 9 S was also visible (Figs. *5A*, *6A*), which was not seen with either subunit expressed alone, using identical conditions (data not shown). In contrast, at 6 hr the majority of the labeled receptor subunits migrated at 9 S (Figs. *5B*, *6B*). There was also a drastic reduction in the total number of receptor subunits at this 6 hr time point, with only ~36% of the total counts present after labeling remaining (Fig. *6B*). At 20 hr, most of the 9 S peak was still evident, with ~31% of the starting counts remaining (Fig. *5C,F*). The 9 S pool presumably forms quickly, because it could be detected at zero time (after a 1 hr labeling period) but was difficult to resolve from unassembled subunits at this time point (Fig. *6A–C*). Also, the 9 S fraction seemed relatively stable, as demonstrated by only a small loss (~10%) of this peak between 6 and 20 hr (Fig. *6B,C*) suggesting a half-life of ~40 hr for receptors composed of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits. Collectively the results suggest that oligomerization of receptor subunits occurs quickly but is relatively inefficient, and that unassembled subunits are degraded. Pulse–chase and immunoprecipitation were used to analyze the half-lives of the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits on homomeric expression, as shown in Figure 7. At all time points a single band of 52 kDa was seen for the $\alpha1^{(9E10)}$ subunit, whereas in contrast two bands of 56 and 50 kDa were evident for the $\beta2^{(9E10)}$ subunit. The lower band for the $\beta2^{(9E10)}$ subunit presumably represents unglycosylated material. These results were quantified using a phosphorimager. In the case of the $\beta2^{(9E10)}$ subunit the signals form both bands were pooled. Half-lives of ~2 hr were determined for both the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits. Similar values were seen in three separate experiments. These half-lives for homomeric receptor subunits are consistent with the dramatic loss of signal seen during the first 6 hr of the pulse–chase experiments shown in Figures 5 and 6.

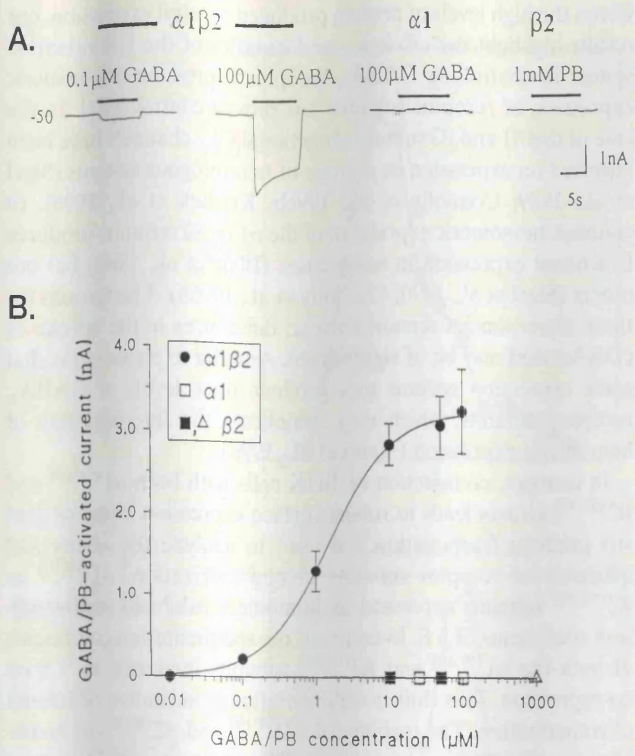

Figure 3. Electrophysiological properties of $GABA_A$ receptors produced in BHK cells. *A*, Whole-cell recordings form BHK cells infected with Semliki Forest viruses expressing $\alpha1^{(9E10)}\beta2^{(9E10)}$. Homomeric $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits were insensitive to GABA, and $\beta2^{(9E10)}$ subunits were also insensitive to pentobarbital (*PB*). The duration of the ligand application is indicated by *solid lines*. Holding potential, −50mV. *B*, GABA (●, □, and ■) and pentobarbital (△) equilibrium concentration responses for BHK cells expressing $\alpha1^{(9E10)}\beta2^{(9E10)}$ or single subunits. data were fitted by $I/I_{max} = [1/(1 + \{[A]/EC_{50}\}^n)]$, where I and I_{max} represent GABA-activated and maximally activated current, n is the Hill coefficient, and A is the GABA concentration. EC_{50} = 1.47 ± 0.12 μM; n = 1.0 ± 0.08. Data points indicate mean ± SEM.

The time dependence of receptor surface expression

To examine the time dependence of surface expression, BHK cells were co-infected with $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ viruses. Infected cells were then fixed after differing periods, and the level of surface receptor expression was determined by fluorescence. Surface receptor levels were then quantified by confocal microscopy, as shown in Figure 8. Surface expression could be first detected 8 hr after infection, which increased rapidly up to 10 hr and leveled off after this period (Fig. 7). Interestingly these results suggest a significant latency between subunit oligomerization, which was evident within 1 hr (Fig. 5), and the appearance of cell surface receptors, which could be detected 8 hr after infection, as determined by fluorescence measurements.

Subunit ratio of $GABA_A$ receptors composed of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits

The ratio of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits present in the 9 S receptor pool was analyzed from expressing cells after labeling with [^{35}S]methionine for 1 hr followed by a 12 hr chase. This will ensure degradation of all unassembled subunits produced during the preceding 1 hr labeling period. Fractions containing the 9 S receptor isolated by sucrose density gradient fractionation were pooled and immunoprecipitated with 9E10 antibody followed by SDS-PAGE. Incorporated counts were then quantified using a Bio-Rad phosphorimager. Signals from the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits were then normalized for relative methionine content. A typical result is shown in Figure 9*A*, where an $\alpha1^{(9E10)}$:$\beta2^{(9E10)}$ subunit ratio of 0.9 was determined. Ratios of 1.2, 1.1, 0.8, and 0.8 were obtained in four separate experiments. To investigate this observation further, the subunit ratio of cell surface receptors was analyzed. Cells expressing both the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits were labeled with [^{35}S]methionine and exposed to 9E10 antibody at 4°C. The surface receptor population was then isolated using protein G–Sepharose resolved by SDS-PAGE, and subunit levels were then quantified. A ratio of 1.1 was found for the $\alpha1^{(9E10)}$:$\beta2^{(9E10)}$ subunits in the experiment shown in Figure 9*B*. Similar ratios were found in two other experiments. Together, these data are consistent with a 1:1 ratio of $\alpha1^{(9E10)}$:$\beta2^{(9E10)}$ subunits, suggesting that receptors are composed of equal numbers of subunits, e.g., a tetrameric or hexameric but, interestingly, not pentameric tertiary structure.

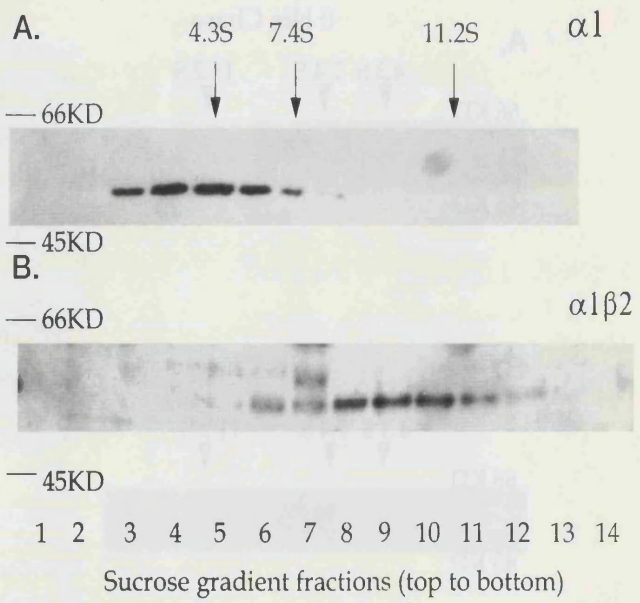

Figure 4. Differential sedimentation of the $\alpha1^{(9E10)}$ subunit dependent on coexpression with the $\beta2^{(9E10)}$ subunit. Cells infected with the $\alpha1^{(9E10)}$ (*A*) or co-infected with both the α1(9E10) and $\beta2^{(9E10)}$ (*B*) subunit viruses were lysed and subjected to sucrose density gradient fractionation 16 hr after infection. Gradient fractions were separated by SDS-PAGE, and the $\alpha1^{(9E10)}$ subunit was detected via Western blotting using 9E10 antibody (*A*) or a rabbit polyclonal antibody against the α1 subunit (*B*). Sedimentation coefficients for the $\alpha1^{(9E10)}$ subunit were determined with reference to proteins with known sedimentation: BSA (4.3 S), aldolase (7.4 S), and catalase (11.2 S).

Surface expression of homomeric and heteromeric $GABA_A$ receptors in cultured neurons using Semliki Forest virus

To determine whether $GABA_A$ receptor assembly in BHK cells is a faithful indicator of neuronal events, cultured SCG neurons were infected with Semliki Forest viruses expressing 9E10-tagged receptor subunits. Infection of neurons with either single $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ virus did not result in the detection of fluorescence in nonpermeabilized cells (Fig. 10*A*). Abundant intracellular staining could be seen for both subunits (Fig. 10*C*). This perinuclear and tubular staining (Fig. 9*A,B*) is consistent with localization of these subunits to the ER in neurons (Krijnse-Locker et al., 1995). In contrast, co-infection of SCG neurons with both viruses led to the detection of robust staining in the absence of detergent (Fig. 10*D*). Staining of 9E10 was evident in neuronal cell bodies, as well as in the neuronal processes. Interestingly "hot spots" (indi-

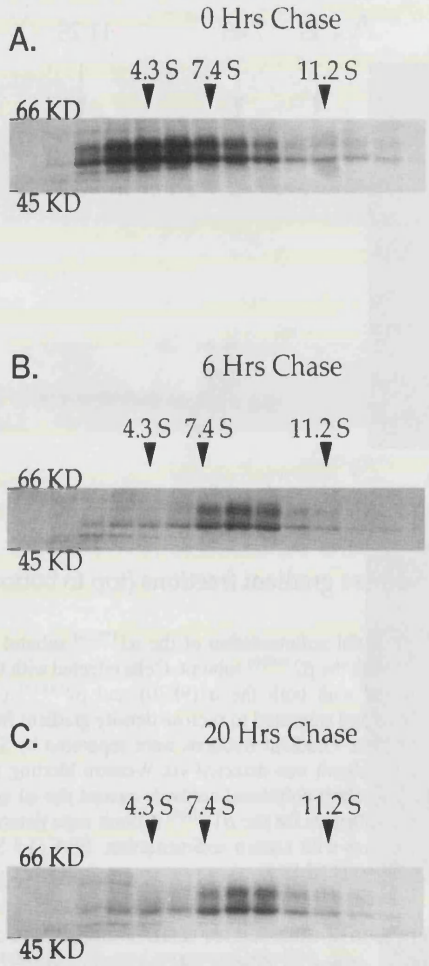

Figure 5. Pulse–chase analysis of $GABA_A$ receptor assembly. Cells co-infected with both subunit viruses were labeled with 100 μCi/ml [^{35}S]methionine 2 hr after infection for 1 hr and chased for 0 (*A*), 6 (*B*), or 20 (*C*) hr excess cold methionine. The cells were then lysed and subjected to sucrose density gradient fractionation. Gradient fractions were then immunoprecipitated with 9E10 antibody, and the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits were resolved by SDS-PAGE. Sedimentation coefficients for the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits were determined as described in Figure 4.

cated in Fig. 10*D* by the *arrow*) of fluorescence were often detected in the neuronal processes. This presumably represent clustering of the virally encoded receptors.

DISCUSSION

Molecular cloning has revealed a large multiplicity of $GABA_A$ receptor subunits, with some neurons, such in the hippocampal dentate gyrus expressing up to 12 differing subunits. Clearly to establish the extent of $GABA_A$ receptor heterogeneity it is important to understand the mechanisms that control receptor assembly.

We have engineered Semliki Forest viruses (Liljestrom and Garoff, 1991) to express epitope-tagged $GABA_A$ receptor subunits (Connolly et al., 1996a,b). Using this system, we have compared the assembly of $GABA_A$ receptors composed of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits in BHK cells and cultured SCG neurons. In BHK cells, homomeric expression resulted in the synthesis of large amounts of protein for each of these respective subunits. However, the subunits were unable to access the cell surface. In common with studies in A293 and MDCK cells these subunits were ER-retained in BHK cells (Connolly et al., 1996b). Given the high levels of protein produced on viral expression, our results highlight the efficiency and capacity of the ER retention system in controlling $GABA_A$ receptor expression. Homomeric expression of receptor subunits has proven controversial. In the case of the $\beta1$ and $\beta3$ subunits functional Cl^- channels have been reported on expression in a range of heterologous systems (Sigel et al., 1989; Connolly et al., 1996b; Krishek et al., 1996). In contrast, homomeric expression of the $\alpha1$ or $\beta2$ subunits produces functional expression in some cases (Blair et al., 1988) but not others (Sigel et al., 1990; Connolly et al., 1996b). The reasons for these discrepancies remain unclear; differences in the species of cDNAs used may be of significance. Another explanation is that some expression systems may produce trace levels of $GABA_A$ receptor subunits, which may complicate the interpretation of homomeric expression (Ueno et al., 1996).

In contrast, co-infection of BHK cells with both $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ viruses leads to robust surface expression. Sucrose density gradient fractionation was used to analyze the ability and efficiency of receptor subunits to oligomerization. $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ subunits expressed as homomers exhibited sedimentation coefficients of 5 S. In contrast, the sedimentation coefficients of both the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits increased to 9 S on co-expression. This shift in sedimentation is indicative of subunit oligomerization. The inability of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ to homo-oligomerize presumably results in ER retention and subsequent degradation, with similar half-lives of 120 min. ER retention of these subunits is presumably mediated via interaction with the chaperone proteins heavy-chain binding protein and calnexin, respectively (Ou et al., 1993; Hammond et al., 1994; Pelham, 1995). Both of these proteins have been previously shown to interact with the $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits, respectively (Connolly et al., 1996a). To study the efficiency of receptor oligomerization the appearance of the 9 S complex containing both subunits was analyzed. Thirty-six percent of available $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ subunits attained this 9 S sedimentation coefficient; the remainder of the subunits were degraded. The majority of the unassembled subunits were degraded within 6 hr, consistent with the short half-lives of either the $\alpha1^{(9E10)}$ or $\beta2^{(9E10)}$ subunit found on homomeric expression. In contrast, assembled receptors seemed relatively stable, with only ~10% of this population being degraded over a 14 hr period. Semliki Forest virus infection results in suppression of host synthesis, and therefore our result may overestimate subunit half-lives. However, receptors composed of $\alpha1$ and $\beta2$ have half-lives of in excess of 45 hr in transfected HEK A293 cells (S. J. Moss and T. G. Smart, unpublished observations), consistent with our findings using Semliki Forest virus expression. The appearance of $\alpha1^{(9E10)}$ and $\beta2^{(9E10)}$ receptors at the cell surface was slow, lagging considerably after the appearance of 9 S receptor complexes. The slow transport of these receptors to the cell surface may be attributable to the absence of $\gamma2$ subunits, or alternatively this may reflect a difference in cellular environment.

There seem to be parallels between the assembly of $GABA_A$ receptors and the muscle AChR subunits stably expressed in fibroblasts. These studies have demonstrated that single AChR α, β, γ, or δ subunits are incapable of oligomerization and are unable to access the cell surface (Claudio et al., 1989; Paulson et al., 1991). Stable cell surface receptors are only produced on co-expression of all four subunits (Claudio et al., 1989; Paulson et al., 1991; Green and Millar, 1995). Again, in common with $GABA_A$ receptor assembly there is a significant lag time (up to 4 hr) between the appearance of AChR receptors at the cell sur-

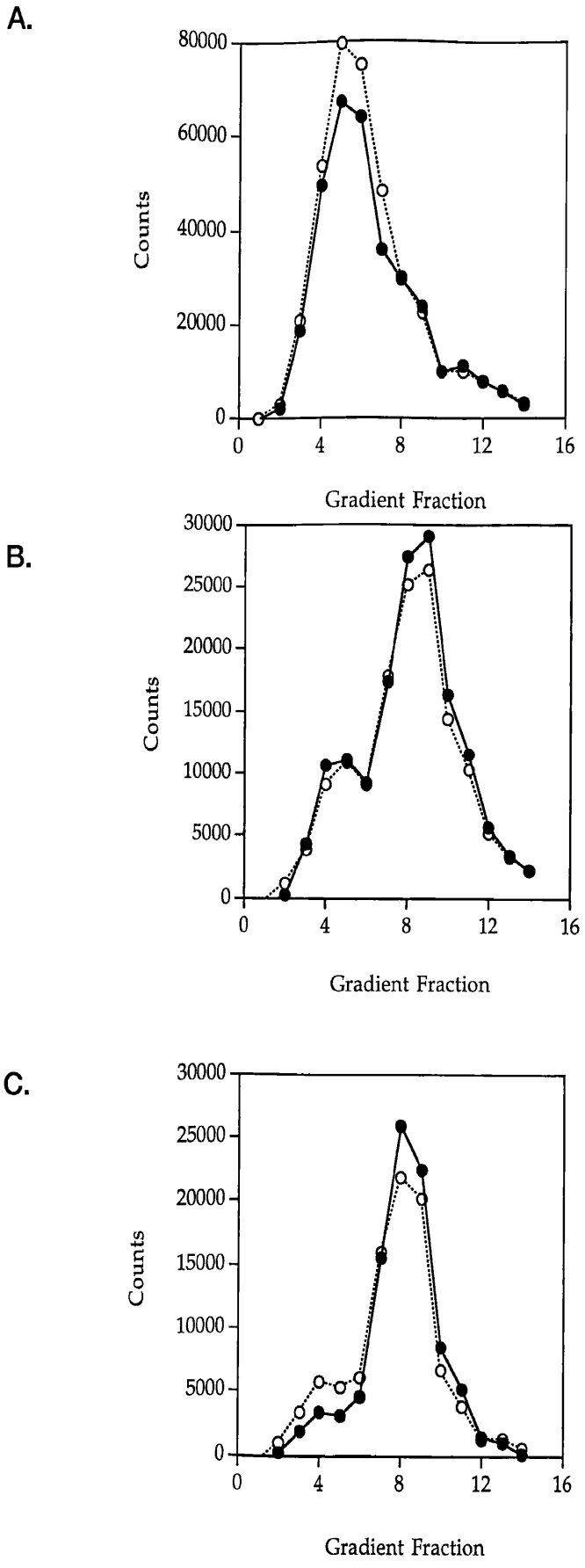

Figure 6. Quantification of receptor assembly. The levels of incorporated methionine in $\alpha 1^{(9E10)}$ (○) and $\beta 2^{(9E10)}$ (●) subunits were quantified in gradient fractions for cells chased for 0 (*A*), 6 (*B*), and 20 (*C*) hr using a Bio-Rad phosphorimager. Background was subtracted using the same volume that was used to integrate the subunit signals.

face and subunit oligomerization. Likewise, the assembly of the AChR seems to be an inefficient process, with only 20–30% of translated subunits being assembled into surface receptors (Ross et al., 1991; Green and Millar 1995).

$GABA_A$ receptors composed of $\alpha\beta$ subunits differ from receptors composed of $\alpha\beta$ and $\gamma 2$ subunits with regard to Zn^{2+} and benzodiazepine sensitivity, in addition to single-channel conductance (Draguhn et al., 1990; Verdoorn et al., 1990; Moss et al.,

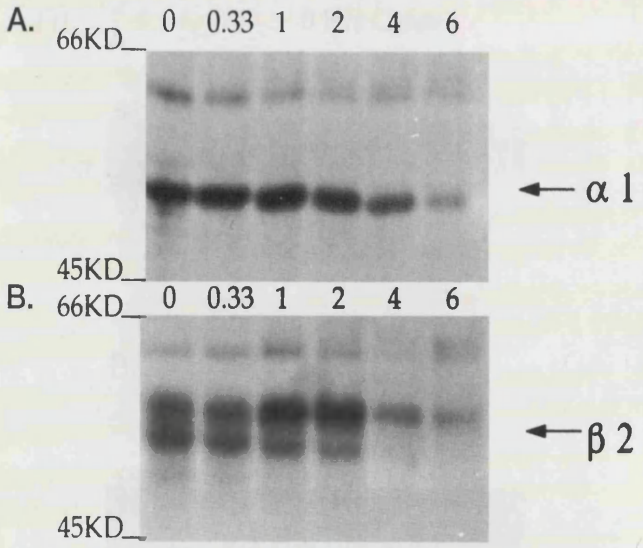

Figure 7. Degradation of single $GABA_A$ receptor subunits expressed in BHK cells. BHK cells were infected with Semliki Forest viruses producing either the $\alpha 1^{(9E10)}$ (*A*) or $\beta 2^{(9E10)}$ (*B*) subunit. Two hours after infection the cells were labeled with [^{35}S]methionine (400 μCi/ml) for 20 min and then chased for differing periods. The cells were then lysed, and receptor subunits were isolated by immunoprecipitation with 9E10 antibody. Precipitated material was then subjected to SDS-PAGE, and the subunit levels were quantified using a Bio-Rad phosphorimager.

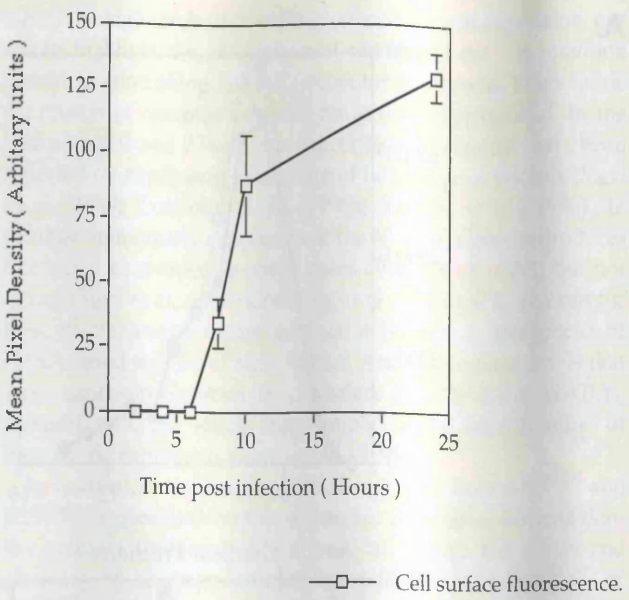

Figure 8. Time dependence of surface expression of receptors composed of $\alpha 1^{(9E10)}$ and $\beta 2^{(9E10)}$ subunits. Co-infected cells were fixed at differing periods after infection and processed simultaneously for fluorescence with 9E10 antibody without permeabilization. Images were collected from cells, and the average intensity of staining is shown. Data were collected from at least five cells at each time point.

1991; Smart et al., 1991; Angelotti et al., 1993). The existence of receptors composed of $\alpha\beta$ subunits in neurons has not been established. However, the high Zn^{2+} sensitivity (Draguhn et al., 1990; Smart et al., 1991) of these receptors coupled with low single-channel conductance (Moss et al., 1991; Angelotti et al., 1993) is reminiscent of some populations of neuronal $GABA_A$ receptors (Smart, 1992; Kaneda et al., 1995). The tertiary structure of these differing receptor isoforms has been examined. Mutagenic and biochemical approaches have suggested that receptors composed of $\alpha\beta 2\gamma 2$ or $\alpha\beta 3\gamma 2$ subunits are pentameric (Backus et al., 1993; Chang et al., 1996; Tretter et al., 1997). The structure of α/β receptors is more controversial. Receptors composed of $\alpha 1\beta 3$ receptors expressed in *Xenopus* oocytes have been reported to be tetrameric (Kellenberger et al., 1996) whereas the same receptor population expressed in A293 cells has been reported to be pentameric (Tretter et al., 1997). These studies may be complicated by the presence of receptors composed of homomeric $\beta 3$ subunits (Connolly et al., 1996b) in addition to receptors containing both the $\alpha 1$ and $\beta 3$ subunits. Studying receptors composed of $\alpha 1\beta 2$ subunits may give more consistent results, because neither of these subunits can access the cell surface on homomeric expression because of ER retention (Connolly et al., 1996a,b). An alternate explanation for the observations on α/β receptors is that two populations of receptors exist, which are pentameric but differ in their subunit ratios, e.g., $2\alpha 3\beta$ and $3\alpha 2\beta$. Given the unitary dose–response curves for α/β receptors, the single IC_{50} value for Zn^{2+} inhibition, and unitary single-channel properties, this seems unlikely (Macdonald and Olsen, 1994).

A tetrameric rather than pentameric structure of receptors composed of $\alpha\beta$ subunits compared with $\alpha\beta\gamma$ may possibly explain the lower single-channel conductance of 15–16 and 28–32 pS, respectively. (Moss et al., 1991; Angelotti and Macdonald, 1993). Analysis of the channel permeability of these two receptor types may help resolve this intriguing possibility. It will be of interest to study the assembly of $GABA_A$ receptors composed of

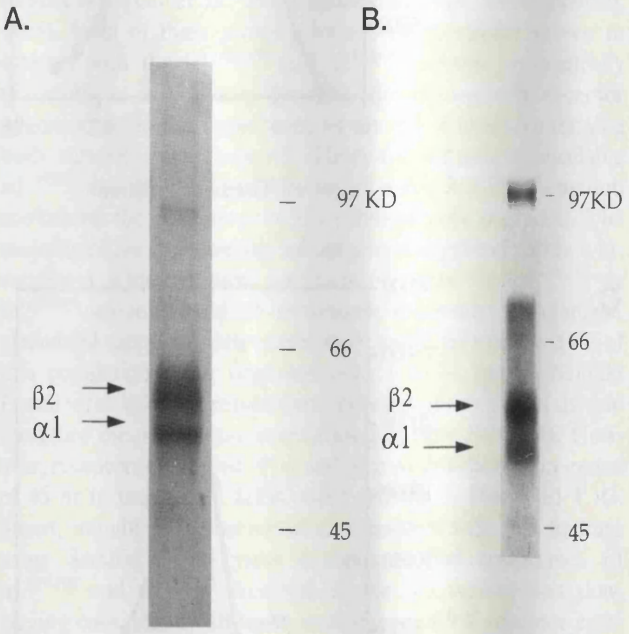

Figure 9. Subunit ratio of $GABA_A$ receptors composed of $\alpha 1^{(9E10)}$ and $\beta 2^{(9E10)}$ subunits. *A*, BHK cells expressing both receptor subunits were pulse-labeled with [^{35}S]methionine (200 μCi/ml) for 2 hr; 3 hr after infection, the cells were then chased for 12 hr with excess cold methionine. Cell lysates were then subjected to sucrose density gradient fractionation, and the fractions corresponding to the 9 S peak were pooled and immunoprecipitated with 9E10 antibody. Receptor subunits were then resolved by SDS-PAGE and quantified using a Bio-Rad phosphorimager. After correction for methionine content ($\alpha 1 = 9$; $\beta 2 = 15$) a ratio of 0.9 was found for the $\alpha 1^{(9E10)}$:$\beta 2^{(9E10)}$ subunits in the experiment shown. *B*, Labeled cells were exposed to 9E10 antibody at 4°C for 30 min. The cell surface receptor population was then isolated via immunoprecipitation with protein G in the presence of excess 9E10 peptide and resolved by SDS-PAGE, and subunit levels were quantified. A ratio of 1.1 was found for the $\alpha 1^{(9E10)}$:$\beta 2^{(9E10)}$ subunits in the experiment shown.

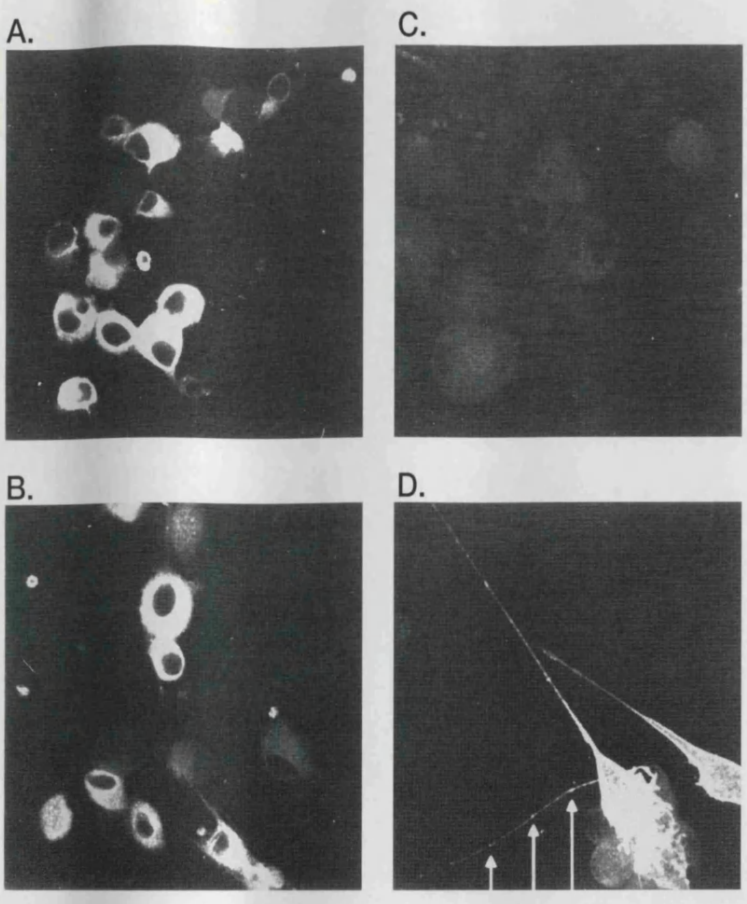

Figure 10. Infection of cultured SCG neurons with Semliki Forest viruses expressing $GABA_A$ receptor subunits. Cultured SCG neurons were infected after 3 d in culture, and receptor expression was determined by immunofluorescence using 9E10 antibody 12 hr after infection with (+) or without (−) permeabilization. Images were then collected using confocal microscopy from cells infected with *A*, $\alpha 1^{(9E10)}$ (+); *B*, $\beta 2^{(9E10)}$ (+); *C*, $\alpha 1^{(9E10)}$ (−); and *D*, $\alpha 1^{(9E10)}$ and $\beta 2^{(9E10)}$ (−).

$\alpha\beta$ and γ subunits using Semliki Forest virus. Routine triple infection of cells with viruses encoding $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits has proven difficult.

We exploited the broad host range of the Semliki Forest virus (Liljestrom and Garoff, 1991) to examine whether the assembly of $GABA_A$ receptors seen in heterologous systems is a faithful model of neuronal events. The use of receptor cDNAs modified with reporter epitopes (Connolly et al., 1996b), allowed the discrimination between recombinant and neuronal receptor pools to be made. Infection of SCG neurons with either single $\alpha 1^{(9E10)}$ or $\beta 2^{(9E10)}$ subunit viruses did not result in surface expression, because these subunits were ER-retained. Co-infection resulted in robust surface expression. SCG neurons express benzodiazepine-sensitive $GABA_A$ receptors, suggesting the presence of α, β, and γ receptor subunits of unknown identity. Presumably, the failure of single subunits to reach the cell surface on viral infection is attributable to low levels of endogenous subunits available for oligomerization. Suppression of host protein synthesis on Semliki infection will exaggerate this problem (Liljestrom and Garrof, 1991). An alternative explanation is that the virally expressed subunits are unable to assemble with the endogenous receptor subunits. Taken together the ER retention of single subunits and robust surface expression seen of the $\alpha 1^{(9E10)}\beta 2^{(9E10)}$ combination in both cell types suggest that receptor assembly occurs via similar pathways in BHK cells and SCG neurons. Whether the efficiency of assembly is similar in BHK cells and SCG neurons remains to be established. Neuronal-specific chaperone proteins that enhance expression of red and green opsins have been reported (Ferreria et al., 1996). Whether a similar mechanism exists to enhance the assembly of $GABA_A$ receptors is unknown. Finally, the ability to infect neurons with multiple Semliki Forest viruses expressing defined epitope-tagged receptor subunits should allow the assembly and trafficking of $GABA_A$ receptors to be studied in their native environments.

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TABLE 1 Effect of cSRC and sodium vanadate on GABA single-channel properties

Channel parameters	Control	+cSRC/$NaVo_3$
Mean open time, τ_0 (ms)	5.14±1.26 (5)	8.03±1.93* (4)
Open time, τ_{01} (ms)	2.55±0.76 (5)	3.9±1.15
Open time, τ_{02} (ms)	13.07±3.48 (5)	13.45±4.88 (4)
Single-channel current, i (pA)	1.79±0.08 (5)	1.73±0.19 (4)
Open probability (%)	0.12±0.09 (5)	0.45±0.19* (4)

Effects of cSRC (75 U ml^{-1}) and sodium vanadate (100 μM) on single GABA ion-channel parameters. Numbers in parentheses represent the number of experiments.

* Parameters are significantly different ($P<0.05$) when analysed with a modified two-tailed unpaired t-test, allowing for unequal variances.

Modulation of $GABA_A$ receptors by tyrosine phosphorylation

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γ-AMINOBUTYRIC acid type-A ($GABA_A$) receptors are the major sites of fast synaptic inhibition in the brain. They are presumed to be pentameric heteroligomers assembled from four classes of subunits with multiple members: α (1–6), β (1–3), γ (1–3) and δ (1)[1–5]. Here, $GABA_A$ receptors consisting of α1, β1 and γ2L subunits, coexpressed in mammalian cells with the tyrosine kinase vSRC (the transforming gene product of the Rous sarcoma virus), were phosphorylated on tyrosine residues within the γ2L and β1 subunits. Tyrosine phosphorylation enhanced the whole-cell current induced by GABA. Site-specific mutagenesis of two tyrosine residues within the predicted intracellular domain of the γ2L subunit abolished tyrosine phosphorylation of this subunit and eliminated receptor modulation. A similar modulation of $GABA_A$ receptor function was observed in primary neuronal cultures. As $GABA_A$ receptors are critical in mediating fast synaptic inhibition, such a regulation by tyrosine kinases may therefore have profound effects on the control of neuronal excitation.

Tyrosine phosphorylation of $GABA_A$ receptors was analysed in human embryonic kidney cells[5–7] (A293; CRL 1573) expressing $GABA_A$-receptor α1, β1 and γ2L subunits. Receptors composed of these subunits can reproduce many of the properties of neuronal $GABA_A$ receptors[1,3,5,7]. Tagging of the γ2L subunit with the 9E10 epitope[8] was used to aid the immunopurification of $GABA_A$ receptors. Addition of this epitope to the γ2L subunit seemed to be functionally silent (S.J.M and T.G.S, unpublished observations). The $\gamma2L^{(9E10)}$ subunit was identified as a protein of relative molecular mass 42,000 (M_r 42K) that assembled efficiently with the α1 (52K) and β1 (56K and 58K) subunits (Fig. 1*a*, lanes 1–3) as judged by coprecipitation with 9E10 antibodies. The identity of the α1 and β1 subunits was confirmed by immunoprecipitation under denaturing conditions, using subunit-specific antisera[9,10] (Fig. 1*a*, lanes 4–7). The multiple bands for the α1 and β1 subunits seemed to result from proteolysis[9,10]. In native immunoprecipitations, a protein of approximately 76K (Fig. 1*a*, lanes 1–3) coprecipitated with the $GABA_A$ receptor subunits. This was identified as the molecular chaperone, heavy-chain-binding protein (C. Connolly and S.J.M., unpublished observations).

To analyse tyrosine phosphorylation, A293 cells were co-transfected with complementary DNAs for $GABA_A$ receptor subunits and a cDNA encoding vSRC, the cellular homologue of which is widely expressed in the central nervous system[11,12]. Western blotting demonstrated that A293 cells have low levels of phosphotyrosine, which could not be significantly enhanced by treatment with vanadate, a tyrosine phosphatase inhibitor (Fig. 1*b*, lanes 1, 2), suggesting that A293 cells have high intrinsic phosphatase and/or low tyrosine kinase activities[13,14]. Expression of vSRC in these cells led to a significant increase in phosphotyrosine content (Fig. 1*b*, lanes 3–5). In accordance with this, receptors composed of α1, β1 and $\gamma2L^{(9E10)}$ subunits, expressed alone in A293 cells, showed undetectable or only low levels of basal phosphorylation[9,10] (Fig. 1*c*, lane 5). Coexpression of receptor subunits with vSRC resulted in phosphorylation of the γ2L subunit (Fig. 1*c*, lane 2) on tyrosine residues only (Fig. 1*d*, lane 1). The β1 subunit was also phosphorylated but to a much lower stoichiometry (approximately eightfold). The predicted major intracellular domain of the γ2L subunit contains a consensus site for tyrosine phosphorylation centred on tyrosines 365 and 367 (refs 6, 12, 15). Mutation of both of these residues to phenylalanines abolished phosphorylation of the $\gamma2L^{(9E10)}$ subunit when it was coexpressed with the α1 and β1 subunits (Fig. 1*c*, lane 3). Mutation of these sites on the $\gamma2L^{(9E10)}$ subunit led to an increased phosphorylation of the β1 subunit (Fig. 1*c*, lane 3) which occurred exclusively on tyrosine residues (Fig. 1*d*, lane 2). The phosphorylation of the β1 subunit was abolished by mutation of tyrosines 384 and 386 within the predicted intracellular domain of this subunit (Fig. 1*c*, lane 4).

The functional effects of tyrosine phosphorylation were determined by whole-cell recording in A293 cells. Intracellular application of the tyrosine kinase inhibitor genistein to cells expressing vSRC and α1β1γ2L receptor subunits led to a time-dependent decrease in the amplitude of the GABA response (Fig. 2*a*). Biochemical analysis of α1β1γ2L $GABA_A$-receptor subunits exposed to genistein also revealed a 10–20% decrease in the phosphorylation of the γ2L subunit (data not shown). Genistein, however, had no effect on receptors incorporating the mutant $\gamma2L^{Y(365/367)F}$ subunit (Fig. 2*b*). Expression of this mutant subunit did not differ significantly from that of the wild-type

$\gamma2L^{(9E10)}$ subunit, as judged by western blotting (Fig. 1*e*) and receptor pharmacology (data not shown). Modulation of $GABA_A$-receptor function was also studied by exposing receptors expressed alone in A293 cells to purified cSRC by means of intracellular dialysis. In cells exposed to both cSRC and sodium vanadate, an enhancement of the GABA-activated whole-cell current was evident (Fig. 2*c*, *e*). The current mediated by $GABA_A$ receptors incorporating the mutant $\gamma2L^{Y(365/367)F}$ subunit was not enhanced by cSRC and sodium vanadate (Fig. 2*d*, *e*). In common with the vSRC cotransfection experiments, we found no functional effects of phosphorylating the two tyrosine residues in the β1 subunit, as the effects of cSRC were totally abolished by mutation of the phosphorylation sites on the γ2L subunit.

To assess whether native neuronal $GABA_A$ receptors are regulated by tyrosine phosphorylation, recordings were made from cultured superior cervical ganglion (SCG) neurons. $GABA_A$ receptor function is modulated in these neurons by benzodiazepines, suggesting the presence of $GABA_A$ receptors incorporating γ2 and/or γ3 subunits[6,7,16–18]. Exposure to genistein by means of intracellular dialysis led to a time-dependent decrease in the amplitude of the GABA response in SCG neurons (Fig. 3*a*, *d*). Intracellular application of sodium vanadate alone caused an increase in the magnitude of the GABA response, suggesting that endogenous tyrosine kinases are more active in these neurons than in A293 cells (Fig. 3*b*, *d*). This was confirmed by biochemical analysis, which revealed that SCGs have approximately fourfold higher levels of kinase activity (data not shown). Treatment with cSRC also caused a significant time-dependent increase in the GABA-activated current after recording for 15 min (Fig. 3*c*, *d*). Overall, these observations show that neuronal $GABA_A$ receptors can be modulated, either directly or indirectly, by tyrosine kinase activity and, in common with the observations with recombinant receptors, this seems to enhance receptor function.

To examine the likely mechanism underlying the enhancement of whole-cell GABA responses, single GABA-channel currents, activated by 10 μM GABA, were studied in outside-out patches taken from SCG neurons in the absence and presence of internal cSRC and sodium vanadate (Fig. 4*a*, *b*). The single-channel current and chord conductances at −70 mV holding potential (control, 25.6 pS, $n=7$; +cSRC/sodium vanadate, 24.7 pS, $n=4$) were unaffected by cSRC/vanadate treatment (Table 1). However, both the mean open time and the probability of channel opening were significantly increased ($P<0.05$; Table 1). Analysis of the distribution of all open times for patches containing few simultaneous openings revealed that two exponential functions were required to fit the dwell time distribution (Fig. 4*c*, *d*). The time constants τ_{01} and τ_{02}, representing respectively the short and long open time distributions, were apparently unaffected by

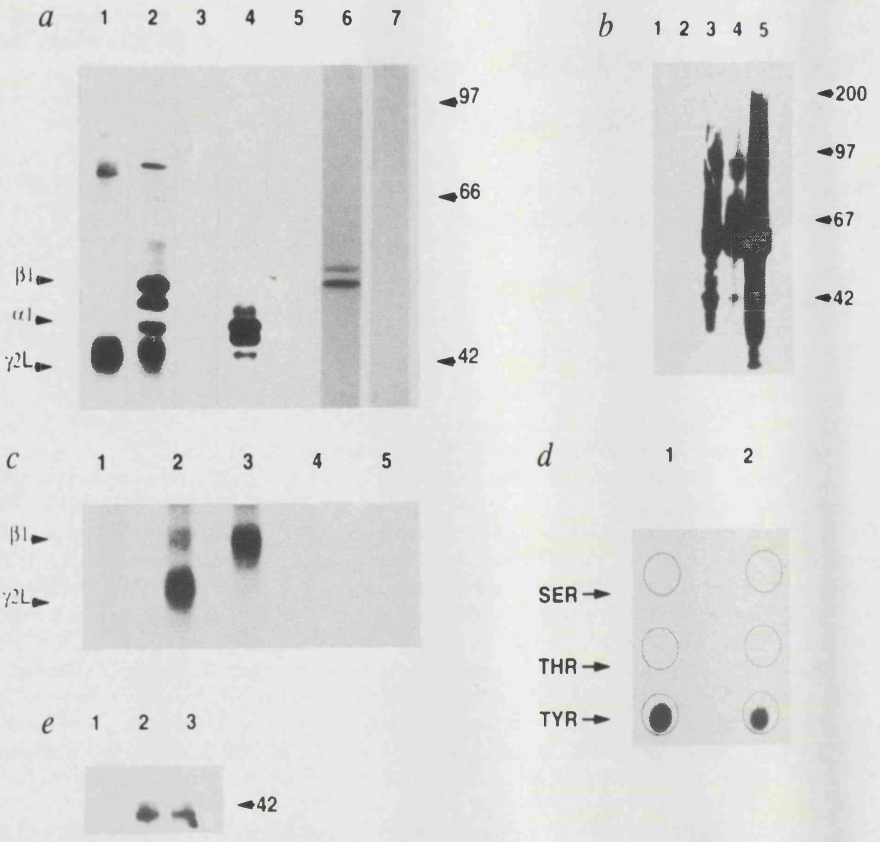

FIG. 1 Tyrosine phosphorylation of $GABA_A$-receptor subunits expressed in A293 cells. *a*, Identification of receptor subunits by immunoprecipitation. Receptors were isolated from expressing cells under native (N) or denaturing (D) conditions after [^{35}S]methionine labelling with either 9E10 antisera or anti-α1 or β1 antibodies, followed by SDS–PAGE (8% gels) and fluorography. Lane 1, $\gamma2L^{(9E10)}$ alone, 9E10 (N); lane 2, $\alpha1\beta1\gamma2L^{(9E10)}$, 9E10 (N); lane 3, control cells, 9E10 (N); lane 4, $\alpha1\beta1\gamma2L^{(9E10)}$, anti-α1 (D); lane 6, $\alpha1\beta1\gamma2L^{(9E10)}$ anti-β1 (D); lanes 5 and 7, $\alpha1\beta1\gamma L^{(9E10)}$, anti-α1 (D) and anti-β1 (D), respectively preadsorbed with the relevant immunogens. Numbers on the right indicate molecular weight standards. *b*, Western blotting of A293 total cell lysates with phosphotyrosine antibodies. Lane 1, control cells; lane 2, +100 μM sodium vanadate for 4 h at 37 °C; lanes 3 and 5, +vSRC and receptor $\alpha1\beta1\gamma2L^{(9E10)}$ subunits; lane 4, vSRC alone, blotted with a monoclonal antiserum against phosphotyrosine. 50 μg of total protein was loaded in lanes 1–4, 250 μg in lane 5. *c*, Phosphorylation of $GABA_A$ receptors by vSRC. Expressing cells were labelled with [^{32}P]orthophosphoric acid and immunoprecipitated with 9E10 antisera. Receptor subunits were resolved by SDS–PAGE and autoradiography. Lane 1, vSRC alone; lane 2, $\alpha1\beta1\gamma2L^{(9E10)}$ + vSRC; lane 3, $\alpha1\beta1\gamma2L^{(9E10)Y(365/367)F}$ + vSRC; lane 4, $\alpha1\beta1^{Y(383/385)F}\gamma2L^{(9E10)Y(365/367)F}$ + vSRC; lane 5, $\alpha1\beta1\gamma2L^{(9E10)}$ alone. *d*, Phosphoamino-acid analysis of $GABA_A$-receptor subunits. Lane 1, $\gamma2L^{(9E10)}$ subunit (*c*, lane 2); lane 2, β1 subunit (*c*, lane 3). The migration of phosphoserine, phosphothreonine and phosphotyrosine standards is indicated. *e*, Detection of wild-type and mutant forms of the $\gamma2L^{(9E10)}$ subunit by western blotting. A293 cells expressing receptor subunits were lysed and blotted with 9E10 antisera. Lane 1, control cells; lane 2, $\alpha1\beta1\gamma2L^{(9E10)}$; lane 3, $\alpha1\beta1\gamma2L^{(9E10)Y(365/367)F}$.

METHODS. Expression and immunoprecipitation of $GABA_A$ receptors in A293 cells, western blotting, phosphoamino-acid analysis and the anti-α1 and anti-β1 sera have been described previously[6–9,10,13,21,22]. The 9E10 epitope, EQKLISDEEL[8], was added to the γ2L subunit between amino acids 4 and 5 by site-specific mutagenesis[9,10]. Mutagenesis was also used to convert tyrosines 365/367 (γ2L subunit) and tyrosines 384/386 (β1 subunit) to phenylalanine residues.

cSRC/vanadate (Table 1). The increased mean open time seemed to result from a shift in the relative areas of the distributions, with more long openings (22% increased to 39%) occurring at the expense of the shorter openings (78% reduced to 61%; $P<0.05$). this effect would also increase the probability of channel opening.

In conclusion, our results suggest that tyrosine phosphorylation may be an endogenous regulatory mechanism for enhancing or maintaining $GABA_A$-receptor function[19,20]. Given the ubiquity of tyrosine kinases in the central nervous system[11,12], and their regulation by growth factors and neurotransmitters, tyrosine phosphorylation may therefore represent a new and diverse

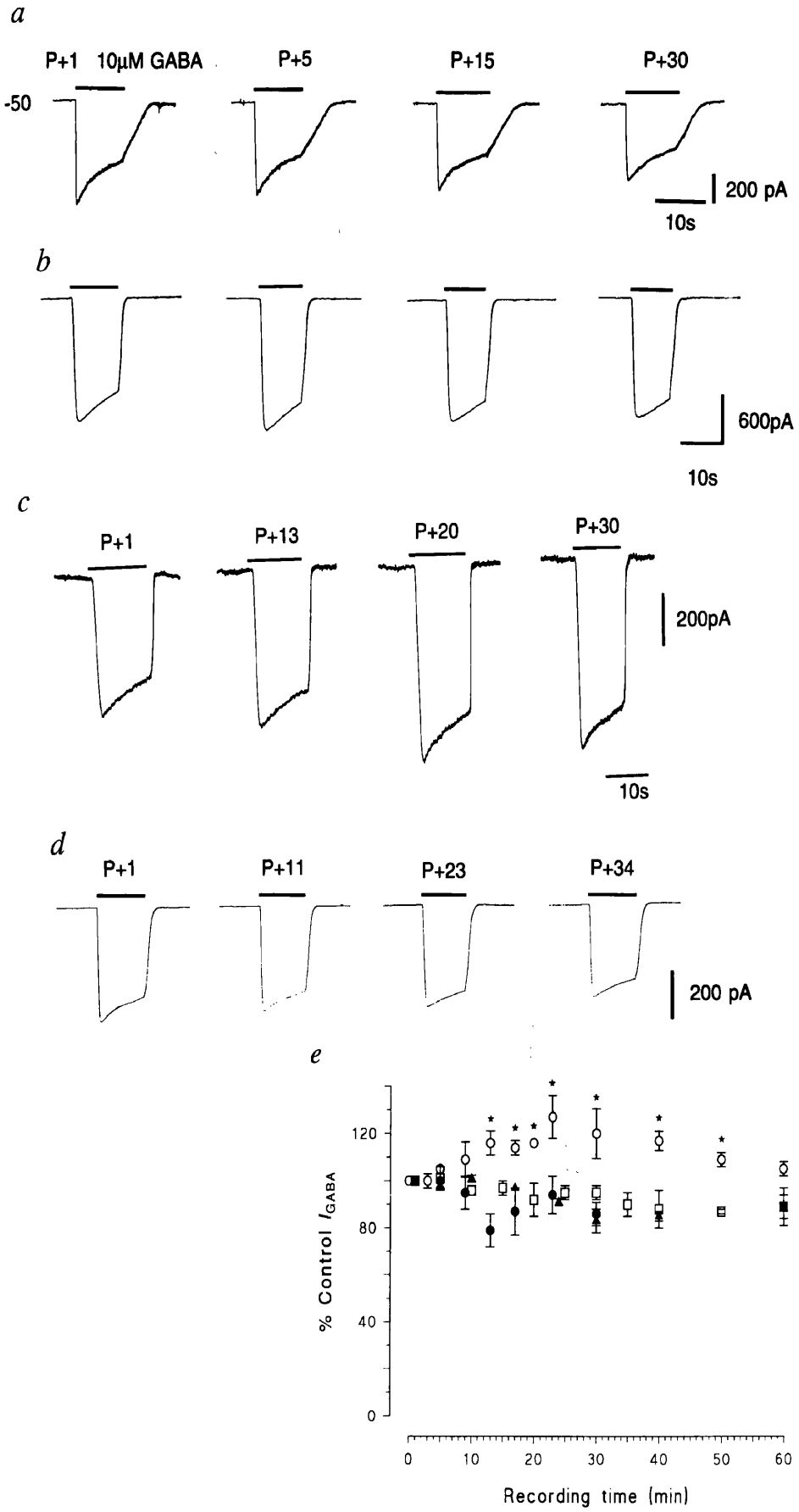

FIG. 2 Whole-cell responses to 10 μM GABA recorded from A293 cells expressing receptors composed of wild-type α1β1γ2L subunits or receptors in which biochemically identified phosphorylation sites had been mutated with or without vSRC. *a*, α1β1γ2L + vSRC and *b*, $\alpha1\beta1\gamma2L^{Y(365,367)F}$ + vSRC exposed to 100 μM genistein by intracellular dialysis. *c*, *d*, Recordings from cells expressing only α1β1γ2L and $\alpha1\beta1\gamma2L^{Y(365/367)F}$, respectively, exposed to cSRC (75 U ml^{-1}) and sodium vanadate (100 μM) by intracellular dialysis. The cells were held at −50 mV and GABA was rapidly applied from a nearby Y-tube for the duration indicated by the bar. Recording times above each trace (P+*x*) indicate the duration in min following the attainment of the whole-cell configuration. *e*, Stability plot of GABA-activated membrane currents (I_{GABA}). The peak amplitudes of membrane currents activated by 10 μM GABA in transfected A293 cells expressing either α1β1γ2L or $\alpha1\beta1\gamma2L^{Y(365,367)F}$ constructs are pooled and plotted against recording time (formation of the whole-cell recording mode is defined as $t=0$ min). The first response to GABA, usually elicited within P+1, was defined as 100%. Points are mean±s.e.m. for : α1,β1,γ2L, ▲, control pipette solution ($n=6$ cells); α1β1γ2L, ●, +100 μM $NaVO_3$ ($n=4$); α1β1γ2L, ○, +75 U ml−1 cSRC and 100 μM $NaVO_3$ ($n=5$); $\alpha1\beta1\gamma2L^{Y(365,367)F}$, □, +75 U ml^{-1} cSRC and 100 μM $NaVO_3$ ($n=6$). * A significant difference from control untreated ($P<0.05$ assessed by ANOVA with Bonferroni, Tukey–Kramer or Student–Newman–Keuls *post hoc* tests). Recordings were made from transiently transfected A293 cells up to 24 h after transfection with receptor expression constructs as described previously[9,10].

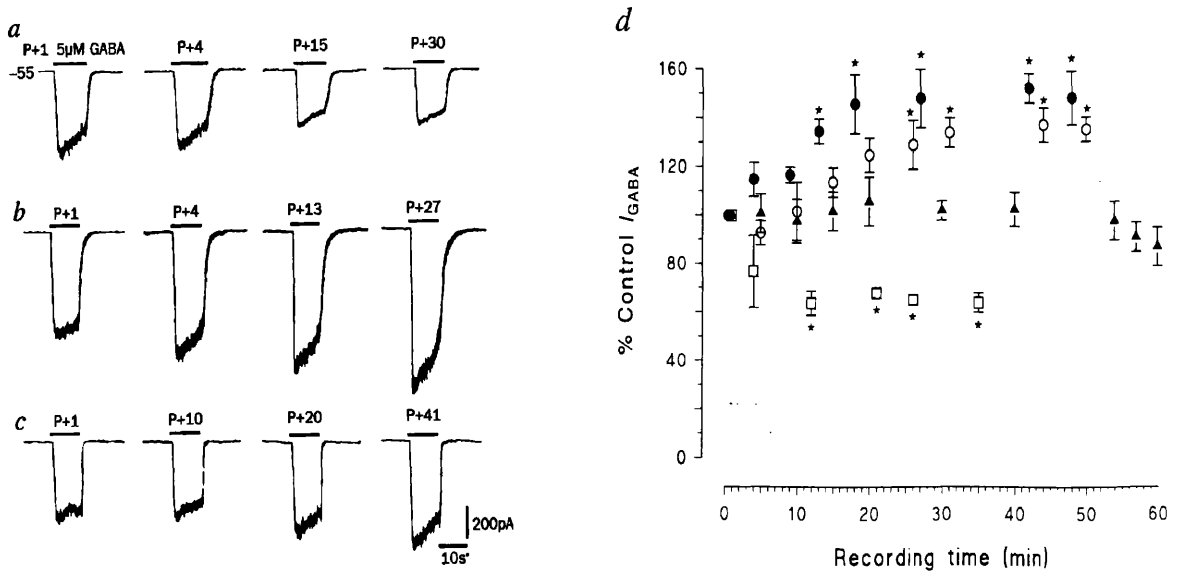

FIG. 3 Superior cervical ganglion neurons in culture were exposed to 5 μM GABA from the Y-tube and internally perfused with 20 μM genistein (*a*), 100 μM $NaVO_3$ (*b*) or 75 U ml^{-1} cSRC (*c*). Peak current amplitudes to GABA were pooled and plotted against the recording time in *d*. Points are mean±s.e.m. for neurons exposed to: ●, 100 μM $NaVO_3$ ($n=4$); ○, 75 U ml^{-1} cSRC ($n=3$); □, 20 μM genistein ($n=4$); and ▲, control pipette solution ($n=7$). * Values significantly different from control ($P<0.05$ assessed by ANOVA). Superior cervical ganglia were removed from postnatal rats (P+2–4 days) and mechanically and enzymatically dissociated as described previously[9,10,18].

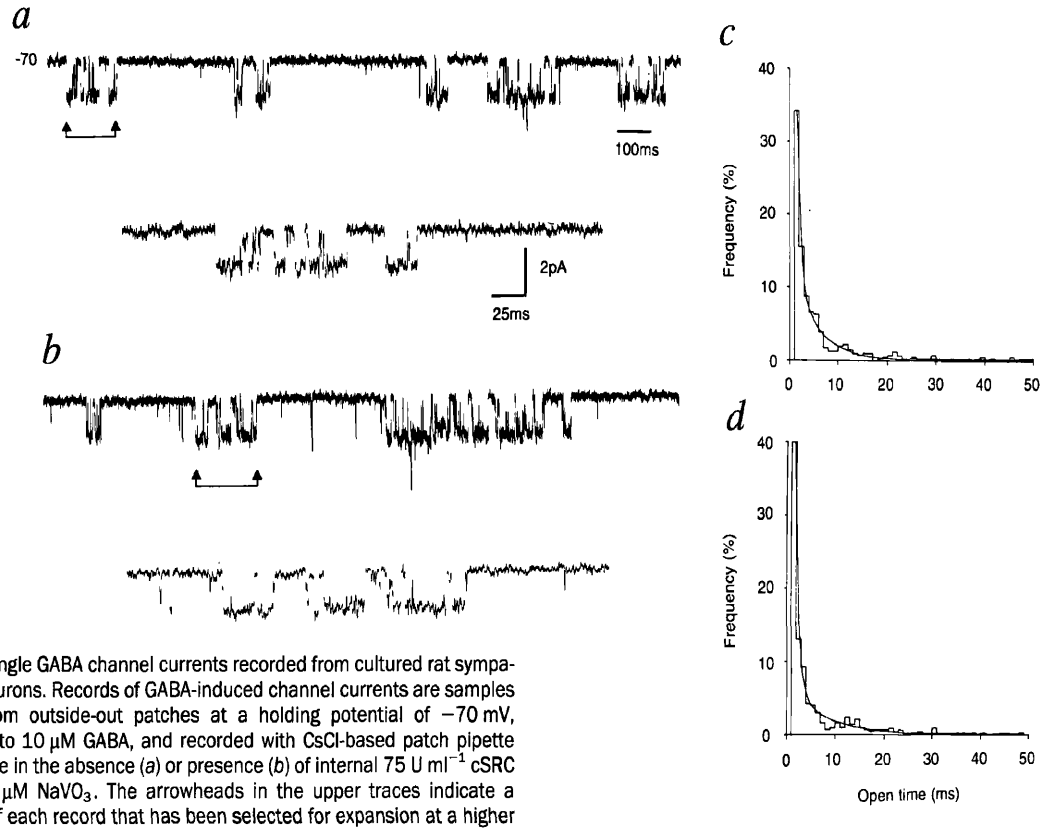

FIG. 4 Single GABA channel currents recorded from cultured rat sympathetic neurons. Records of GABA-induced channel currents are samples taken from outside-out patches at a holding potential of −70 mV, exposed to 10 μM GABA, and recorded with CsCl-based patch pipette electrolyte in the absence (*a*) or presence (*b*) of internal 75 U ml^{-1} cSRC and 100 μM $NaVO_3$. The arrowheads in the upper traces indicate a portion of each record that has been selected for expansion at a higher time resolution and is shown in the lower traces. In the absence (*c*) or presence (*d*) of cSRC and $NaVO_3$ the mean open time distributions, for all openings longer than 100 μs, were described by the sum of two exponential components.

METHODS. Outside-out patches were obtained from cultured rat sympathetic neurons bathed in control Krebs solution as described previously[18]. To induce GABA-activated single-channel currents, the excised patch was positioned juxtaposed to a Y-tube from which 10 μM GABA was rapidly applied. The single-channel currents were resolved using an EPC7 amplifier and stored on a Racal tape recorder (d.c. to 5 kHz). Currents were filtered at 1–3 kHz (−3dB; 6-pole Bessel) and digitized at 5–15 kHz before analysis using software provided by J. Dempster (PAT ver. 7; University of Strathclyde). The measurements of open durations were made using a 50% threshold cursor to the main conductance state of 25 pS. The transition detection of open and closed states was used to form an idealized record from which individual open durations were measured and collated into the frequency distributions. The distributions were fitted with the sum of exponential functions using a nonlinear least squares, Levenberg–Marquadt fitting routine as described previously[18]. Single-channel currents were accepted for kinetic analysis if there was apparently only one channel active, or when the number of multiple channel openings never exceeded 2% of the total number of open events[18].

process for modulating inhibition mediated via $GABA_A$ receptors and consequently cell excitability.

Note added in proof: Similar modulation of $GABA_A$ receptor function has recently been reported elsewhere[23]. □

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