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Cellular Distribution and Immobilisation
of GABA_A Receptors

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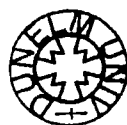
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A thesis submitted to the Department of Biological Sciences,

University of Durham in accordance with the requirements

for the degree of Doctor of Philosophy

2000



19 JUN 2001

To Roberto and my kids Robertito and Ritilla

Abstract

Synaptic inhibition in the vertebrate central nervous system is largely mediated by type A GABA receptors (GABA_AR). The clustering of GABA_AR at discrete and functionally significant domains on the nerve cell surface is an important determinant in the integration of synaptic inputs. To discern the role that specific GABA_AR subunits play in determining the receptor's cell surface topography and mobility, recombinant GABA_ARs, comprising different GABA_AR subunit combinations, were transiently expressed in COS7, HEK293 and PC12 cells. In addition, a series of domain swapping experiments were performed in order to elucidate which regions of the protein are important in mobility/anchoring of receptors. The cellular localization and lateral mobility of the recombinantly expressed GABA_ARs were determined by immunocytochemistry and Fluorescence Photobleach Recovery (FPR), respectively.

The results presented in this thesis show that GABA_AR α 1 subunits are recruited by the β 3 subunits from an internally sequestered pool and assembled into a population of GABA_ARs that are spatially segregated into clusters and also immobilised on the cell surface. FPR experiments on recombinant GABA_AR containing α 1- α 6 subunits expressed in COS7 cells showed restricted mobilities consistent with mobility constants determined for native GABA_ARs expressed on cerebellar granule cells. Furthermore, the intracellular loop domain M3/M4 of the α 1 subunits was found to be required for anchoring recombinantly expressed GABA_ARs in COS7 and cerebellar granule cells in culture, but not for GABA_AR clustering at the cell surface.

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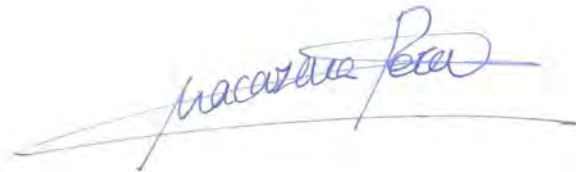
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Declaration

No part of this thesis has been submitted previously for a degree at this or any other institution. All the material is my own original work unless otherwise stated.

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A handwritten signature in blue ink, appearing to read 'Mucawana Peru', is written over a horizontal line.

Most used abbreviations

AMPAR	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
CNS	Central Nervous System
Dc	Lateral Diffusion coefficient
DOPE	L-dioleoyl phosphatidylethanolamine
DOTAP	N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
F	Mobile Fraction
FBS	Foetal Bovine Serum
FPR	Fluorescence Photobleaching Recovery
GABA	Gamma-aminobutyric acid
GABA _A R	Type A GABA receptor
GABA _B R	Type B GABA receptor
GABA _C R	Type C GABA receptor
GABARAP	GABA _A R-associated protein
GlyR	Glycine receptor
GPI	Glycosylphosphatidylinositol
HIGS	Heat-Inactivated Goat Serum
IPSP	Inhibitory postsynaptic potentials
MEM	Modified Eagle Medium
mGluR	Glutamate metabotropic receptor
NA	Numerical Aperture
NGF	Nerve Growth Factor
NMDAR	N-methyl-D-aspartate receptor
PBS	Phosphate Buffered Saline
PDZ	PSD-95, discs large and ZO-1

Chapter 1

Introduction

Chapter one

Introduction

1.1. GABA receptors

The mammalian central nervous system (CNS) is a highly complex integrative centre, processing a vast number of positive and negative inputs. Proper functioning of the brain depends on a delicate interplay between excitatory and inhibitory neurotransmission between neurons. The major inhibitory neurotransmitter in the vertebrate brain is gamma-aminobutyric acid (GABA).

GABA was originally identified as the principal agent in brain extracts capable of inhibiting crayfish stretch-receptor neurons, an effect mediated by an increase in the membrane permeability to Cl^- . This was followed by studies into the inhibitory role of this amino acid in crustacea, in vertebrate, and finally in the mammalian CNS, where it may function at up to 40% of the synapses in brain, (for review see Bennett and Balcar, 1999).

GABA mediates its inhibitory effect through its interaction with a variety of receptors in all areas of the central nervous system. Two major types of GABA receptors have been well described: Type A GABA receptor (GABA_AR) and type B GABA receptor (GABA_BR , Hill and Bowery, 1981). These receptors are quite different, not only from a pharmacological point of view but also structurally and functionally.

GABA_ARs are bicuculline-sensitive, ligand-gated Cl^- channels. Following the binding of GABA to the GABA_AR , a conformational change results in the opening of an intrinsic Cl^- pore which, in most cases, results in the hyperpolarisation of the recipient neuronal cell leading to stabilisation of the



resting membrane potential (Olsen and Venter, 1986; Stephenson, 1988; Farrant *et al.*, 1990).

GABA_BRs bind baclofen, are bicuculline-insensitive (Sivilotti and Nistri, 1988), and their mode of action is metabotropic. GABA_BRs mediate their effects through potassium and calcium channels via a guanine-nucleotide-binding (G) protein (Bowery, 1993). Both GABA_AR and GABA_BR have inhibitory roles in the CNS, although the activation of GABA_AR induces fast inhibitory postsynaptic potentials (IPSP) in contrast with the slow IPSP induced by GABA_BR activation (Nakayasu *et al.*, 1995).

In addition, a third type of GABA receptor, which are exclusively composed of $\rho(1-3)$ subunits, has recently been identified in retina, type C GABA receptor (GABA_CR, Lukasiewicz, 1994). GABA_CRs are also ligand-gated Cl⁻ channels, however, there are differences in the pharmacological characteristics of GABA_AR and GABA_CR that allow them to be discriminated in a separate type of GABA receptor (Johnston, 1986), although discrepancies have emerged (discussed in Barnard *et al.*, 1998). GABA_CRs are not sensitive to bicuculline blockade and not modulated by barbiturates, benzodiazepines or neuroactive steroids. These receptors are activated at lower concentrations of GABA and their mean channel open times in response to GABA are greater (Johnston, 1996; Cherubini and Strata, 1997).

The studies carried out in this thesis have focussed on GABA_AR.

1.2. GABA_AR pharmacology

The GABA_AR is important not only because of its fundamental role in the regulation of brain excitability but also by the fact that its function is allosterically regulated by pharmaceutically significant drugs (Turner and Whittle, 1983; Olsen, 1987). The GABA_AR response is positively modulated by benzodiazepine agonists such as flunitrazepam and clonazepam (anxiolytics) or negatively modulated by inverse agonists, such as the β -carboline, methyl-4-ethyl-6,7-dimethoxy- β -carboline-3-carboxylate (anxiogenic). GABA_ARs also have binding sites for a variety of other clinically important substances, including barbiturates (anti-convulsant), certain neuroactive steroids, in either a positive or a negative manner (Majewska *et al.*, 1986, 1990) and picrotoxin (convulsant agents). Although both barbiturates and benzodiazepine agonists potentiate the effect of GABA, they do so by different mechanisms. Barbiturates prolong the mean open time of the channel while benzodiazepine agonists increase the frequency of channel opening (McDonald and Olsen, 1994).

These mentioned compounds, in most cases, do not interact directly with the GABA binding site but exert their action by binding to allosterically-coupled sites on the GABA_A receptors. Thus, binding of these agents induce conformational changes in the GABA_A receptor that may influence its binding properties and modulate GABA-induced chloride ion flux (Sieghart, 1992). GABA_ARs are believed to have at least five different binding sites: the GABA, the picrotoxin/convulsant, the benzodiazepine, the barbiturate and the steroid binding sites. From binding studies using specific radioligands only three of these different binding sites have been fully characterized, the picrotoxin/convulsant site (Ticku *et al.*, 1978; Squires *et al.*, 1983), the benzodiazepine site (Squires and

Braestrup, 1977) and the GABA binding site (Zukin *et al.*, 1974). However, all the other compounds that interact with GABA_ARs are either not available as radioligands or their potency for modulating GABA_ARs is too low to allow direct binding studies. Thus, their site(s) of action has been investigated by studying their interaction with the three well-characterized binding sites on GABA_ARs previously described.

A schematic model of a mammalian GABA_AR showing the chloride channel and the five proposed distinct functional binding domains for various ligands and modulators that act on the receptor complex is shown in Figure 1.1.

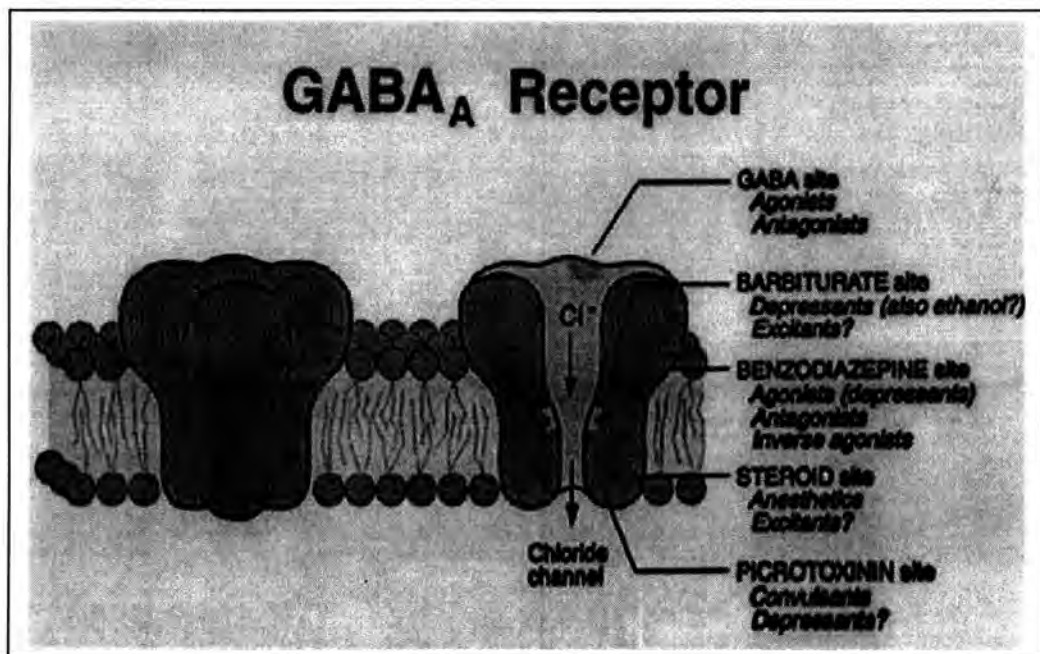


Figure 1.1. Structural model of the GABA_A/benzodiazepine receptor-chloride ionophore complex. The cut-away view demonstrates targets for a variety of compounds that influence the receptor complex. (From: DeLorey and Olsen, 1994)

1.2.1. The GABA binding site.

GABA, by binding to GABA_ARs, opens channels that are selectively permeable to Cl⁻. Depending on the prevailing electrochemical membrane potential for Cl⁻, the effect of GABA can be excitatory or inhibitory. In developing, immature neurons, activation of GABA_AR results in an efflux of Cl⁻ that causes a membrane depolarisation (Cherubini *et al.*, 1991). In terminally differentiated neurons, the Cl⁻ electrochemical membrane potential is such that following GABA_ARs activation Cl⁻ flows into the cell and induces a slight hyperpolarisation of the membrane and a reduced neuronal excitability of the cells.

The affinity of GABA to GABA_ARs is very low, micromolar concentrations of GABA being required to activate the chloride ion channel in electrophysiological experiments (Segal and Barker, 1984). It has been suggested from electrophysiological experiments that at least two molecules of GABA must bind to the receptor to induce full activation of channel activity (Sakmann *et al.*, 1983). Sieghart (1995) discuss the apparent existence of several distinct GABA binding sites on a single GABA_AR. These sites show high, low and very low affinity for GABA and its agonist.. Up to five GABA binding sites might be present on a single GABA_AR. These binding sites in the unoccupied state might have a similar high affinity for GABA agonist. On increasing occupation these sites with GABA, the affinity of the remaining unoccupied sites might allosterically become reduced. The high and possibly the low affinity GABA sites probably are constantly occupied under the physiological GABA concentration present in the synaptic cleft. Occupation of these sites does not cause an opening of the chloride channels. Thus, the existence of the remaining binding site with

very low affinity ensures that GABA-activated chloride channels can only be opened under conditions of synaptic transmission where a large amount of GABA is released into the synaptic cleft.

1.2.2. The benzodiazepine binding site

Benzodiazepines, such as diazepam or flunitrazepam, enhance GABAergic function by increasing the frequency of Cl⁻ channel opening with little effect on the channel open time or GABA_AR affinity (Edgar and Schwartz, 1992). Benzodiazepines, however, are not able to open directly the GABA_AR chloride channel in the absence of GABA (Study and Barker, 1981).

1.2.3. The picrotoxin/convulsant binding site

Picrotoxin and cage convulsants are allosteric inhibitors of GABA_AR activity by directly binding to the GABA_AR chloride pore and hence, blockade of the channel (Inoue and Akaike, 1988). These substances do not displace benzodiazepines from their high affinity binding sites (Olsen, 1982), but allosterically modulate benzodiazepine receptor binding (Karobath *et al.*, 1981).

1.2.4. Barbiturate interaction with GABA_AR

Barbiturates can exert two different effects on GABA_AR that are differentially dose dependent. Electrophysiological studies revealed that these substances enhance the actions of GABA by increasing the mean channel open duration and not altering receptor conductance or opening frequency (Study and Barker, 1981). At higher concentrations (>50μM) barbiturates are able to open directly the GABA_AR chloride channel in the absence of GABA (Bormann, 1988).

1.2.5 Steroids interaction with GABA_AR

Several steroids, at low concentrations (30 to 300 nM), enhance GABA-stimulated chloride conductance (Majewska, 1992; Kokate *et al.*, 1994) and at higher concentrations (1 μM) produce a direct opening of the Cl⁻ channel that is inhibited by the GABA_AR antagonist bicuculline (Majewska, 1992).

1.3. GABA_AR gene diversity

Since the initial cloning in 1987 of cDNAs encoding two different subunits of the GABA_AR from bovine brain (Schofield *et al.*, 1987), the molecular biology of this receptor has become increasingly complex. The ligand-gated ion channel is proposed to be an hetero-oligomer composed of five subunits (Nayeem *et al.*, 1994). GABA_AR subunits are classified, with respect to their amino acid sequence homology and designated as α , β , γ , δ , ϵ , and π (Whiting *et al.*, 1997; reviewed in Mehta and Ticku, 1999). Recently, a novel GABA_AR subunit, named as Θ , has been found in rat brain (Chadha *et al.*, 1999). The α , β , and γ subunits contain multiple members or isoforms that arise from separate genes that have diversified from an evolutionary origin, so that there are, $\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$ and $\gamma 1$ – $\gamma 3$ (reviewed in McDonald and Olsen, 1994 and Mehta and Ticku, 1999). The existence of this wide range of subunits and subunit variants implies a vast diversity of potential GABA_AR subtypes. Further heterogeneity is introduced by the existence of alternative splice variants for mammalian GABA_AR $\alpha 6$, $\beta 2$ and $\gamma 2$ subunit genes (McKernan and Whiting, 1996). The $\gamma 2$ subunit, for instance exists in two forms, $\gamma 2$ short ($\gamma 2s$) and $\gamma 2$ long ($\gamma 2l$). The latter contains an extra 8 amino acids in its intracellular loop domain (Whiting *et al.*, 1990; Kofuji *et al.*,

1991; Glencorse *et al.*, 1992), which has been shown to be a target for protein kinase C-mediated phosphorylation and for reported differences in receptor pharmacology (Whiting *et al.*, 1990; Wafford *et al.*, 1991).

Figure 1.2. illustrates the six different GABA_AR subunits and their relationship in terms of amino acid sequence homology.

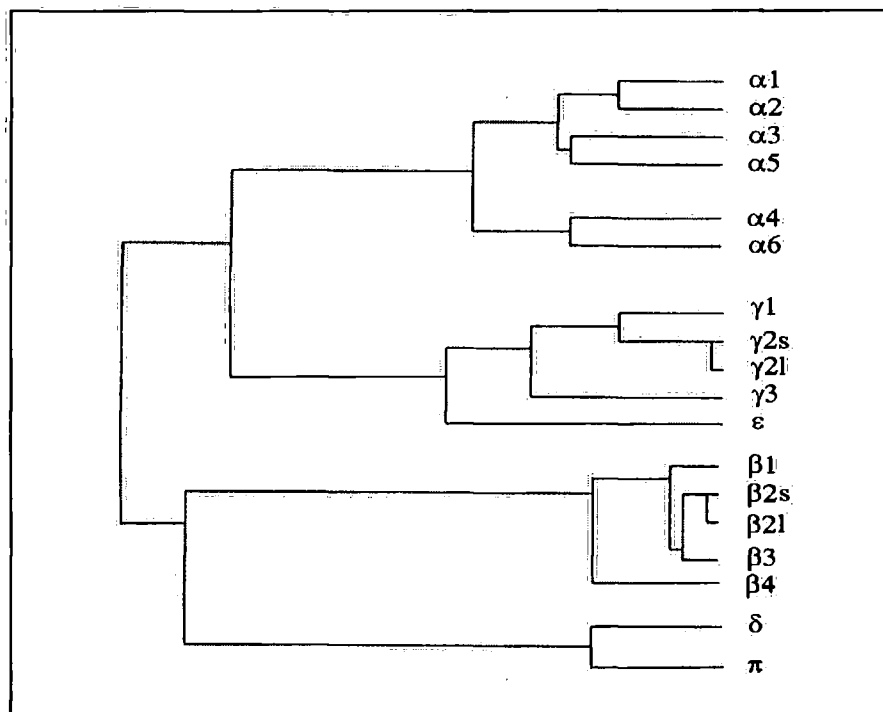


Figure 1.2. *The human GABA_A receptor polypeptide family.* The dendrogram to the left of the figure indicates the homologies between the deduced amino acid sequences of each subunit; the length of the line separating the subunits represents the distance between their sequences. γ_{2s}/γ_{2l} and β_{2s}/β_{2l} are the alternative splice isoforms of these subunits. (Modified from: McKernan and Whiting, 1996, and Barnard *et al.*, 1998).

1.4. Structural model of GABA_AR

GABA_AR is proposed to be a heteropentameric glycoprotein of about 275 kDa, composed of combinations of multiple subunits, polypeptides of about 50 kDa. (Figure 1.3.). The subunits form a quasi-symmetric structure around the ion channel. Each GABA_AR subunit is predicted to have four hydrophobic membrane-spanning domains, designated M1-M4. These putative hydrophobic domains are predicted to span the membrane and form the integral chloride ion channel. It is postulated that the M2 domain contributes to the inner wall of the channel (Schofield *et al.*, 1987, DeLorey and Olsen, 1992). The N-terminal, hydrophilic domain, of each subunit protein is predicted to be located extracellularly. This region contains several potential sites for N-linked glycosylation, thus, *in vivo* carbohydrate attachment would account for the differences between the observed and predicted molecular weights of the natural and cDNA-deduced GABA_AR subunit masses, respectively (Schofield *et al.*, 1987). A sequence of 15 amino acids, highly conserved across all the members of the ligand-gated ion channel superfamily receptor, is found in this N-terminal region. This sequence is predicted to form a Cys-Cys β loop. The C-terminal end of the protein follows the M4 domain and, as with the N-terminus, is thought to be extracellular.

The transmembrane-spanning domains (M1-M4) and defined regions within the N-terminus are highly conserved among different subunit types. In contrast, sequence identity is poorly conserved in the cytoplasmic loop region, which connects M3 and M4. This domain, designated the M3/M4 cytoplasmic loop, is hydrophilic and contains consensus sequences for phosphorylation by various kinases. Figure 1.3. represents the predicted structural model of the GABA_AR.

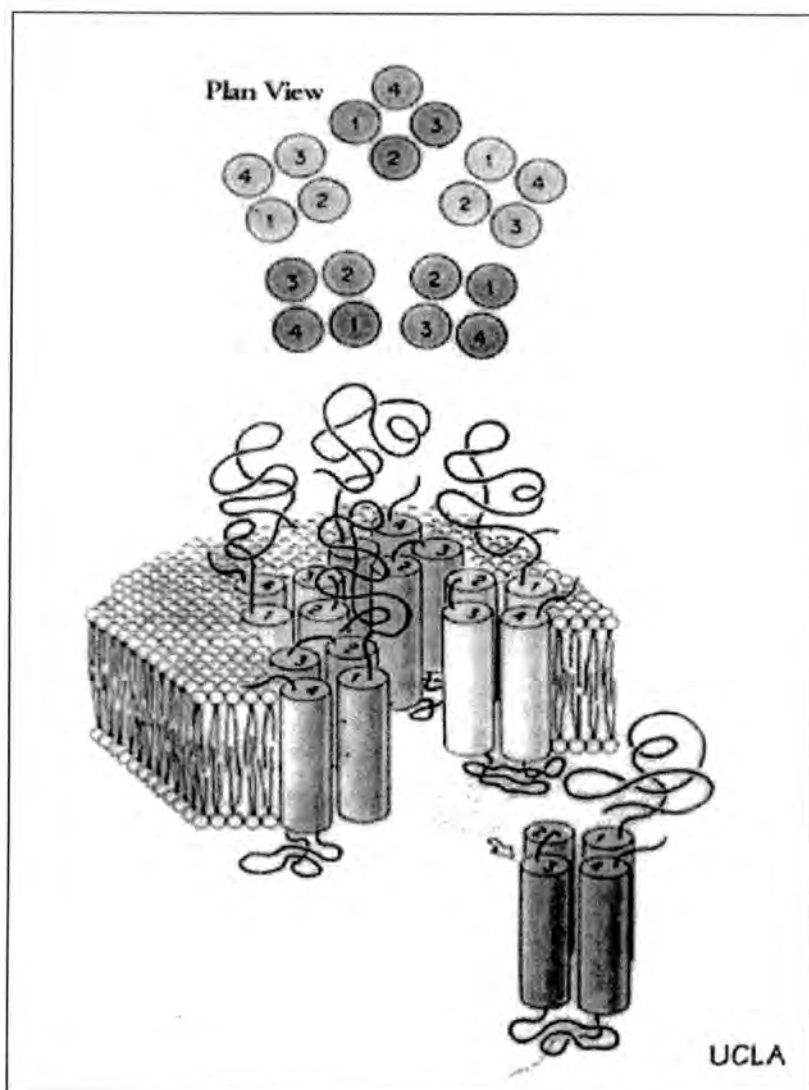


Figure 1.3. Model of the structure of the $GABA_A R$. Each subunit has four membrane-spanning domains (cylinders numbered 1-4). Taken from DeLorey and Olsen, 1992.

1.5. GABA_AR heterogeneity

The variety of genes discovered to encode the different subunits and subunit isoforms of the GABA_AR and the assumption of a pentameric stoichiometry (Nayeem *et al.*, 1994), imply a tremendous heterogeneity of GABA_AR subunit composition. Permutation analysis predicted that there were 151887 possible different receptor combinations (Burt and Kamatchi, 1991), new subunits have been discovered since then enhancing this number. This theoretical heterogeneity of GABA_ARs has not been experimentally observed, in fact only a restricted number of GABA_ARs have been shown to be functionally expressed (reviewed in Barnard *et al.*, 1998). This raises the question as to why there is such a structural diversity of GABA_AR subunits and why this exceeds the currently known functional diversity of GABA-mediated currents in neurons. Furthermore, why has such GABA_AR structural diversity been created and maintained during evolution?

It is conceivable that GABA_AR heterogeneity has evolved so as to meet the specific requirements of distinct neuronal cells. Evidence has emerged that the expression of different GABA_AR genes differ in distinct areas of the CNS (Levitan *et al.*, 1988; Wisden *et al.*, 1992), during development (Killisch *et al.*, 1991; Laurie *et al.*, 1992) and within individual neurons (Meinecke *et al.*, 1989).

1.6. Stoichiometry of GABA_AR

Immunoaffinity purification and immunoblotting approaches have been used to identify which of the subunits co-assemble to form native receptors, however different groups have obtained contradictory results. An example of these discrepancies are the arguments both in favour and against whether more than

one different α subunit can exist within the same receptor complex. Duggan *et al.* (1991) showed, by western blotting of immunoaffinity-purified GABA_ARs from detergent-solubilised bovine cerebral cortex, that subpopulations of GABA_ARs contained both $\alpha 1:\alpha 2$, $\alpha 2:\alpha 3$ and $\alpha 1:\alpha 3$ subunits in the same receptor complex. McKernan *et al.* (1991), also reported that a subpopulation of rat GABA_ARs contained both $\alpha 1$ and $\alpha 3$ subunits. Immunoprecipitation studies on detergent-solubilised rat cerebellar GABA_ARs showed that in a subpopulation of receptors, both $\alpha 1$ and $\alpha 6$ subunits coexist within the same GABA_AR complex (Pollard *et al.*, 1995; Khan *et al.*, 1996 and Jechlinger *et al.*, 1998). However, Quirk *et al.* (1994) reported that the $\alpha 6$ subunit was not co-assembled with any other α subunit. In addition, double-immunolabelling of clustered receptors expressed on the surface of cultured cerebellar granule cells suggested that $\alpha 1$ and $\alpha 6$ subunits do not co-localise in the same receptor complex (Caruncho *et al.*, 1993). A similar discrepancy has arisen regarding the γ subunit. Quirk *et al.* (1994), have reported the co-purification, from rat brain, of GABA_ARs that contain both $\gamma 2$ and $\gamma 3$ subunits. Tögel *et al.* (1994), however, found no evidence for association of $\gamma 2$ with $\gamma 3$ subunits.

These contrasting results obtained from different laboratories may be explained by the sensitivity of detection of the antibodies used. Although the conclusions are confusing most of the GABA_ARs appear to contain at least one α , one β and either one γ or one δ subunit, but receptor stoichiometry has not been unambiguously established. Comparisons of conductances of wild-type and mutated cloned receptors predicted a polypeptide stoichiometry of $(\alpha 1)_2(\beta 1)(\gamma 2)_2$ as more likely than others (Backus *et al.*, 1993), in agreement with the data of

Khan *et al.* (1994), that predicted receptors with two α , one β and two γ . Although, once again conflicting data have emerged with reports from several laboratories predicting that the majority of receptors have a subunit stoichiometry of $(\alpha)_2(\beta)_2(\gamma)_2$ (Chang *et al.*, 1996; Tretter *et al.*, 1997). Thus, perhaps native GABA_AR do not have an unique subunit stoichiometry. Rather, subunit assembly may be characteristic of individual neuronal cells, and may be governed by the specific demands made upon that neuron to produce an appropriate inhibitory response to external or internal stimuli.

1.7. Brain distribution of GABA_ARs

GABA_ARs are widely distributed throughout the CNS. By in-situ hybridisation studies, several groups have attempted to elucidate the localisation of the different GABA_AR subunit mRNAs through out the brain. These studies have revealed a pattern of expression of GABA_AR mRNAs in the adult brain that are summarised in Table 1.1. (taken from Darlinson and Albrecht, 1995). The highest levels of GABA_AR mRNAs found in the brain encode α_1 , α_2 , α_3 , β_2 , β_3 and γ_2 subunits, α_1 being the most abundant and wide distributed. Since the presence of a mRNA transcript within a cell is not proof that a translated product is actually expressed, a fundamental question that remains to be answered is what is the subtype composition that actually exists in neurons.

Table 1.1. Some mammalian GABA_AR subunit associations and their locations

(Taken from Darlinson and Albrecht, 1995)

Subunits	Prominent brain region
α1 and γ2	Cerebral cortex and cerebellum
α3 and γ2	Cerebral cortex and brainstem
α1 and β3	Cerebral cortex
α2 and β3	Cerebral cortex, amygdala and hypothalamus
α3 and β3	Cerebral cortex
α2 and γ1	Cerebellum (Bergmann glia)
α4 and δ	Forebrain and certain thalamic nuclei
α5 and β1	Hippocampus
α6 and γ2	Cerebellum
α6 and δ	Cerebellum
α1, β1 and γ2	Olfactory bulb (mitral cells), hippocampus and dentate gyrus
α1,β2 and γ2	Olfactory bulb (mitral cells) and cerebellum
α1,β2/β3 and γ2	Olfactory bulb (mitral cells), forebrain, brainstem and cerebellum
α2,β3 and γ2	Spinal cord (motor neurons)
α3, β2/β3 and γ2	Olfactory bulb (granule cells)

1.8. Why GABA_AR heterogeneity?

The existence of different subunit isoforms that have unique but overlapping distributions throughout the mammalian brain, may endow a diversity of physiological properties. From recombinant studies it has been shown that there are slight functional differences between receptors containing distinct subunit isoforms (Verdoorn *et al.*, 1990). Levitan and co-workers suggested differences in receptor sensitivity to GABA depending on the α subunit included in the recombinant receptor when expressed in *Xenopus* oocytes (Levitan *et al.*, 1988a, 1988b). In addition, on the basis of receptor binding and electrophysiological studies, it has been suggested that recombinant GABA_ARs of defined subunit composition have differential sensitivity to allosteric modulation by endogenous molecules found in the brain which include neurosteroids (Puia *et al.*, 1990), and Zn²⁺ (Draguhn *et al.*, 1990).

From the pharmacological point of view, differential expression of the receptor subunit genes generates a great diversity of GABA_AR responses to some

psychoactive drugs. While GABA, bicuculline, and barbiturates act on receptors formed from only α or β subunits (Pritchett *et al.*, 1988; Levitan *et al.*, 1988b), the co-expression of these subunits with the γ subunit is required for the positive or negative allosteric modulation of GABA-evoked Cl^- currents through the GABA_A/benzodiazepine receptor (Pritchett *et al.*, 1989a). Furthermore, the type of α or γ subunit expressed imparts distinct benzodiazepine pharmacology on the receptors of which they are a part (Pritchett *et al.*, 1989a, 1989b; Pritchett and Seeburg, 1990; Herb *et al.*, 1992).

In addition to physiological and pharmacological differences, the composition of the receptor might determine its distribution and maintenance on the nerve cell surface. In the CNS GABA_ARs are distributed on cell bodies, dendrites, and in some cells at axon hillocks, at both synaptic and extrasynaptic sites (Somogyi *et al.*, 1989; Baude *et al.*, 1993; Craig *et al.*, 1993, Nusser *et al.*, 1996). The segregation of GABA_AR to strategic locations on the nerve cell surface is an important determinant in the integration of synaptic inputs (Vu and Krasne, 1992). Therefore, knowledge of the precise localisation of GABA_AR at the cellular and subcellular level may provide insight into inhibitory mechanisms, drug, and perhaps disease states involving GABAergic pathways in the CNS. Numerous studies have centred on localising the sites on cells and processes at which these receptors occur with the aid of autoradiographic techniques in which radiolabelled molecules such as GABA, benzodiazepines, and others with high affinity for the GABA_AR are used as markers (Kuhar *et al.*, 1986). While autoradiographic techniques have proved highly useful for the analysis of regional distributions of GABA_ARs, the cellular and subcellular localisation of the receptors demand higher resolution techniques. The post-synaptic localisation

of GABA_AR has been confirmed using subunit-specific antibodies (Baude *et al.*, 1992). In addition, extra-synaptic labelling has also been detected using monoclonal antibodies which are specific for antigenic sites on both the α (bd24) and β (bd17) subunits (Baude *et al.*, 1992; Nusser *et al.*, 1995).

Previous studies employing fluorescently labelled GABA/benzodiazepine receptors on cultured neurons have shown that while GABA_ARs are localised in clusters of high density on the cell body and in patches on dendrites, more than 85% of the GABA_AR on processes and 70% on cell bodies are immobile (Velazquez *et al.*, 1989). The results suggest that even in the absence of synaptic contact, there are specific mechanisms that segregate and immobilise GABA/benzodiazepine receptors on the cell body and dendrites.

The relation between GABA_AR subunit composition and the lateral mobility of the complex on the membrane has not yet been determined. Receptors containing different subunit complements may have different rates of motion. This property would provide a neuron with a new type of synaptic plasticity, that is, the ability to route specific complexes to different neuronal domains according to their subunit composition.

1.9. Plasma membrane dynamics

Plasma membrane proteins are not totally free to drift randomly on the “lipid sea” were they are embedded, but instead are subjected to restraining influences that restricts their mobility. Three non excluded models have been proposed for the transient confinement of membrane proteins (reviewed in Sheets *et al.*, 1995), briefly:

a) The membrane-skeleton fence model

In this model the membrane spectrin and ankyrin based cytoskeleton compartmentalise the membrane into small domains ($0.1-1 \mu\text{m}^2$) providing a barrier to the free diffusion of membrane proteins. The cytoplasmic domains of membrane proteins interact sterically with the cytoskeletal network beneath the cell surface, thus membrane proteins with large cytoplasmic domains will experience more restrictions in their mobility than proteins with small cytoplasmic regions. The membrane proteins can escape from one domain and move to adjacent compartments as a result of dynamic properties of the cytoskeleton.

b) High localised concentration of proteins

High local concentrations of proteins, directly or indirectly bound to the cytoskeleton, can act as obstacles to the free diffusion of membrane proteins. Here, the diffusing proteins interact with other proteins either sterically or specifically through direct chemical interactions. The protein obstacles may form large networks *via cis* interactions forming a physical barrier for diffusion. Such a mechanism may explain the transient confinement of GPI-anchored proteins (GPI: glycosylphosphatidylinositol) which can not interact directly with the cytoskeletal meshwork.

c) Localised lipid domains

Local differences in the lipid composition of the bilayer, forming highly viscous microdomains, may also account for protein confinement.

1.10. Protein anchoring

In addition to the restriction in mobility of membrane proteins due to domain confinement, as described above, more specific mechanisms are needed to ensure the total anchoring or immobilisation of some proteins. The clustering of membrane proteins into discrete and functionally significant domains is an important feature of neuronal cell biology. The placement of Ca²⁺ channels at dendritic spines for amplification and at nerve endings for neurotransmitter release (Cohen *et al.*, 1991), and the sequestration of AMPA and NMDA receptors at dendritic spines (Baude *et al.*, 1995) are striking examples of how cell surface components are localised and maintained in discrete membrane domains of the neuron.

Neurotransmitter receptors rely on varied complex mechanisms to regulate their surface distribution. Although some neurotransmitter receptors, such as glycine receptors (GlyR) and glutamate receptors (AMPA and NMDAR), require activation for cluster formation at postsynaptic sites, GABA_AR do not (reviewed in Kirsch, 1999).

Inhibitory GlyR are clustered opposite the presynaptic terminal by interaction with the anchoring protein gephyrin. The subsynaptic aggregation of gephyrin is induced by calcium influx (Kirsch and Betz, 1998). Gephyrin has been shown to interact with polymerising tubulin *in vitro* (Kirsch *et al.*, 1991), and the postsynaptic localisation of gephyrin/GlyR clusters seems to be mediated by microtubules and microfilaments (discussed by Kirsch, 1999).

The β subunit of the GlyRs has been proven to mediate the binding to gephyrin via its cytoplasmic loop region. In overlay and transfection experiments this binding was shown to involve a motif of 33 amino acids in the central region

of the M3-M4 cytoplasmic loop of GlyRs β subunit, with an 18- amino acid core sequence harbouring the dominant binding determinants (Meyer *et al.*, 1995). Recently, site-directed mutagenesis studies of this binding motif have revealed that the gephyrin binding activity of the GlyRs β subunit can be assigned to hydrophobic amino acid residues located on one side of a potentially imperfect amphipathic helix. (Kneussel *et al.*, 1999).

The ionotropic glutamate receptors NMDAR and AMPAR, and the metabotropic glutamate receptor, mGluR, are clustered on the postsynaptic side of an excitatory synapse by PDZ (or functionally PDZ-like) protein-interaction domains, (PDZ is named after the proteins in which these domains were first identified: PSD-95, discs large and ZO-1). PDZ domains are motifs of about 90 amino acids that mediate protein-protein interactions by interacting with the C-termini of proteins (Dong *et al.*, 1997).

Different anchoring proteins have been shown to interact with each of the three different glutamate receptors. NMDARs are concentrated at specific domains by interaction with PSD-95 proteins (discussed in Sheng, 1997), AMPAR bind the synaptic protein GRIP (glutamate receptor interacting protein), Dong *et al.*, (1997) and mGluR are clustered *via* interaction with a protein named Homer (Brakeman *et al.*, 1997).

The interaction between the glutamate receptors and the anchoring proteins seems to be mediated by the intracellular domains of some of the subunits that form the receptor complex. In fact, some specific amino acid sequences contained in the intracellular region of NMDA and AMPA receptors have been implicated in the spatially distinctive clustering of the complex (Ehlers *et al.*, 1995; Kornau *et al.*, 1995; Dong *et al.*, 1997). Ehlers *et al.* (1995) have identified a sequence of amino

acids contained in the C-terminal domain of the NR1 subunit (proposed to be intracellular), that is responsible for the targeting or anchoring of the NR1 to structures associated with the plasma membrane. They determined that the 37-amino acid C1 exon cassette (found in some of the four different NR1 splice variants) was necessary for the formation of NR1-enriched domains. Thus, alternative splicing can regulate the subcellular distribution of the NR1 subunits.

In addition, the use of the two-hybrid system has shown that a tSXV motif (where S is serine, X is any amino acid and V is valine) contained in the C-terminal of the NR2 subunit and certain NR1 splice forms interact with the PDZ domain of the PDS-95 protein (Kornau *et al.*, 1995). Additional studies have shown that the NMDAR NR1 subunit interacts directly with the 68 kDa neurofilament subunit in a manner regulated by alternative splicing. This interaction occurs between the cytoplasmic C-terminal domain of NR1 and the rod domain of the neurofilament and requires the presence of the alternatively spliced C1 exon cassette in the NR1 subunit (Ehlers *et al.*, 1998).

Moreover, interactions between AMPA and GRIP are mediated by the association of the C-terminal motif SVKI* (*denotes a stop codon) of GluR2, GluR3 and possibly GluR4c (Dong *et al.*, 1997).

The association of glutamate receptors with intracellular proteins is widely reviewed in Dingledine *et al.* (1999).

The machinery employed to anchor GABA_AR at postsynaptic specific domains of the membrane is less clear. Gephyrin has been shown to indirectly interact with $\gamma 2$ subunit-containing GABA_AR complexes (Essrich *et al.*, 1998). Furthermore, a GABA_AR-associated protein (GABARAP) has recently been identified that interacts selectively with $\gamma 2$ subunits and colocalises with

GABA_AR in cultured cortical neurons (Wang *et al.*, 1999). Sequence analysis of this protein reveals that is similar to light chain 3 (LC-3) of microtubule-associated proteins MAP-1A and MAP-1B. Interestingly, the heavy chain of MAP-1B has been shown to interact with the GABA_CR (Hanley *et al.*, 1999).

Several groups are involved in the study of how receptors are anchored and maintained at specific membrane domains. Approaches such as chemical cross-linking (Burden *et al.*, 1983); immunofluorescent labelling (Phillips *et al.*, 1991); Triton X-100 solubilisation and immunoblotting (Phillips *et al.*, 1993); and the use of the yeast two-hybrid system (Wang *et al.*, 1999) have been used to identify cytoskeletal protein candidates that mediate neurotransmitter receptor anchoring. Each of these approaches, however, depends on an *in vitro* or biochemical reconstitution of the system and does not reveal whether these associations contribute to the functional anchoring of the receptor complexes. In addition, it is known that the interaction of membrane proteins with the cytoskeleton is complex and can involve interactions or mechanisms of restriction or compartmentalisation that are above interactions at the secondary or tertiary structural levels that are not commonly revealed in reconstituted systems.

The study carried out in this thesis has approached the problem of receptor anchoring and identification of the molecular interactions in a different manner. Thus, attention was focused on the membrane dynamics of the receptor, measuring its lateral mobility by using the Fluorescence Photobleach Recovery (FPR) method. Although this approach has not been used to identify receptor anchoring mechanisms or the cytoskeletal associations, FPR has been used to investigate which subunits may be involved in receptor anchoring, and their

function in restricting receptor mobility in reconstituted systems. This technique allows measurement of the lateral mobility of membrane components. Direct measurement of the rates of protein lateral diffusion help to characterise the physical forces and interactions between cell surface components and other cellular structures.

Aims and Objectives

The overall objective of this thesis was to elucidate whether nerve cells use the structural diversity of GABA_AR subunits to regulate receptor mobility and location on the cell surface. Fluorescence Photobleach Recovery (FPR) was used to measure the lateral mobility of GABA_AR in transfected cells and cultured neurons. Concurrently, immunocytochemistry was used to determine the cellular localisation of these GABA_ARs.

The following hypothesis were tested:

- 1) The segregation and lateral mobility of GABA_ARs is determined by the subunit composition of the receptor.

- 2) The inclusion of a $\beta 3$ subunit in a GABA_AR is required for transport of the complex to the cell surface.

The sorting properties of the $\alpha 1$, $\beta 3$ and $\gamma 2s$ GABA_AR subunits were studied. The final localisation of GABA_ARs composed of specific subunits was determined by immunocytochemistry in transfected COS7, HEK293 and PC12 cells. The role of the $\alpha 1$ subunit in controlling lateral mobility of GABA_AR was tested by FPR.

- 3) GABA_ARs lateral mobility is determined by the α subunit included in the complex. Different α subunit isoforms imply differences in receptor mobility.

The lateral mobility of recombinant GABA_AR complexes containing different α subunits expressed in COS7, HEK293 cells and native α subunit-containing GABA_ARs expressed in primary cultured neurons was measured by FPR.

4) The M3/M4 cytoplasmic loop of the $\alpha 1$ subunit controls GABA_ARs mobility.

The lateral mobility of receptors containing $\alpha 1$ subunits in which the M3/M4 domain was deleted ($\alpha 1_{CD}$) and chimeras of the $\alpha 1$ subunit ($\alpha 1_{CH}$) where the cytoplasmic loop domain was replaced by that found in the $\alpha 2$, $\alpha 3$ and $\alpha 6$ subunits, was measured by FPR in transfected COS7 and HEK293 cells. These experiments were repeated for $\alpha 1_{CH}$ and $\alpha 1_{CD}$ -containing receptors expressed in cultured cerebellar granule neurons.

This study provides important information on how this family of ligand-gated receptors might be distributed and maintained on nerves in the CNS.

Chapter 2

Material and Methods

Chapter two

Materials and Methods**2.1. Material**

Cell culture consumables were purchased from GIBCO BRL, (Paisley, Scotland).

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Mammalian expression vectors were obtained from the following sources:

pSVK3	Pharmacia, Herts, U.K.
pCDM8	Invitrogen, San Diego, USA
pcDNA I/Amp	Invitrogen, San Diego, USA

Wizard Maxipreps and DNA related products were purchased from Promega (Madison, USA).

Transfection reagents: Lipofectamine was obtained from GIBCO BRL, (Paisley, Scotland); ESCORT was obtained from the Sigma Chemical Company (St. Louis, MO, USA); TfxTM-50 Reagent was from Promega (Madison, USA); The Electro Cell Manipulator was purchased from BIO RAD (California, USA).

The antibodies used throughout this investigation, monoclonal anti- α 1 (bd24) and anti- β 2/3 (bd17) were obtained from Boehringer, (Germany); TRITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit were from

Calbiochem, (Nottingham, U.K.); Cascade blue-conjugated goat anti-mouse antibody was purchased from Molecular Probes, (Leiden, The Netherlands).

Fluorescent compounds for FPR, Bodipy-Ro-1986, were purchased from Molecular Probes, (Leiden, The Netherlands).

Molecular mass standards were obtained from the Sigma Chemical Company (St. Louis, MO, USA).

Bio-Rad microradiance confocal microscope was purchased from BIO RAD (California, USA).

Gel Doc 1000 imaging system was from BIO RAD (California, USA).

2.2. Methods

2.2.1. Cell culture procedures

2.2.1.1. Initiating cultures from frozen stocks

An aliquot of frozen cells (see 2.2.1.5) was thawed with gentle agitation in a 37°C water bath. The vial was rinsed with 70% ethanol and the cells transferred to a sterile tube and resuspended in 10 ml of complete growth medium. The cells were then pelleted at 600g for 5 minutes and subsequently seeded into a culture flask containing the appropriate amount of culture medium ($1-2 \times 10^6$ cells/ml).

2.2.1.2. Maintenance and propagation of cells

All cells were maintained at 37°C in a 5% CO₂ incubator for a up to 4 months of continuous culture, after which a fresh culture from frozen stocks was initiated.

HEK293 cells were cultured in Modified Eagle Medium (MEM) supplemented with 10% foetal bovine serum (FBS). Cells were subcultured at a 1:10 split ratio (1×10^6 cells/ml) following trypsinisation for 1 minute at room temperature (described below in section 2.2.1.3).

COS7 cells were grown in Dulbecco's MEM (high glucose) supplemented with 10% FBS. The culture was trypsinised for 5 minutes at 37°C and subcultured at a 1:10 split ratio (1×10^6 cells/ml).

PC12 cells were cultured in RPMI medium supplemented with 10% horse serum and 5% FBS. Differentiation was induced by the addition of 50 ng/ml nerve growth factor (NGF, 7S form) and 1µM dibutyryl cyclicAMP for 7 days. Undifferentiated cells were subcultured as described below in 2.2.1.3.

Cerebellar granule cells were derived from the cerebella of 10 days old rats (P₁₀). The cerebella were washed with MEM once and trypsinised for 25 minutes at 37°C, with continuous shaking. The tissue was then triturated 20 times and centrifuged at 100g for 5 minutes to pellet the cells. The supernatant was carefully removed, fresh media was added to the pellet and trituration was repeated a further 60 times. Dissociated cells (250×10^3 cells/cm²) were plated onto 35 mm poly-lysine coated dishes. The cells were maintained in MEM supplemented with 10% FBS, 20mM glutamine and 0.04 M KCl.

2.2.1.3. Subculturing cells

Adherent cells: When cells reached 70-80% confluence they were subcultured. Cells were detached from culture flasks by enzymatic treatment with 0.25% trypsin. Culture medium was removed and the cells were washed once with phosphate-buffered saline (PBS, 8g NaCl, 2.9g Na₂HPO₄-12H₂O, 0.2g KH₂PO₄, 0.2g KCl, 12g sucrose per litre, pH 8.0). For a 25 cm² culture flask, 2ml trypsin (0.25g per 100 ml of PBS) was added and incubated 1-5 minutes at 25-37°C depending on the cell type. 10 ml of culture medium was then added and the cells transferred to a sterile tube. Cells were pelleted by centrifugation at 500g for 5 minutes. The cell pellet was resuspended in 1ml of culture medium by gently pipetting further 9 ml of culture medium was then added. For a 1:10 split ratio, 1ml of the cell suspension (1×10^6 cells/ml) was seeded in a 25 cm² flask with 9 ml of culture medium.

Suspension Cells: Cells were pelleted by centrifugation at 500g for 5 minutes and resuspended in 1 ml of culture medium by gentle pipetting. 9 ml of culture medium was then added and for a 1:10 split ratio, 1ml of this cell suspension ($1 \times$

10^6 cells/ml) was seeded in a 25 cm² flask with 9 ml of culture medium.

2.2.1.4. Counting cells

100 μ l of cell suspensions were diluted with 100 μ l of 6% trypan blue vital dye and counted on a Neubaumer haemocytometer using a Nikon TMS inverted microscope.

2.2.1.5. Freezing cells

Cells from a healthy log-phase culture were harvested as described above in 2.2.1.3. and resuspended in a minimal volume of culture medium. Cells were counted and diluted to 1×10^6 cells/ml in culture medium with 10% DMSO and 40% FBS. 1ml aliquots of cells were dispensed into pre-labelled 2ml cryovials and then frozen in liquid nitrogen. The frozen aliquots were transferred to liquid nitrogen for long term storage.

2.2.2. Molecular biological procedures

RNA was removed from DNA solutions by the addition of RNase A to a final concentration of 20 μ g/ml.

2.2.2.1. Subcloning GABA_AR cDNAs into expression vectors

GABA_AR cDNAs were donated by Pr. P. Seeburg, Dr. H. Luddens, Dr. D. Burt, and Dr. R. Joho. Bovine α 1, rat β 3 and β 2 and murine γ 2s GABA_AR subunit cDNAs were obtained cloned into the mammalian expression vector pCDM8, an expression vector under the control of the constitutive cytomegalovirus (CMV) promoter and SV40 intron/ Poly A tail sequence, thus,

were used with no further modification. On the other hand, rat GABA_AR α 2–6 and β 1 subunit cDNAs were obtained cloned into the non-expression vector pBluescript SK- and were subsequently re-cloned into the mammalian expression vectors, pCDNA I/Amp for rat GABA_AR α 2–6 subunits and pSVK3 for β 1 subunit. Briefly: approximately 5 μ g of DNA (cDNA in non-expression vector) was digested with the appropriate restriction enzymes (see Table 2.1 below and 2.2.2.9.) to liberate the required cDNA fragment. cDNA fragments were gel purified (2.2.2.3.), ligated into digested pCDNA I/Amp vector (2.2.2.4.) and transformed into competent bacteria (2.2.2.6.). Positive colonies were selected and DNA mini/midi-preps made (2.2.2.7.). Orientation and sequence of constructs were verified by restriction digests and DNA sequencing. Figure 2.1., 2.2., 2.3., 2.4, 2.5. and 2.6. show the subcloning strategy and representative agarose gels for the α 2, α 3, α 4, α 5, α 6 and β 1 subunit cDNAs respectively.

Table 2.1. *Subcloning GABA_AR cDNAs into Mammalian Expression Vectors*

GABA _A R subunit	cDNA subclone	Vector Preparation
Rat α 2	1.6kb Hind III- EagI fragment	Hind III- NotI cut pcDNAIamp
Rat α 3	2.7kb XhoI fragment	XhoI cut pcDNAI amp
Rat α 4	2.3kb BamHI- Bsp120I fragment	BamHI-NotI cut pcDNAIamp
Rat α 5	1.5kb HindIII-BamHI fragment	HindIII- BamHI cut pcDNAIamp
Rat α 6	1.7kb BamHI- XhoI fragment	BamHI-XhoI cut pcDNAIamp
Rat β 1	3.0kb XhoI fragment	XhoI cut pSVK3

Figure 2.1. $\alpha 2$ cDNA subcloning strategy

The entire coding region of GABA_AR $\alpha 2$ subunit cDNA (1.6kb) was excised (↓) from pSK⁺ using *HinD* III (H) and *Eag* I (E) and ligated into H and E double digested pCDNAI Amp. The location of restriction enzymes recognition sequences (*EcoR* I (RI) and *Sph* I (S)) used to characterise the construct and the size of digestion products are shown.

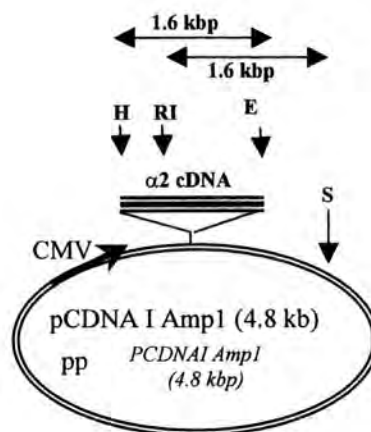


Table 2.1.1. Restriction enzymes used to characterise GABA_AR $\alpha 2$ subunit cDNA subcloned into pCDNA I Amp.

The location of sites (within the vector or insert) and the expected size of digestion products are given. The result of digestions are also shown (also see Fig. 2.1.1.)

GABA _A R subunit	Restriction endonuclease	Sites in		Fragments (kbp)		Notes	Lane
		vector	insert	expected	result		
$\alpha 2$	/	/	/	/	+	uncut	1
$\alpha 2$	<i>EcoR</i> I	0	1	6.4	+	linearised	2
$\alpha 2$	<i>HinD</i> III	cloning site		6.4	+	"	3
$\alpha 2$	<i>Sph</i> I	1	0	6.4	+	"	4
$\alpha 2$	<i>EcoR</i> I + <i>Sph</i> I			4.9 + 1.5	+	orientation of insert	5

Figure 2.1.1. Characterisation of subcloned GABA_AR $\alpha 2$ subunit cDNA

Restriction digestions were separated on 0.7% TAE buffered agarose gels and visualised with ethidium bromide. M: 1.0 μ g *Pst* I digested λ DNA (fragments in kbp); Lanes 1-5: 1.0 μ g of $\alpha 2$ GABA_AR pCDNA I Amp cut with 1: no enzyme; 2: *EcoR* I; 3: *HinD* III; 4: *Sph* I; 5: *EcoR* I and *Sph* I.

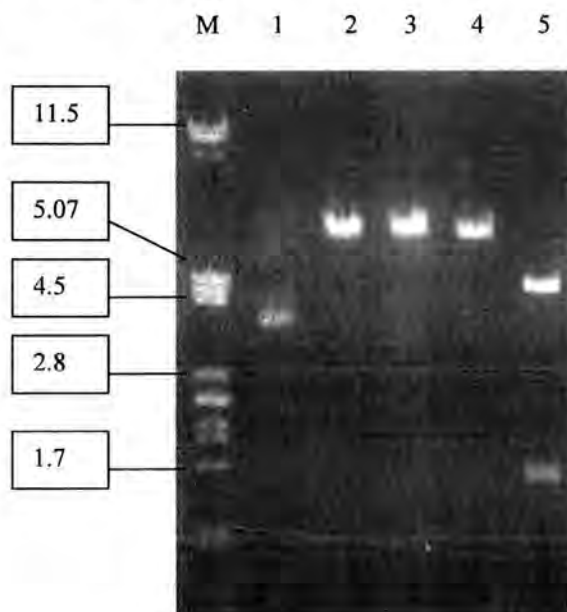
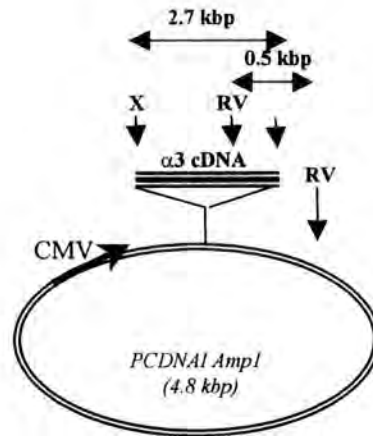


Figure 2.2. $\alpha 3$ cDNA subcloning strategy

The entire coding region of GABA_AR $\alpha 3$ subunit cDNA (2.7kbp) was excised (\downarrow) from pSK⁻ using *Xho* I (X) and ligated into X digested pCDNAI Amp. The location of restriction enzymes recognition sequences (*EcoRV* (RV)) used to characterise the construct and the size of digestion products are shown.

**Table 2.2.1.** Restriction enzymes used to characterise GABA_AR $\alpha 3$ subunit cDNA subcloned into pCDNA I Amp.

The location of sites (within the vector or insert) and the expected size of digestion products are given. The result of digestions are also shown (also see Fig. 2.2.1.)

GABA _A R subunit	Restriction endonuclease	Sites in		Fragments (kbp)		Notes	Lane
		vector	insert	expected	result		
$\alpha 3$	/	/	/	/	+	uncut	1
$\alpha 3$	<i>EcoRV</i>	1	1	7.0 + 0.5	+	orientation of insert	2

Figure 2.2.1. Characterisation of subcloned GABA_AR $\alpha 3$ subunit cDNA

Restriction digestions were separated on 0.7% TAE buffered agarose gels and visualised with ethidium bromide. M: 1.0 μ g *Pst* I digested λ DNA; ; Lane: 1, 1.0 μ g of uncut $\alpha 3$ GABA_AR pCDNA I Amp; 2, 1.0 μ g of *EcoRV* cut $\alpha 3$ GABA_AR pCDNA I Amp

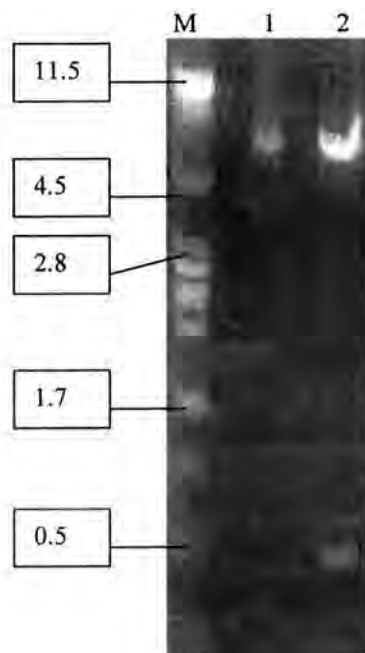


Figure 2.3. $\alpha 4$ cDNA subcloning strategy

The entire coding region of GABA_AR $\alpha 4$ subunit cDNA (2.3kbp) was excised (▼) from pSK⁻ using *Bam* HI (B) and *Bsp* 120I (Bs) ligated into B and *Not* I (N) double digested pCDNAI Amp. The location of restriction enzymes recognition sequences (*Xba* I (Xb) and *EcoRV* (RV)) used to characterise the construct and the size of digestion products are shown.

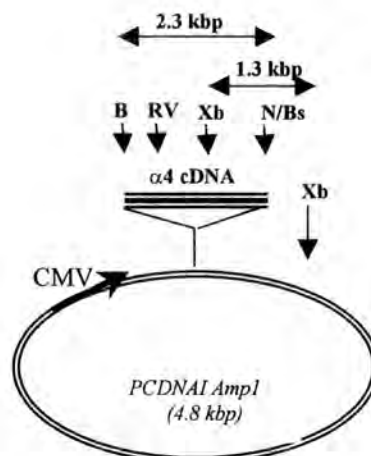


Table 2.3.1. Restriction enzymes used to characterise GABA_AR $\alpha 4$ subunit cDNA subcloned into pCDNA I Amp.

The location of sites (within the vector or insert) and the expected size of digestion products are given. The result of digestions are also shown (also see Fig. 2.3.1.)

GABA _A R subunit	Restriction endonuclease	Sites in		Fragments (kbp)		Notes	Lane
		vector	insert	expected	result		
$\alpha 4$	/	/	/	/	+	uncut	1
$\alpha 4$	<i>Xba</i> I	1	1	5.8 + 1.3	+	orientation of insert	2
$\alpha 4$	<i>EcoRV</i>	0	1	7.1	+	linearised	3

Figure 2.3.1. Characterisation of subcloned GABA_AR $\alpha 4$ subunit cDNA

Restriction digestions were separated on 0.7% TAE buffered agarose gels and visualised with ethidium bromide. M: 1.0 μ g *Pst* I digested λ DNA; : Lanes 1-5: 1.0 μ g of $\alpha 4$ GABA_AR pCDNA I Amp cut with 1: no enzyme; 2: *Xba* I; 3: *EcoRV*.

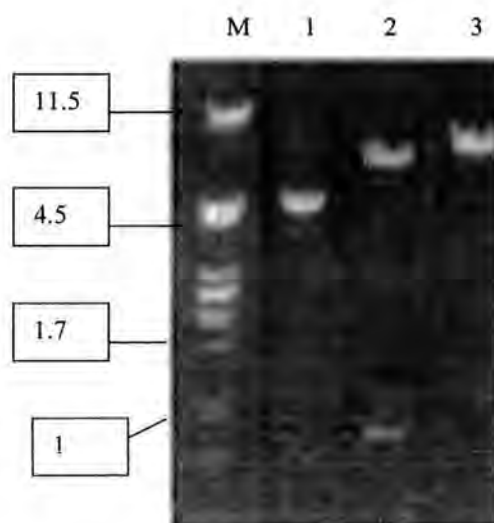
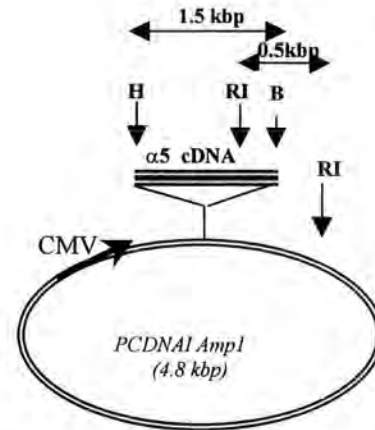


Figure 2.4. $\alpha 5$ cDNA subcloning strategy

The entire coding region of GABA_AR $\alpha 5$ subunit cDNA (1.5kbp) was excised (▼) from pSK⁻ using *Hind* III (H) and *Bam* HI (B) and ligated into H and B double digested pCDNAI Amp. The location of restriction enzymes recognition sequences (*Eco*R I (RI)) used to characterise the construct and the size of digestion products are shown.

**Table 2.4.1.** Restriction enzymes used to characterise GABA_AR $\alpha 5$ subunit cDNA subcloned into pCDNA I Amp.

The location of sites (within the vector or insert) and the expected size of digestion products are given. The result of digestions are also shown (also see Fig. 2.4.1.)

GABA _A R subunit	Restriction endonuclease	Sites in		Fragments (kbp)		Notes	Lane
		vector	insert	expected	result		
$\alpha 5$	/	/	/	/	+	uncut	1
$\alpha 5$	<i>Eco</i> RI	1	1	5.8 + 0.5	+	orientation of insert	2

Figure 2.4.1. Characterisation of subcloned GABA_AR $\alpha 5$ subunit cDNA

Restriction digestions were separated on 0.7% TAE buffered agarose gels and visualised with ethidium bromide. M: 1.0 μ g *Pst* I digested λ DNA; : Lanes 1-5: 1.0 μ g of $\alpha 5$ GABA_AR pCDNA I Amp cut with 1: no enzyme; 2: *Eco*R I.

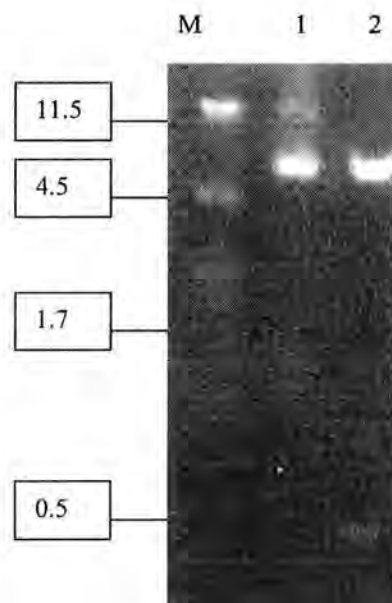
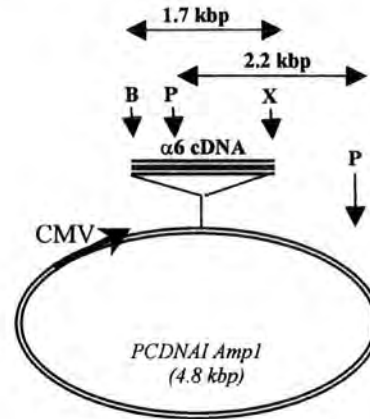


Figure 2.5. $\alpha 6$ cDNA subcloning strategy

The entire coding region of GABA_AR $\alpha 6$ subunit cDNA (1.7 kbp) was excised (↓) from pSK⁻ using I *Bam* HI (B) and *Xho* I (X) ligated into B and X double digested pCDNA1 Amp. The location of restriction enzymes recognition sequences (*Bam* HI (B) and *Pvu* II (P)) used to characterise the construct and the size of digestion products are shown.

**Table 2.5.1.** Restriction enzymes used to characterise GABA_AR $\alpha 6$ subunit cDNA subcloned into pCDNA 1 Amp.

The location of sites (within the vector or insert) and the expected size of digestion products are given. The result of digestions are also shown (also see Fig. 2.5.1.)

GABA _A R Subunit	Restriction endonuclease	Sites in		Fragments (kbp)		Notes	Lane
		vector	insert	expected	result		
$\alpha 6$	/	/	/	/	+	uncut	1
$\alpha 6$	<i>Bam</i> HI	cloning site		6.5	+	linearised	2
$\alpha 6$	<i>Pvu</i> II	1	1	4.3 + 2.2	+	"	3

Figure 2.5.1. Characterisation of subcloned GABA_AR $\alpha 6$ subunit cDNA

Restriction digestions were separated on 0.7% TAE buffered agarose gels and visualised with ethidium bromide. M: 1.0 μ g *Pst* I digested λ DNA; : Lanes 1-5: 1.0 μ g of $\alpha 5$ GABA_AR pCDNA 1 Amp cut with 1: no enzyme; 2: *Bam* HI; 3: *Pvu* II.

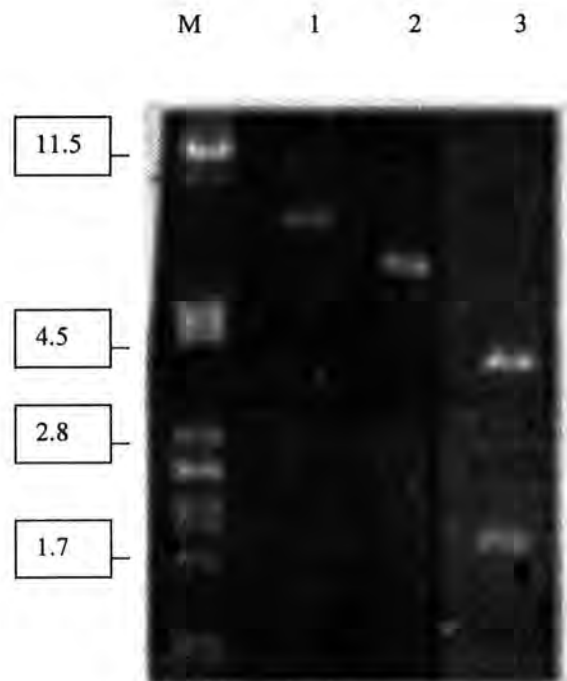
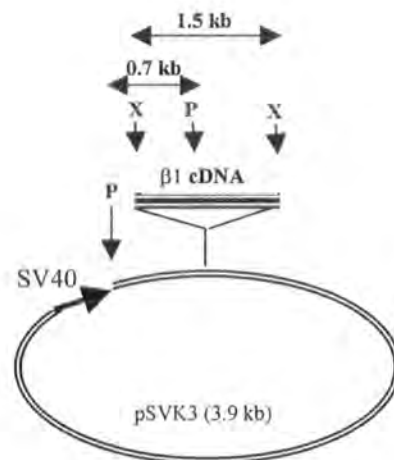


Figure 2.6. $\beta 1$ cDNA subcloning strategy

The entire coding region of GABA_AR $\beta 1$ subunit cDNA (1.5 kb) was excised (\downarrow) from pSK⁺ using *Xho* I (X) and ligated into X digested pSVK3. The location of restriction enzymes recognition sequences (*Pst* I (P) and *Xho* I (X)) used to characterise the construct and the size of digestion products are shown.

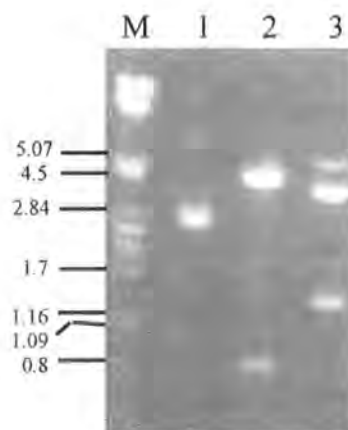
**Table 2.6.1.** Restriction enzymes used to characterise GABA_AR $\beta 1$ subunit cDNA subcloned into pSVK3.

The location of sites (within the vector or insert) and the expected size of digestion products are given. The result of digestions are also shown (also see fig 2.6.1.)

GABBAR Subunit	Restriction endonuclease	Sites in		Fragments (kbp)		Notes	Lane
		vector	insert	expected	result		
$\beta 1$	/	/	/	/	+	uncut	1
$\beta 1$	<i>Xho</i> I	cloning site	0	3.9 + 1.5	+	excision of insert	2
$\beta 1$	<i>Pst</i> I	1	1	4.6 + 0.7	+	orientation of insert	3

Figure 2.6.1. Characterisation of subcloned GABA_AR $\beta 1$ subunit cDNA

Restriction digestions were separated on 0.7% TAE buffered agarose gels and visualised with ethidium bromide. M: 1.0 μ g *Pst* I digested λ DNA; Lanes 1-3: 1.0 μ g of $\alpha 2$ GABA_AR pSVK3 cut with 1: no enzyme; 2: *Pst* I; 3: *Xho* I.



2.2.2.2. Agarose gel electrophoresis

Gel electrophoresis of DNA samples was carried out as described by Sambrook *et al.* (1989). Briefly, samples were electrophoretically separated in 1x TAE buffered gels (50x stock- 242 g Tris, 100 ml EDTA pH 8.0, 57.1 ml glacial acetic acid per litre) at 5-10 V.cm⁻¹ using Pharmacia GNA-100 electrophoresis tanks. The concentration of agarose within a gel was varied depending on the size of DNA to be separated, but usually 0.7% agarose (separation of 10-0.8kb linear DNA) was used. Ethidium bromide was added to a final concentration of 0.2 µg/ml. *Pst*I or *Hind*III digested λ-DNA were used as molecular weight markers.

DNA fragments were visualised using a trans-illuminator (UVP Inc.) or Gel Doc 1000 imaging system.

2.2.2.3. DNA fragment isolation

DNA to be recovered following electrophoresis was removed in the smallest volume of agarose possible and isolated by the following protocol.

Qiaex II Agarose Gel Extraction

The Qiagen Gel Extraction kit was used following the manufacturers guidelines. Briefly, 3 volumes of buffer QX 1 was added to 1 volume of agarose. This was incubated at 50°C for 10 minutes with 10 µl of Qiaex II particles. The sample was centrifuged for 30 seconds and the supernatant removed. The pellet was resuspended in 500 µl of buffer QX1 and spun for 30 seconds at top speed in the bench top microfuge. The pellet was then washed twice in buffer PE and allowed to air dry for 10-15 minutes. The pellet was then resuspended in 20 µl of

TE (10 mM Tris; 1mM EDTA) spun for 30 seconds and the DNA solution removed to a clean eppendorf. This was repeated to ensure maximum recovery of DNA.

2.2.2.4. Ligation

Gel purified vector and restriction fragments were ligated using bacteriophage T4 DNA ligase according to the manufacturers instructions. Briefly 1 μ l of 10x ligase buffer (200 mM Tris-Cl (pH 7.6), 50 mM MgCl₂, 50 mM dithiothreitol and 500 μ g/ml bovine serum albumin) was added to a sterile microfuge tube. Equimolar amounts of insert and vector DNA, to a total concentration of 100 ng DNA were added. ddH₂O was added to 9 μ l followed by 1 μ l (1 U) of bacteriophage T4 DNA ligase. The reactions were incubated overnight at 4°C. Controls that contained the plasmid vector and insert DNA alone were performed in tandem. 1-2 μ l of each of the ligation reactions were used to transform competent *E.coli* as described in 2.2.2.6.

2.2.2.5. Preparation of competent cells

5 ml of LM broth (10 g Bacto triptone, 5 g Bacto yeast extract and 10 g NaCl per litre) was inoculated with a single bacterial colony, grown overnight at 37°C, with appropriate antibiotics and subcultured 1:100 into fresh LM broth. The cells were grown to an OD₆₀₀ of 0.3-0.35, chilled for 5 minutes on ice then harvested at 4000g, 4°C for 7 minutes. Cell pellets were resuspended in $\frac{2}{5}$ of the original culture volume in solution A (30 mM K-acetate, 100 mM RbCl₃, 10 mM CaCl₂,

50 mM MnCl₂ and 15% glycerol). The solution was adjusted to pH 5.8 with 0.2 M acetic acid and incubated on ice for 5 minutes. Centrifugation was repeated and the pellets resuspended in $1/25$ of the original culture volume of solution B (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂ and 15% glycerol). The pH was adjusted to pH 6.5 with KOH and incubated on ice for 15 minutes, after which 200 µl aliquots were transferred to cryovials and snap frozen in liquid nitrogen and stored at -80°C.

2.2.2.6. Transformation of competent bacteria

An aliquot of cells was thawed on ice for 10 minutes. DNA was added and incubation continued for 45-60 minutes. The cells were heat shocked at 43.5°C for 45 seconds, held on ice for 3 minutes and then 800 µl of pre-warmed (to 37°C) LM broth (10 g Bacto triptone, 5 g Bacto yeast extract and 10 g NaCl per litre)was added. The tube was incubated for 1 hour at 37°C, with occasional shaking. Finally 100-200 µl aliquots of the tubes contents were spread onto selective agar plates.

2.2.2.7. Extraction and purification of plasmid DNA

a) Mini Preparation:

A single colony of transformed bacteria was grown overnight in 5 ml of LB with appropriate antibiotic selection. 1.5 ml of the overnight culture was taken and the cells harvested by centrifugation at 12,000g for 1 minute. The supernatant was discarded and the cells resuspended in 200 µl of resuspension solution 1 (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). 200 µl of lysis buffer

2 (0.2 M NaOH, 1% SDS) was then added and the solutions mixed by inversion until the suspension became viscous indicating cell lysis. 200 µl of neutralising solution 3 (1.32 M K-acetate pH 4) was added and mixed by gentle vortexing. The sample was centrifuged at top speed in a microfuge for 1 minute to pellet bacterial debris. The supernatant was transferred to a clean eppendorf and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) added. Following centrifugation at 14,000g for 10 minutes the aqueous phase was removed and DNA precipitated by the addition of 2 volumes of 100% ethanol and 0.1 volume of 3 M K-acetate.

Alternatively 500 µl of Promega Magic Mini-Prep DNA purification resin was added to the supernatant removed after the addition of solution 3. This was incubated at room temperature for 5 minutes with occasional inversion. The solution was then pipetted into a syringe barrel attached to a Magic Mini-Prep column. The solution was injected into the column then washed with 4 ml of column wash solution 4 (final concentrations of 80 mM K-acetate, 8.3mM Tris-HCl pH 7.5, 40 µM EDTA were initially made up in a volume of 125 ml. 170 ml of 95% ethanol were then added to a final volume of 295 ml). The column was removed to a clean eppendorf and centrifuged for 1 minute at 12,000g and then air dried for 5 minutes. The DNA was eluted with 100 µl sterile milli Q water pre-heated to 70°C. This was added to the column and incubated for 5 minutes prior to elution by centrifugation at 12,000g for 1 minute.

b) Midi preparation: DNA was purified using the Wizard Midipreps kit (Promega) according to the manufacturers instructions. The method utilises a modified alkaline lysis protocol and anionic purification resin.

Briefly: A single colony of transformed bacteria was grown overnight in 5ml of LB (10 g Bacto triptone, 5 g Bacto yeast extract and 10 g NaCl per litre) with appropriate antibiotic selection. The overnight culture was subcultured into 250 ml fresh LB and grown overnight with appropriate antibiotic selection. Cells were harvested by centrifugation at 5,000g for 10 minutes at 22-25°C. The cell pellet was resuspended in 25 ml of solution 1 (50 mM glucose; 25 mM TrisCl (pH 8); 10 mM EDTA (pH 8) and gently lysed by inversion in 15 ml of solution 2 (0.2 N NaOH in an equal volume of 1% SDS). The lysed cells were neutralised by adding 15 ml of solution 3 (60 ml of 5M K-acetate; 11.5 ml of glacial acetic acid; 28.5 ml of double distilled H₂O) and inverting several times. The bacterial lysate was then centrifuged at 20,000g for 15 minutes at 4°C and the supernatant transferred to a fresh centrifuge tube following filtration through sterile Miacloth, 0.5 sample volume of isopropanol was added and centrifuged at 14,000g for 15 min at 4°C, to precipitate the DNA. The DNA pellet was resuspended in 2ml of TE (10 mM Tris; 1mM EDTA), 10ml of Wizard resin was added and the solution transferred to a column and washed twice with solution 4 (125ml of ddH₂O, 80 mM K-acetate; 8.3 mM Tris (pH 7.5); and 40 M EDTA followed by the addition of 170 ml of 95% ethanol), then once with 5 ml of 80% ethanol. The resin was centrifuged to dryness at 1300g for 5 minutes and DNA eluted in 1.5 ml of preheated 60°C TE after centrifugation at 1300g for 5 minutes.

2.2.2.8. Ethanol precipitation of DNA.

DNA was precipitated by the addition of 0.1 sample volume of 3 M NaOAc pH 4.8 and 2.5 sample volume of 100% ethanol. The sample was mixed and incubated at -20°C for 1 hour. DNA was recovered by centrifugation at 13,000g

for 5 minutes. The DNA pellet was washed twice with 70% ethanol, with 5 minute centrifugation at 12,000 g in between each wash. The pellet was air dried and subsequently resuspended in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0).

2.2.2.9. Restriction Endonuclease Digestion

Plasmid DNA was digested at concentrations of 10–100 ng/μl in a total volume of 10-50 μl with 5 units of restriction endonuclease, 0.1 volume of the supplied 10x enzyme buffer and made up to volume with sterile water. The reaction was incubated at the recommended temperature for most restriction enzymes this is 37°C, for one hour.

For further analysis by gel electrophoresis the digest was subsequently concentrated by ethanol precipitation as described in 2.2.2.8. and resuspended in a volume of 40 μl T.E.

2.2.2.10. Construction of chimeric GABA_AR α1 subunits

The following truncated α1 subunits were constructed and kindly donated by Dr. H.T. Hooper:

a) A chimera α1 subunit (α1_{CH}) composed of the 5' end of the bovine α1 subunit cDNA and the 3' end of the rat α1 subunit cDNA. This was done for several reasons. First, the monoclonal antibody, bd24, recognises a stretch of three amino acids located at the N-terminus of the bovine and human α1 subunits, that is not faithfully conserved in the rat α1 sequence. Therefore bd24 does not recognise the rat α1 subunit (Ewert *et al.*, 1990). The remaining bovine/human and rat α1 subunit amino acid sequences are identical. The

chimeric $\alpha 1$ subunit was engineered such that the rat $\alpha 1$ subunit cDNA, used in the previous studies, now consisted of the 5' end of the bovine $\alpha 1$ subunit cDNA ligated to the 3' end of the rat $\alpha 1$ subunit cDNA. This construct results in a translated $\alpha 1$ subunit that has an amino acid sequence that is identical to that of the wild-type rat $\alpha 1$ subunit with the sole exception of a single amino acid at the N-terminus that is an essential component of the epitope for the monoclonal antibody bd 24.

Secondly, the rat $\alpha 1$ nucleotide sequence had restriction sites that allowed the construction of the other truncated $\alpha 1$ subunits.

b) A chimera $\alpha 1$ subunit lacking the M3/M4 cytoplasmic loop ($\alpha 1_{CD}$). The bovine/rat chimera $\alpha 1$ subunit was treated with restriction enzymes to delete the M3/M4 cytoplasmic loop. Thus, the construct $\alpha 1_{CD}$ is an $\alpha 1$ subunit with the cytoplasmic loop M3/M4 deleted that contained the monoclonal antibody, bd 24, epitope.

c) Series of truncated $\alpha 1$ subunits where the cytoplasmic loop of the $\alpha 1$ subunit was replaced with the cytoplasmic loops of $\alpha 2$, $\alpha 3$ and $\alpha 6$ subunits ($\alpha_{CH-\alpha x(x=2,3,6)}$). These domain swap chimeras bear the distinguishing "bovine/human" N-terminal epitope.

Figure 2.7. Subcloning strategy in the production of $GABA_{A}R$ α_{CH} , α_{CD} and $GABA_{A}R$ $\alpha_{CH-\alpha}$

In order to investigate the role of the cytoplasmic domain (CD) lying between transmembraneous domains M3 and M4 of $GABA_{A}R$ α_{1-6} subunits on receptor mobility, a series of $GABA_{A}R$ α_1 clones were constructed:

- 1) the CD was deleted to give $GABA_{A}R$ α_{CD}
- 2) the CDs from $GABA_{A}R$ α_{2-6} were individually inserted into $GABA_{A}R$ α_{CD} (ie: to create a $GABA_{A}R$ α_1 in which the cytoplasmic domain was swapped to give $GABA_{A}R$ $\alpha_{CH-\alpha(2-6)}$. All constructs were verified with restriction digests and DNA sequencing.

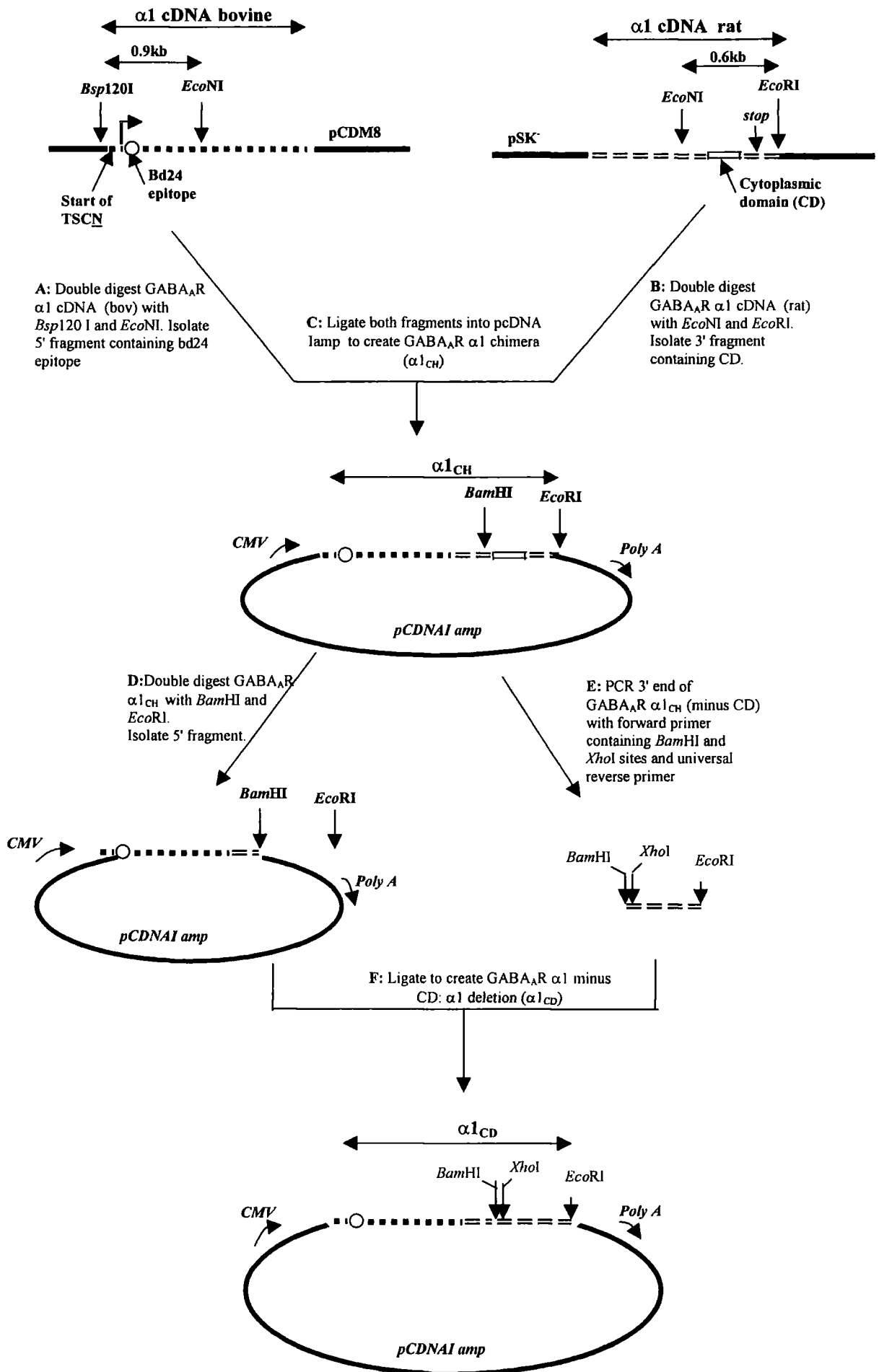
In order to maintain the epitope bd24, but facilitate subcloning, the first step was to make a $GABA_{A}R$ α_1 chimera: $GABA_{A}R$ α_{CH} (panels A-D). A 0.9kb *Bsp120I* - *EcoNI* 5' restriction fragment (encoding the N' terminal bd24 epitope) of the bovine $GABA_{A}R$ α_1 (panel A) was ligated to a 0.6kb 3' *EcoNI*- *EcoRI* restriction fragment of the rat (panel B) into the mammalian expression vector pCDNA1amp (panel C).

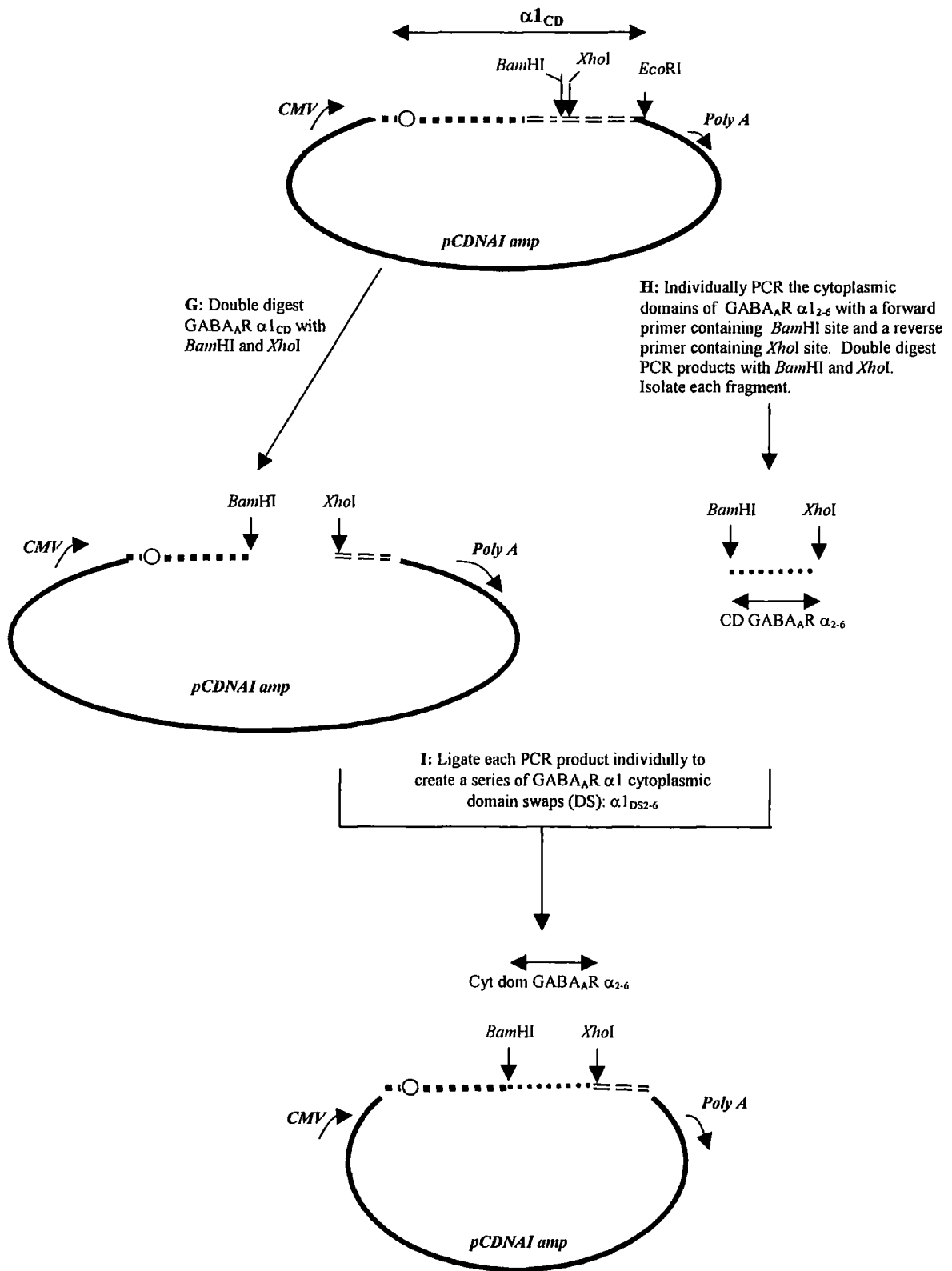
The 3' end of rat $GABA_{A}R$ α_1 was amplified from codon 414 (in effect deleting codons 362-413) using an universal reverse primer (sp6) and a forward primer (5' **aggatcctctctcgcgagcgtcagcaaatcgaccg** 3') which encodes two unique restriction sites for *Bam*HI (in bold) and *Xho*I (underlined) at its 5' end. The 3' end of $GABA_{A}R$ α_{CH} was removed by digestion with *Bam*HI and *Eco*RI (panel D) and replaced with the *Bam*HI - *Eco*RI double digested rat PCR product (panel F) to create $GABA_{A}R$ α_{CD} .

$GABA_{A}R$ α_{CD} was double digested with *Bam*HI and *Xho*I (panel G). The CDs from $GABA_{A}R$ α_{2-6} were individually amplified using forward primers encoding a *Bam*HI restriction site and reverse primers encoding a *Xho*I restriction site (See table 2.7.1. for primer sequences). Amplified CDs were double digested with *Bam*HI and *Xho*I (panel H) and individually ligated into *Bam*HI and *Xho*I digested $GABA_{A}R$ α_{CD} to create $GABA_{A}R$ $\alpha_{CH-\alpha(2-6)}$ (panel I).

Table 2.7.1. Primers used to amplify CDs from $GABA_{A}R$ α_{2-6}

GABA _A R α_x	PCR Primer Sequences 5' - 3'	
	Forward (<i>Bam</i> HI in bold)	Reverse (<i>Xho</i> I underlined)
α_2	aggatccaggctccgtcatgata	<u>gctcgagttgaaagtttcttgg</u>
α_3	aggatccagcagcccaaccaa	<u>gctcgagttgtaggtcttggctct</u>
α_4	aggatcctctccagaagtcc	<u>gctcgaggatccagaagtggtg</u>
α_5	aggatcctgaactcactactaaa	<u>gctcgagttgtaggtctttttac</u>
α_6	aggatccgtcccagaagccga	<u>gctcgagccaatggctgtaag</u>





2.2.3. Transfections

Controls were performed transfecting the cells with the vector only (mock transfections) and each transfection was performed in duplicate.

2.2.3.1. Liposome mediated transfections

Lipofectamine:

Lipofectamine is a liposome-mediated transfection reagent, with a formulation of 3:1 (w/w) of the polycationic lipid DOSPA and the neutral lipid DOPE in membrane filtered water. This reagent was used to transiently transfect GABA_AR subunit cDNAs into COS7, HEK293 and cerebellar granule cells using the manufactures guidelines. COS7 and HEK293 cells were plated at 50% confluency on poly-lysine coated glass cover-slips or onto poly-lysine coated dishes, so that on the day of the transfection cells were approximately 70% confluent. Dissociated granule cells (250×10^3 cells/cm²) were plated onto 35 mm poly-lysine coated dishes. For each transfection two 12 x 75 mm sterile tubes containing the following were prepared: Tube 1: 1-2 µg DNA was diluted in 100 µl of serum-free growth medium. Tube 2: 5 µl (10 µg) of Lipofectamine (GIBCO BRL) diluted into 100 µl of serum-free growth medium. The two solutions were combined, gently mixed and incubated at room temperature for 45 minutes to allow DNA-liposome complexes to form after which 800 µl of serum-free medium was added. Cells were washed once with 2 ml serum-free medium and the DNA-liposome mixture was then dropwise. The cells were incubated for 3-5 hours at 37°C in a 5 % CO₂ incubator. Cells were then washed once with complete growth medium and incubated for 12-24 hours with serum-complete medium.

ESCORT- Transfection reagent:

ESCORT is a liposome formulation comprising the cationic lipid DOTAP and DOPE at a ratio of 1:1 (w/w).

This reagent was employed as described for lipofectamine, with the sole exception that the incubation time for forming the DNA-liposome complex was 15 minutes.

TfxTM-50 Reagent:

TfxTM-50 Reagent is a mixture of a synthetic cationic lipid molecule and DOPE supplied as dried lipid films. Upon rehydration with water, these lipids form multilamellar vesicles that associate with nucleic acids.

TfxTM-50 Reagent was used to transfect cerebellar granule cells maintained in culture or after dispersion. The procedure was as recommended by the manufacturer with some modifications, briefly:

The day before the transfection a vial of TfxTM-50 Reagent was warmed to 37°C, reconstituted with 400 µl of nuclease-free water and vigorously vortexed for 10 seconds to resuspended the lipid film. The vial was then placed in a 65°C water bath for 1 minute, vortexed again and stored at -20°C overnight. 2 µg of the cDNA was added to 200 µl of serum-free medium. 9 µl of TfxTM-50 Reagent was then added to the tube. The mixtures were vortexed and placed at room temperature for 45 minutes to allow the lipid and DNA to associate. Cerebellar granule cell cultures were washed twice with 2 ml of serum-free medium. Most of the final wash medium was removed from the cultures. 800 µl of serum-free medium was added to the lipid/DNA. The diluted lipid/DNA mixture was then

applied to each dish of cells. Cells and lipid/DNA mixtures were incubated at 37°C for 2 hours. The cells were then gently overlaid with 2-3 ml of complete medium and returned to the incubator for 48 hours, thereafter the cells were used in the experiments described.

2.2.3.2. Electroporation

Electroporation was used to transfect COS7, HEK293, PC12 cells and primary cultures of cerebellar granule cells just after dissociation.

Electroporation of COS7 and HEK293 cells:

Cells were harvested by trypsination, counted and maintained in serum-free medium at room temperature. 20 µg of cDNA was placed into an ice cold cuvette to which 400 µl of cells ($1-5 \times 10^7$) were added. The cuvette was then replaced on ice for 3-5 minutes. Just prior to electroporation cells were resuspended with a sterile Pasteur pipette. Following electroporation at 260 V in a Electro Cell Manipulator the cells were incubated again on ice for 1-2 minutes and a further 400 µl of serum-free medium was added. Cells were plated at a density of 100-150 cells/cm² and incubated for 2-4 hours after which the media was replaced for complete medium. Cells were maintained in the 5% CO₂ incubator for 48 hours prior to the experimentation.

Electroporation of cerebellar granule cells

Cerebellar granule cells were transfected by the electroporation technique, just after dissociation, as described above for HEK293 and COS7 cells.

2.2.3.3. Transfection of PC12 cells

PC12 cells were "Primed" by treatment with 50 ng/ml NGF (nerve growth factor) for one week (as described in section 2.2.1.2.). Cells were harvested from the culture flasks and transfected by electroporation as above in section 2.2.3.2. Once the cells had adhered to the culture dish (4 hours), the medium was removed and replaced with complete medium supplemented with NGF (50 ng/ml) and dibutyryl cyclic AMP (1 μ M). The transfected "primed" cells extended processes to acquire a polarised phenotype which were then used for fluorescence microscopy and FPR.

2.2.3.4. Calcium phosphate precipitation

Calcium phosphate precipitation was used to transfect cerebellar granule cells just after they were dissociated or following maintenance in primary culture, as described below:

For each transfection two tubes with the following solutions were prepared: tube 1: 5 μ g of DNA diluted in 50 μ l of sterile 0.1x TE to which 169 μ l of ddH₂O, 5 μ l of CaCl₂ (2 M) was added and mixed by pipetting up and down twice. Tube 2: 250 μ l of 2x HBS. Solution I was dropwise to solution II with gentle agitation, and then incubated for 20-30 minutes at room temperature to allow the calcium phosphate:cDNA precipitate to form. The precipitate was then dropwise to the medium covering the cells. The cells were then returned to the incubator and incubated overnight. Cells were then washed with complete medium and incubated for a further 24 hours prior to experimentation.

2.2.4. Immunocytochemistry

Immunocytochemistry was performed on both fixed and live cells. The antibodies used for immunocytochemical experiments are detailed in Table 2.2. together with the working dilutions.

2.2.4.1. Fixed and permeabilised cells

Cells to be fixed and immunolabelled were cultured on coated glass coverslips (coverslips were coated with poly-D-lysine when studying COS7, HEK293 and cerebellar granule cells and collagen/poly-D-lysine when studying PC12 cells). The cellular morphology of the cultured cells was sufficiently retained following paraformaldehyde fixation to permit identification of intracellular compartments. Briefly: 24-48 hours post-transfection, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes. Cells were washed again twice with PBS, incubated for 20 minutes in 0.34% L-lysine: 0.05% Na-m-periodate and then washed twice in PBS. Cells were then blocked with 200 μ l of 10% heat-inactivated goat serum (HIGS) for 15 minutes. Cell-coated coverslips were then incubated for 1-24 hours in primary antibody, diluted in antibody dilution buffer (PBS containing: 1 μ g/ μ l of bovine serum albumin, 10% heat-inactivated goat serum and 0.5% Triton X-100), as detail in Table 2.2. After incubation with primary antibody, cell-coated coverslips were washed three times for 5 minutes in PBS and then incubated for 30 minutes with secondary antibody diluted in antibody dilution buffer, as detailed in Table 2.2. Cell-coated coverslips were then washed three times for 5 minutes in PBS and mounted with mowiol on glass slips.

2.2.4.2. Labelling of live cells

Cells which were to be viewed live were plated in coverslip-bottom chambers after transfection. Cells were washed twice in PBS then incubated at 4°C in primary antibody for 45 minutes and secondary antibody for 20 minutes, both antibodies were diluted in PBS.

Table 2.2. Summary of the antibodies used for immunocytochemistry, their specificity, working concentrations and some specifications.

<i>Antibody name</i>	<i>Specificity</i>	<i>Working concentration</i>	<i>Specifications</i>
bd24	α 1-subunit of GABA _A R from bovine and man	2 μ g/ml	Mouse Monoclonal Antibody
bd17	β 2/3-subunit of GABA _A R from bovine, rat and man	2 μ g/ml	Mouse Monoclonal Antibody
β (102/103)*	β 1/2/3-subunit of GABA _A R	5 μ g/ml	Rabbit Polyclonal Antibody
anti-spectrin	Spectrin	10 μ g/ml	Rabbit Polyclonal Antibody
m-TRITC r-TRITC	Mouse Ig (L+H) Rabbit Ig (L+H)	1 μ g/ml	Goat Polyclonal Antibody
m-FITC r-FITC	Mouse Ig (L+H) Rabbit Ig (L+H)	1 μ g/ml	Goat Polyclonal Antibody
CascadeBlue	Mouse Ig (L+H)	1 μ g/ml	Goat Polyclonal Antibody

* β -antibody (102/103), prepared against the N-terminal sequence of the rat β 3 sequence (amino acids QSVNDPGNMSFVKET). This antibody was characterized by immunoblot analysis and immunocytochemistry of transfected cells. This antibody recognises all three β subunit isoforms as determined by recombinant expression of the β subunit isoforms followed by immunocytochemical analysis. The β -antibody (102/103) was a gift from Dr. Ramiro Salas.

2.2.4.3. Control analyses

Control analyses included cells that were incubated with secondary antibodies alone, and mock transfected cells (cells transfected only with the vector) that were analysed as above to ensure immunofluorescence was a result of a specific interaction of the primary antibody with the antigen. These control tests were carried out routinely before each experiment. All results were negative, i.e. no fluorescence was detected, as illustrated in Figure 2.8.

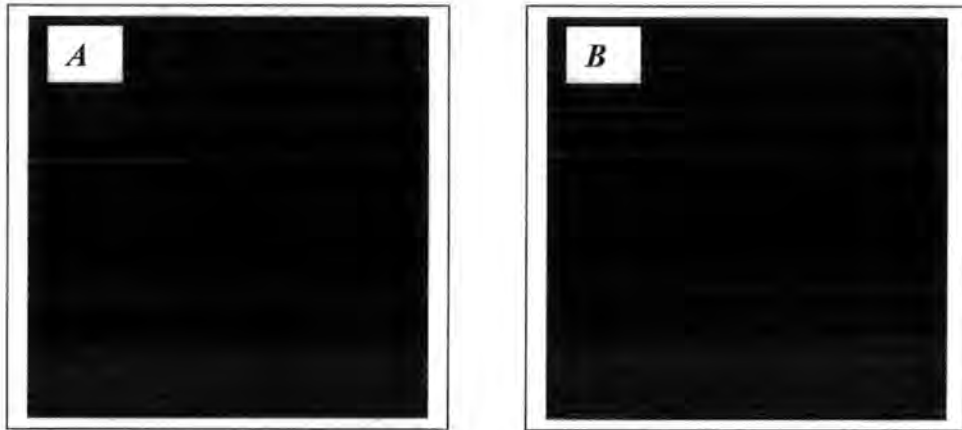


Figure 2.8. *Representative immunocytochemical control experiments.* Panel A: mock transfected HEK293 cells. Cells were labelled live with the monoclonal bd24 antibody specific for the $\alpha 1$ subunit and visualised with TRITC-conjugated anti-mouse specific antibody. Panel B: $\alpha 1$ -transfected HEK293 cells labelled live only with the secondary antibody TRITC-conjugated anti-mouse.

Immunocytochemical controls with cerebellar granule cells

Due to the difficulties in transfecting cerebellar granule cells no control analyses with mock transfected cells could be carried out. The low efficiency of transfection meant that only a few cells per dish expressed the transfected $\alpha 1_{CH}$ and $\alpha 1_{CD}$ subunits, and in some experiments no immunoreactivity could be

detected in any of the cells. For this reason the lack of fluorescence staining in mock transfected cell could be due to problems with the transfection itself and not to the specificity of the antibody. To control the specificity of the antibody non transfected cerebellar granule cells were labelled with the fluorescently labelled Fab' fragments of the GABA_AR α 1 subunit specific-antibody (see 2.2.5.-6) and no fluorescence was detected (Figure 2.9, Panel A). In addition, the immunoreactive cells found were also a proof of the specificity of the antibody, as these few positive cells exhibited a high fluorescence that contrasted with the black background of non transfected cerebellar granule cells (Figure 2.9, Panel B).

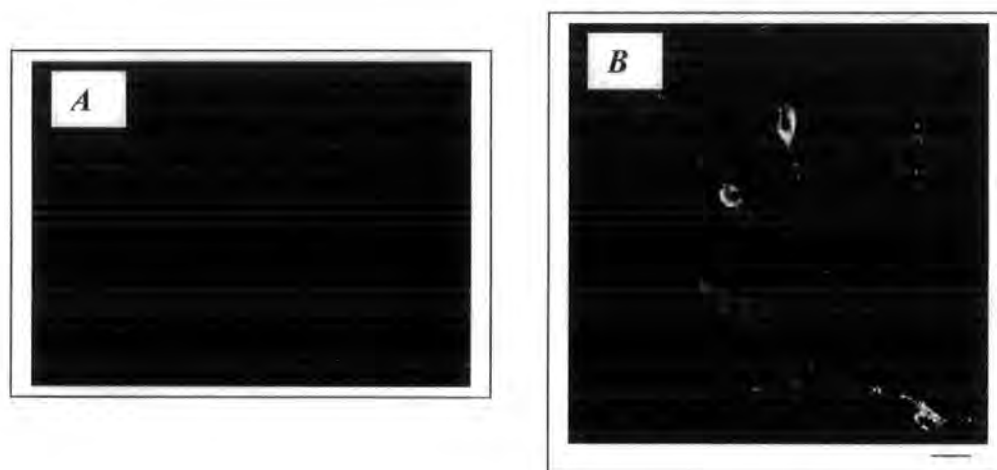


Figure 2.9. Immunocytochemical controls with cerebellar granule cells. Cerebellar granule cells labelled live with the fluorescent-Fab' fragments of the GABA_AR α 1 subunit specific-antibody. Panel A: non transfected cells. Panel B: α 1_{CD} subunit transfected cells, three positive cells are shown against the background. Scale bar: 20 μ m.

2.2.4.4. Photography

Images were obtained through a 100x, 1.3 numerical aperture (NA), 63x, 1.2 NA, water immersion objective or 40x, 1.3 NA objective on a Nikon UFX-II microscope. The image was captured through a Nikon camera FX-35A, or with a confocal microscope (BIORAD).

2.2.5. Generation of Fab' fragments of the GABA_AR α 1 subunit specific-antibody

Fab' fragments of the GABA_AR α 1 subunit specific-antibody were generated by papain digestion of the bd24 antibody, as described by Hallow and Lane, (1988). Digestion of the mouse monoclonal bd24 antibody was proved to be difficult. Preliminary tests to assess the optimum protocol were carried out with purified mouse IgG1 monoclonal antibody (Chemicom). Different times of digestion and different amounts of papain were tested. The products of every digestion were run on an electrophoresis gel, under reducing and non reducing conditions, to determine the amount of IgG1 digested. After these experiments the following protocol was accepted as the optimal one.

Briefly: 10 μ g of the bd24 antibody was digested with 1 μ g of papain in 990 μ l Na-acetate (0.2 mM, pH 5), 5 μ l cysteine (1 M) and 5 μ l EDTA (20 mM), for 15 hours at 37°C. Iodoacetamide at a final concentration of 75 mM, was added and incubated at room temperature for 30 minutes. The Fab' fragments were purified from any remaining intact antibodies by chromatography on a protein A column. Final Fab' fragments concentration was approximately of 0.01 μ g/ μ l in a volume

of 500 μ l. Fragment purity was determined using gel electrophoresis and silver stain (Figure 2.10.). One sample was run with dithiothreitol and one without. Under reducing conditions Fab' fragments yield a doublet of bands at about 25,000 daltons (Figure 2.10., Line 1). Nonreduced Fab' fragments migrate at approximately 50,000 daltons (Figure 2.10., Line 2).

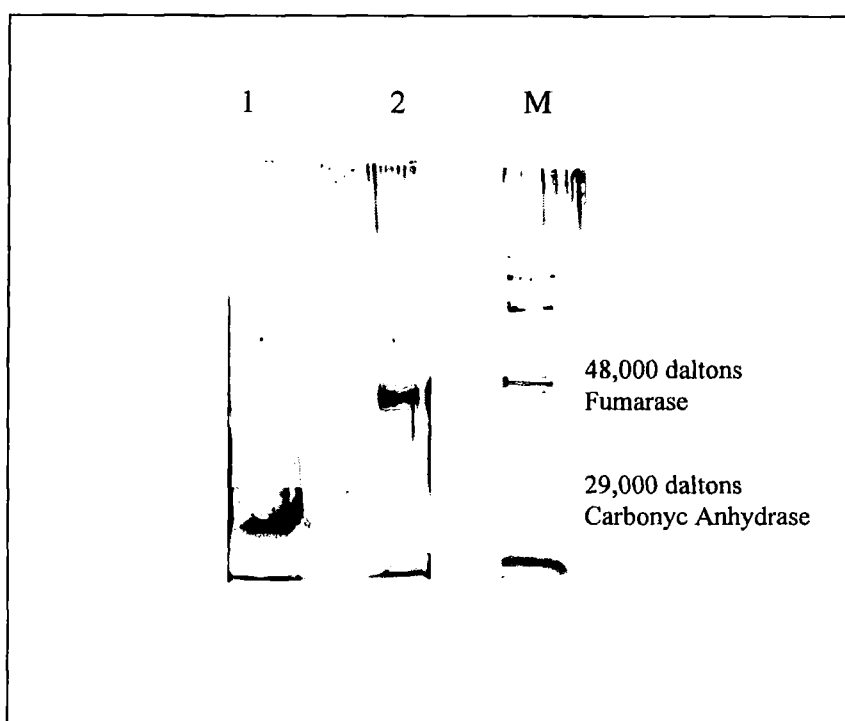


Figure 2.10. Electrophoresis gel of bd24-Fab' fragments stained with silver. Samples were run under reducing (1) and non-reducing (2) conditions, M= Molecular weight standard mixtures (Sigma).

2.2.6. Fluorescent labelling of $\alpha 1$ and $\alpha 6$ Fab' fragments.

Both $\alpha 1$ (bd24) and $\alpha 6$ Fab' fragments (gift from Professor F.A. Stephenson) were labelled with Bodipy. For direct labelling of the Fab' fragments with Bodipy fluorescent groups, the $\alpha 1$ and $\alpha 6$ Fab' fragments were diluted in 900 μ l of sodium carbonate (0.1 M, pH 9.2). To this was added 50 μ l of Bodipy

succinimidyl ester (1 mg/ml) in 5 μ l aliquots, with gentle but continuous stirring. The reaction was incubated in the dark for 8 hours at 4 °C. Unbound dye was separated from Bodipy conjugated Fab' fragments by passing the mixture through a G-10 column.

2.2.7. Fluorescence Photobleach Recovery

FPR is based on measuring the concentration of specific, fluorescently-tagged molecules in a restricted membrane domain as a function of time. To do this, living cells are first labelled by linkage to a fluorescent dye, membrane proteins can be labelled using specific probes, such as a fragment of a fluorescently-tagged antibody. Once labelled, the cells are placed under the microscope, a small area of the cell surface, about 1-4 μm^2 , is briefly exposed to an intense laser pulse which bleaches, irreversibly, the fluorescent molecules in its path, leaving a circular spot, on the surface of the cell that is devoid of fluorescence. If the labelled proteins in the membrane are mobile, then the random movements of these molecules produce a gradual reappearance of fluorescence in the irradiated circle (see Figure 2.11.).

The microscope optics are arranged so that the fluorescence emission from the bleached area is monitored at subsequent times by a photomultiplier tube using the laser, now attenuated, for excitation. The rate of fluorescence recovery provides a direct measure of the rate of diffusion (expressed as a diffusion coefficient) of the mobile molecules. It is the half-time for recovery that is measured from which the diffusion coefficient can be calculated. The extent of fluorescence recovery (expressed as a percentage of the original intensity)

provides a measure of the percentage of the labelled molecules that are free to diffuse. If the fluorescence fails to recover to the same intensity observed before bleaching this can be attributed to a fraction of fluorophores that are immobile on the time-scale of the experiment.

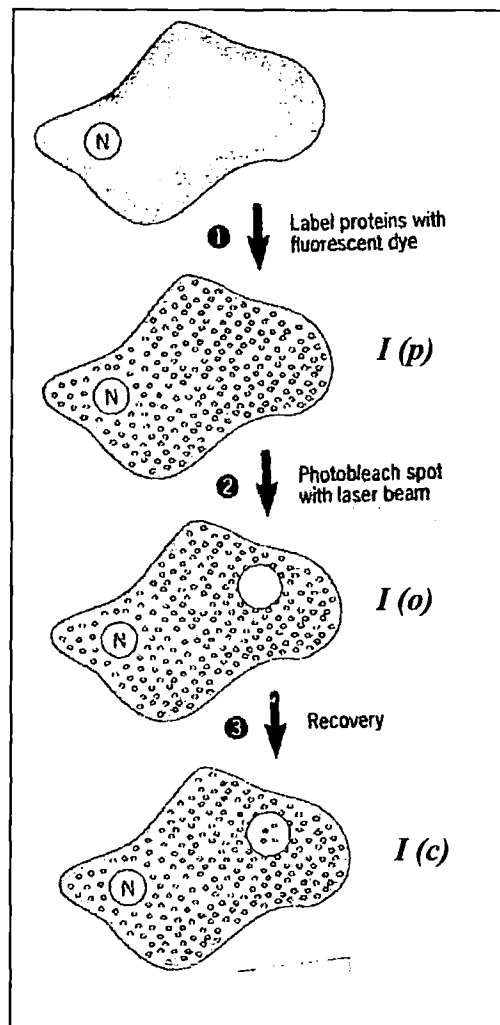


Figure 2.11. Representative illustration of a FRAP experiment. After labelling the cell with the molecule of interest initial levels of fluorescence $I(p)$ are measured. The target area is then bleached $I(o)$ and the recovery of the fluorescence is measured over time $I(c)$

FPR was used to measure the lateral mobility of recombinant GABA_ARs transiently expressed in COS7, HEK293, PC12 and cultured cerebellar granule cells. In addition native GABA_ARs expressed by cerebellar granule cells were analysed.

2.2.7.1. Labelling of recombinant GABA_ARs for FPR analysis

GABA_ARs expressed on living cells were labelled with either the fluorescent benzodiazepine Bodipy-Ro-1986 (Velazquez *et al.*, 1989), or with fluorescently labelled Fab' fragments prepared from subunit-specific antibodies as described in 2.2.6.

Labelling with the fluorescent benzodiazepine, Bodipy-Ro-1986

All experiments using Bodipy-Ro-1986 were performed on cells expressing native GABA_AR or recombinant GABA_AR with subunit combinations of $\alpha x \beta 3 \gamma 2_s$, where αx represents $\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6$ and the truncated $\alpha 1$ subunits $\alpha 1_{CH}, \alpha 1_{CD}$ and $\alpha 1_{CH} - \alpha x_{(x=2,3,6)}$. Because every combination tested included the $\gamma 2$ subunit all of them acquired benzodiazepine binding. Cells were labelled with Bodipy-Ro-1986 at 40 nM or 100 nM in PBS-sucrose. Although at these concentrations not all receptors are labelled, these concentrations were chosen based upon the K_d of the fluorescent benzodiazepine to help minimise any non-specific binding or lipid partitioning of the fluorescent probe. The signal from the unbound fluorophore is negligible because the fluorescence of the Bodipy conjugates are enhanced upon binding to the receptor. Non-specific binding of Bodipy-Ro-1986 was determined by including chlorazepate (1 mM) in

the FPR assay. The non-specific labelling, based upon photon counts obtained under identical experimental conditions, was found to be less than 10% of total.

Labelling with fluorescently tagged Fab' fragments

To label $\alpha\beta\gamma_2$ s receptors containing the $\alpha 1$ or $\alpha 6$ subunits, cells were labelled with the Bodipy-fluorescent Fab' fragment of the monoclonal antibody bd24, which recognises the $\alpha 1$ or with Bodipy-labelled Fab' fragment of a polyclonal GABA_AR $\alpha 6$ subunit-specific antibody, respectively. The $\alpha 1$ subunit-specific Fab' fragments were also used to label recombinant receptors containing the truncated $\alpha 1$ subunit, $\alpha 1_{CH}$ and $\alpha 1_{CD}$ in COS7, HEK293 and cerebellar granule cells. All labelling procedures were performed at room temperature for 20 minutes. Cells were then washed three times with PBS-sucrose.

2.2.7.2. Photobleach recovery

Transfected cells expressing recombinant receptors were identified based on fluorescence labelling as visualised through a Zeiss Photomicroscope III, using a 63x, 1.2 NA water immersion objective (placed directly into the dish containing the cells) with a beam radius of 1.2 μm . The microscope used for FPR experiment is illustrated in Figure 2.12.

The experimental procedure is briefly explained as follows:

From the laser source the appropriate wavelength was chosen, the laser beam was directed using a mirror (M) and passed through the beam shutter (Sr1), which was always open during the experiment. A second mirror (M) drove the laser beam to a diaphragm (D1) which was adjusted to let pass the beam. Then, the

beam went through the first beam splitter (BS1), which split the laser beam into two, one with a high laser power (the "bleacher beam") and the other with an attenuated power.

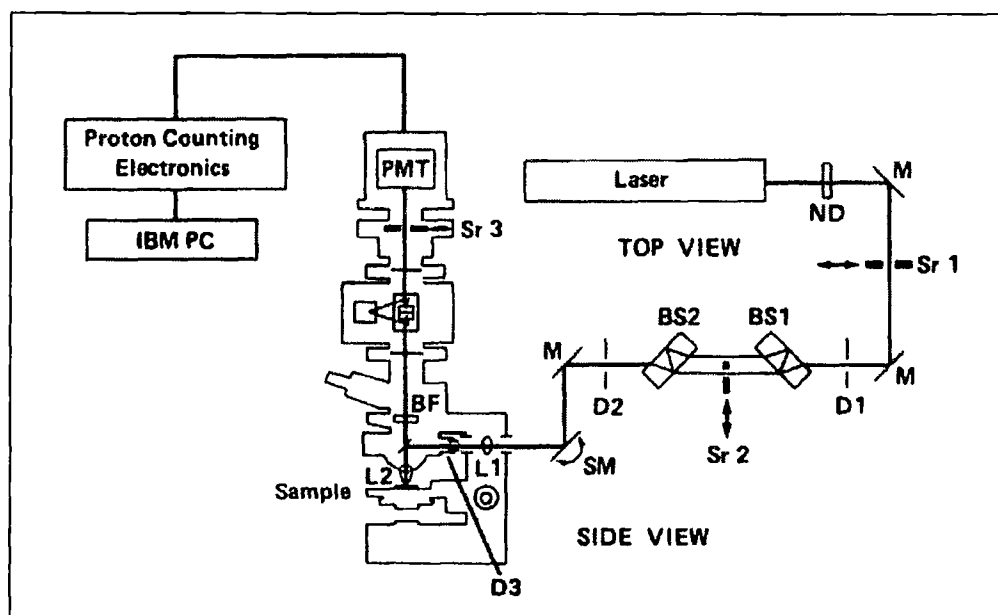


Figure 2.12. Schematic figure of the optical apparatus used for fluorescence photobleach recovery. ND: neutral density filter; M: mirrors; Sr1: beam shutter, Sr2: bleach shutter; D: diaphragms; BS: beam splitters; L: lens; BF: barrier filter.

The attenuated laser beam passed the second beam splitter (BS2), and was focused by a second diaphragm (D2) and a 90/10 mirror (SM) through the microscope to hit the cell preparation with a beam radius of 1.2 μm . Before the bleaching, cells were localised under bright field optics and positive cells were selected using a fluorescence lamp. Cells expressing the recombinant receptors produced a large fluorescence signal, and selection of cells for photobleaching was made on the basis of this fluorescence intensity. Only one measurement was made for each cell. The illuminated region was then bleached by a brief laser

pulse (5 mW), bleaching around 75% of the fluorescence. To do this, the bleach shutter (Sr2), which controls the pass of the "bleacher beam", opened for a period of milliseconds (10-200) to allow the intense laser pulse to bleach the cell. After the bleached pulse the attenuated laser beam continued to excite the sample promoting fluorescence emission, which travelled up to a photomultiplier tube where the signal was monitored. The excited electrons were translated to the IBM monitor as counts, which were displayed graphically on the screen as a function of time (Figure 2.13.).

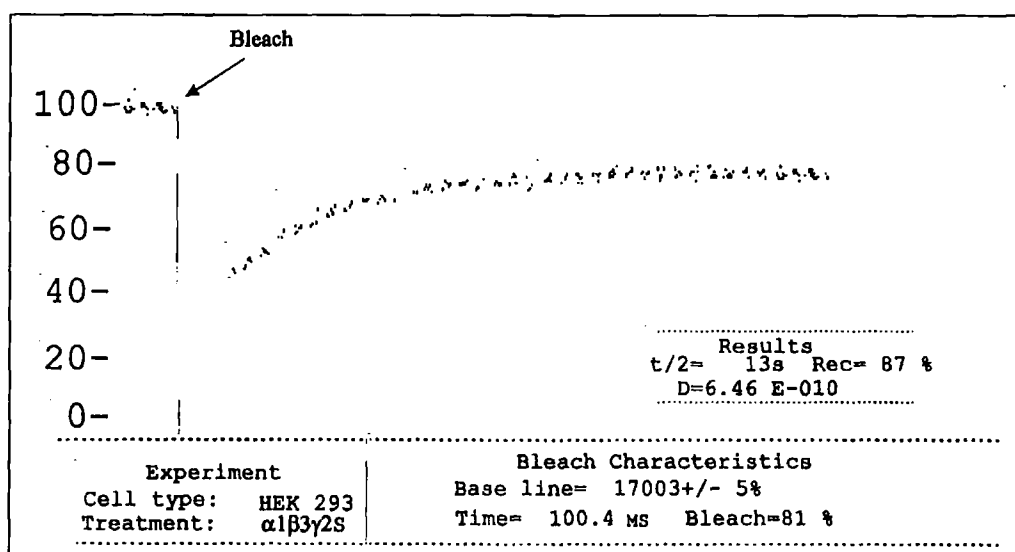


Figure 2.13. Recovery curve for mobile species. The arrow shows the point of photobleaching; y-axis, light intensity as a percentage of normalised pre-bleach levels; x-axis, time.

Once the experiment was completed and the recovery curve stored the data were analysed. The data program enters the multiple curvilinear regression routine, the count curve is fitted and the parameters of diffusion coefficient and percentage of recovery are obtained. Lateral diffusion coefficients (D_c) and

mobile fractions (F, % Recovery) of fluorescently labelled receptors were measured by the spot photobleaching technique deduced by Axelrod *et al.* (1976).

$$Dc=w^2/4T$$

Where w is the e^{-2} radius of the beam and T is the halftime for recovery of the fluorescent signal. The e^{-2} radius of the beam is used because the intensity of the laser light across the circular bleached region is not homogeneous, the laser light is more intense in the middle of the spot than it is toward the edges. For the 63x water immersion objective used in these experiments with a 1.2 μm beam radius the bleached area corresponded to $\sim 2\mu\text{m}^2$.

The fraction of the labelled molecules that are mobile, F , may be calculated from the recovery curve:

$$F = (I_c - I_o) / (I_p - I_o)$$

Where I_p , I_o and I_c are the fluorescence intensities at the pre-bleach, post-bleach and steady state levels respectively, see Figure 2.11.

FPR experiments performed with the same recombinant receptor combinations were repeated on different days and little variation in the parameters were found. The standard mean deviations presented here are the result of repeated measurements on these cultures. The number of recordings made for each cell preparation were in the range 10-30.

2.2.7.3. FPR limitations

The relative size of the bleach spot to the diffusion domain of the fluorescently marked protein dramatically limits the resolution of the FPR experiments. If the bleached area is larger than the domain in which the labelled molecule can freely

diffuse, that molecule will always appear immobile by FPR, even if it is actually diffusing freely in a small confined domain.

The diffusion coefficient is calculated by fitting the recovery curve obtained from every FPR measurement. When the receptors are immobile on the cell surface the recovery curves are almost flat. The curve fitting procedure under these circumstances is difficult and inaccurate. Estimated mobile fractions or percentage recovery in the order of 20% is considered to represent total immobility of all the labelled receptors due to background light and a contribution to fluorescence by unlabelled molecules of the fluorescent dye used to label the cells. Following this rationale, the coefficient of diffusion data obtained from non-recovery curves have to be considered with caution. The small recovery (<25%) measured for some receptors can be considered to be background noise due to these experimental limitations. Thus, receptors which show little recovery are considered to be linked to mobility restricting elements. These receptors are regarded as totally immobile with no rate of motion and diffusion coefficients of zero.

2.2.8. Statistical analysis

Statistical analyses were routinely performed using the statistical package Minitab version 11. A Kolmogorov-Smirnov test of normality was performed and the significance of the difference between data was tested using a Student's t-test. Data are presented throughout the study as mean +/- standard deviation of the mean. In all cases, a probability of less than 0.05 was regarded as statistically significant.

2.2.9. Analysis of predicted secondary structures

Analysis and comparison of predicted secondary structures was done using the network protein sequence @nalysis at IBCP, France: servise@bchs.uh.edu.

Chapter 3

*Lateral mobility and cellular localisation of
recombinant GABA_ARs*

Chapter three

***Lateral mobility and cellular localisation of recombinant
GABA_A receptors expressed in COS7, HEK293 and PC12
cells***

3.1. Introduction

Of all the classes of GABA_AR subunit isoforms, it is the α subunits that exhibit the greatest diversity both in terms of the unique kinetic and pharmacological properties that they confer upon their receptors, as well as their cellular distribution (Wisden *et al.*, 1992). The β subunits have also been shown to be essential for channel function, they have an influence on receptor physiology and pharmacology, as noted for recombinant receptors containing different β subunits (Sigel *et al.*, 1990; Hadingham *et al.*, 1993) though not as dramatically as for the different α subunit isoforms. Inclusion of the γ subunit into the pentameric complex confers benzodiazepine sensitivity to the receptor with some differences noted between alternatively spliced forms of the $\gamma 2$ subunit (Wafford *et al.*, 1991). The relatively limited number of distinct GABA-mediated currents recorded in neurons suggests that the observed structural diversity of GABA_AR subunits may have evolved to satisfy other cellular requirements other than to provide functional versatility (McKernan and Whiting, 1996). Differential receptor sorting could represent one such role. The subunit composition of GABA_A receptors has been reported to regulate their sorting and cell surface distribution (Velazquez and Angelides, 1993; Connolly *et al.*, 1996b), with receptors of different subunit composition selectively clustered and sequestered in different domains (Nusser *et al.*, 1996).

The aim of the work described in this chapter was to gain insight into the role that individual subunits play in receptor clustering and immobilisation and to elucidate the mechanisms by which these occur. Thus, recombinant GABA_AR subunits were expressed in COS7, HEK293 and PC12 cells and their cellular localisation and mobility were determined by immunocytochemistry and by fluorescence photobleach recovery (FPR), respectively. HEK293 and COS7 cells were used because they can be transfected with high efficiency and their internal compartments easily visualised by light microscopy. PC12 cells were used as a model to examine whether compartmentalisation of the receptor occurred following neurite extension and the acquisition of its polarised morphology.

3.2. Results

COS7, HEK293 and PC12 cells were transfected with cDNA of various GABA_ARs subunits in different combinations as described in methods (2.2.3.). In order to define the final cellular localisation of the recombinant GABA_ARs expressed, immunocytochemistry, using subunit-specific antibodies, was performed on both live and fixed cells as described in methods (2.2.4.). Experiments performed on live cells showed the receptors expressed on the cell surface, while fixed and permeabilised cells were used to identify the receptors expressed in intracellular domains. Controls were routinely carried out with mock transfections and with non transfected cells. After labelling the control cells with α 1, and β 3 specific antibodies no immunostaining was detected.

3.2.1. Expression of GABA_AR α , β , and γ subunits in COS7 cells

COS7 cells singly transfected with GABA_AR α 1 subunit cDNA were fixed and permeabilised 48 h post transfection and analysed for GABA_AR α 1 subunit expression by immunocytochemistry using a GABA_AR α 1 subunit-specific antibody. Using this approach it was found that the GABA_AR α 1 subunit protein was retained in an intracellular compartment that had the morphological characteristics of the endoplasmic reticulum (ER) (Figure 3.1. Panel A). The flat morphology of COS7 cells helped to clearly highlight retention of the α 1 subunit in the ER, a pattern which is identical to cells stained with the ER markers DiOC6 and BiP (Terasaki and Reese, 1992). The GABA_AR α 1 subunit-specific antibody, bd24, recognises an N-terminal sequence of the GABA_AR α 1 subunit (Ewert *et al.*, 1990), which when expressed at the cell surface is proposed to be

extracellular. When COS7 cells singly transfected with GABA_AR α 1 subunit cDNA were labelled live, no fluorescence was detected on the cell surface. The absence of labelling on live cells confirms that homomeric GABA_AR α 1 complexes are not transported to, or inserted into, the cell surface plasma membrane (Figure 3.1. Panel B). In contrast to the intracellular retention of the expressed GABA_AR α 1 subunits, when COS7 cells were transfected with GABA_AR β 1 subunit cDNA or GABA_AR β 3 subunit cDNA, the resulting homomeric complexes were found to be sorted to the cell surface (Figure 3.1. Panels C and D) and distributed in clusters. The pattern of cell surface distribution and clustering of the expressed GABA_AR β 1 subunit (Panels C) was similar to the β 3 subunit (Panel D).

When the α 1 subunit was co-transfected with the β 3 subunit, the α 1 subunit was re-routed from its intracellular location, transported to the cell surface, and localised in clusters with the β subunit on the cell surface. Both subunits are clearly expressed on the surface as shown by labelling of live cells (Figure 3.1. Panels E and F). Cotransfection of the γ 2s subunit with the α 1 subunit followed by staining of live cells using the monoclonal antibody bd24, did not reveal any fluorescent signal on the cell surface (Figures 3.1. Panel G), only after fixation and permeabilisation to allow access to the intracellular compartment was the α 1 subunit detected (Figures 3.1. Panel H), suggesting that unlike the β subunits, the γ 2s subunit was not capable of rescuing the α 1 subunit from its intracellular location. When the γ 2s subunit was cotransfected with both the α 1 and the β 3 subunits the cell surface expression and clustering of α 1 β 3 complexes was not altered. Figure 3.1. shows a live cell labelled with the monoclonal antibody bd24

(Panel I) and with the fluorescent benzodiazepine Bodipy-Ro-1986 (Panel J). A clear pattern of clustered receptors is shown on the cell surface.

Figure 3.1. *Localisation of GABA_AR subunits expressed in COS7 cells.*

Panels A and B: COS7 cells transfected with the GABA_AR α 1 subunit cDNA. **Panel A:** Fixed and permeabilised cells were immunostained with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with FITC-conjugated anti-mouse IgG specific-antibody. **Panel B:** live cells were immunolabelled with GABA_AR α 1 subunit-specific monoclonal antibody, bd24 and visualised with FITC-conjugated anti-mouse IgG specific-antibody.

Panel C: COS7 cells transfected with the GABA_AR β 1 subunit cDNA and stained live with the rabbit polyclonal GABA_AR β (102/103) subunit-specific antibody which recognises all three GABA_AR β subunit isoforms, GABA_AR β 1, β 2 and β 3 subunits, and visualised with TRITC-conjugated anti-rabbit IgG specific-antibody.

Panel D: COS7 cells transfected with the GABA_AR β 3 subunit cDNA, were immunostained, live, with the GABA_AR β 3 subunit-specific monoclonal antibody, bd-17, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody.

Panels E and F: Cell surface expression of GABA_AR α 1 β 3 complexes immunolabelled with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and with the GABA_AR β subunit-specific polyclonal antibody for the β 3 subunit. **Panel E:** COS7 cells cotransfected with GABA_AR α 1 and β 3 subunits were immunostained live for GABA_AR α 1 subunit and visualised with cascade blue conjugated anti-mouse secondary antibody; **Panel F,** same cell as Panel E stained live for the β 3 subunit and visualised with TRITC-conjugated anti-rabbit secondary antibody.

Panels G and H: Localisation of recombinantly expressed GABA_AR α 1 γ 2s receptors in COS7 cells. Cells were immunostained live (**Panel G**) and fixed-permeabilised (**Panel H**) with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody.

Panels I and J: Live COS7 cells cotransfected with GABA_AR α 1, β 3, and γ 2s subunit cDNAs, were immunolabelled for the α 1 subunit with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody (**Panel I**) and with Bodipy-Ro-1986 for the α 1 β 3 γ 2s complex (**Panel J**).

Scale bars for all panels: 20 μ m.

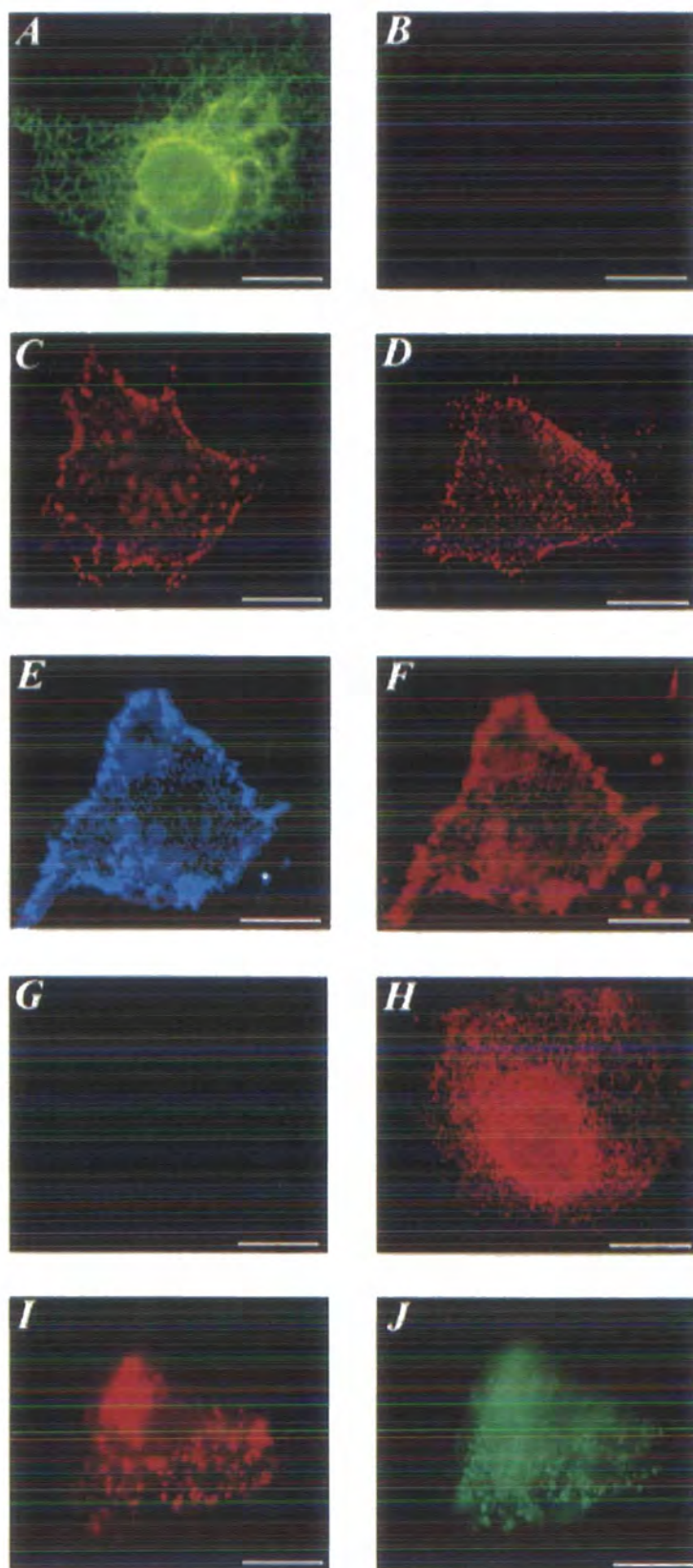


Figure 3.1. Localisation of GABA_AR subunits expressed in COS7 cells.

3.2.2. Expression of GABA_AR α , β , and γ subunits in HEK293 cells

Live HEK293 cells, transfected with the GABA_AR α 1 subunit alone, were immunostained with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24. As with COS7 cells, no surface immunostaining was detected (Figure 3.2. Panel B). When the cells were fixed and permeabilised, an intracellular staining pattern that matched the morphology of the ER was observed (Figure 3.2. Panel A). On the other hand, when HEK293 cells were transfected with the GABA_AR β 1 or β 3 subunit cDNAs alone and subsequently immunostained live, GABA_AR β 1 or β 3 subunits were found expressed, in clusters, at the cell surface as shown in Figure 3.2. Panels C and D.

Experiments performed on cells cotransfected with both the GABA_AR α 1 and the β 3 subunit cDNAs showed that now both the GABA_AR β 3 and α 1 subunits were found expressed at the cell surface. Cells were double labelled in order to determine the distribution of each subunit. GABA_AR α 1 and β 3 subunits were colocalised in the same clusters on the cell surface (Figure 3.2. Panel E and F).

Experiments were then performed in order to determine whether the GABA_AR γ subunit played a role in receptor targeting. Following coexpression of GABA_AR α 1 and γ 2s subunits, cells were immunostained live with the GABA_AR α 1 subunit-specific antibody, bd24. No GABA_AR α 1 subunit immunoreactivity was detected at the cell surface (Figure 3.2. Panel G). GABA_AR α 1 subunit immunoreactivity was detected only when the transfected cells were fixed and permeabilised (Figure 3.2. Panel H). The GABA_AR α 1 subunit was only targeted to the cell surface when the cells were cotransfected with the GABA_AR β 3 subunit cDNA. HEK293 cells transfected with GABA_AR α 1, β 3, and γ 2s subunit

cDNAs were probed live with both the GABA_AR α 1 subunit-specific antibody, bd24 (Figure 3.2. Panel I) and with the fluorescent benzodiazepine receptor ligand, Bodipy-Ro-1986 (Figure 3.2. Panel J).

Figure 3.2. Localisation of GABA_AR subunits expressed in HEK293 cells.

Panels A and B: HEK293 cells transfected with the GABA_AR α 1 subunit cDNA. **Panel A:** Fixed and permeabilised cells were immunostained with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with the TRITC-conjugated anti-mouse IgG specific-antibody. **Panel B:** live cells were immunolabelled with GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody.

Panel C: HEK293 cells transfected with the GABA_AR β 1 subunit cDNA and immunostained, live, with the rabbit polyclonal GABA_AR β subunit-specific antibody, anti- β -102/103, which recognises all three GABA_AR β subunit isoforms, GABA_AR β 1, β 2 and β 3 subunits, and visualised with TRITC-conjugated anti-rabbit IgG specific-antibody.

Panel D: HEK293 cells transfected with the GABA_AR β 3 subunit cDNA, were immunostained, live, with the GABA_AR β 3 subunit-specific monoclonal antibody, bd-17, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody.

Panels E and F: Cell surface expression of GABA_AR α 1 β 3 subunit complexes immunolabelled with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and with the rabbit polyclonal GABA_AR β subunit-specific antibody, anti- β -102/103, for the β 3 subunit. **Panel E:** GABA_AR α 1 β 3 cotransfected HEK293 cells immunostained, live, for GABA_AR α 1 subunit and visualised with cascade blue conjugated anti-mouse IgG specific-antibody; **Panel F:** same cell as Panel E but immunostained, live, for the GABA_AR β 3 subunit and visualised with TRITC-conjugated anti-rabbit IgG specific-antibody.

Panels G and H: Localisation of GABA_AR α 1 γ 2s receptors expressed in HEK293 cells. Cells were immunostained, live (**Panel G**), and fixed-permeabilised (**Panel H**), with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody.

Panels I and J: HEK293 cells cotransfected with GABA_AR α 1, β 3, and γ 2s subunit cDNAs, immunolabelled live for the GABA_AR α 1 subunit with the antibody bd24 and visualised with TRITC-conjugated anti-mouse IgG specific-antibody (**Panel I**) and with Bodipy-Ro-1986 for the GABA_AR α 1 β 3 γ 2s complex (**Panel J**).

Scale bars for all panels: 10 μ m.

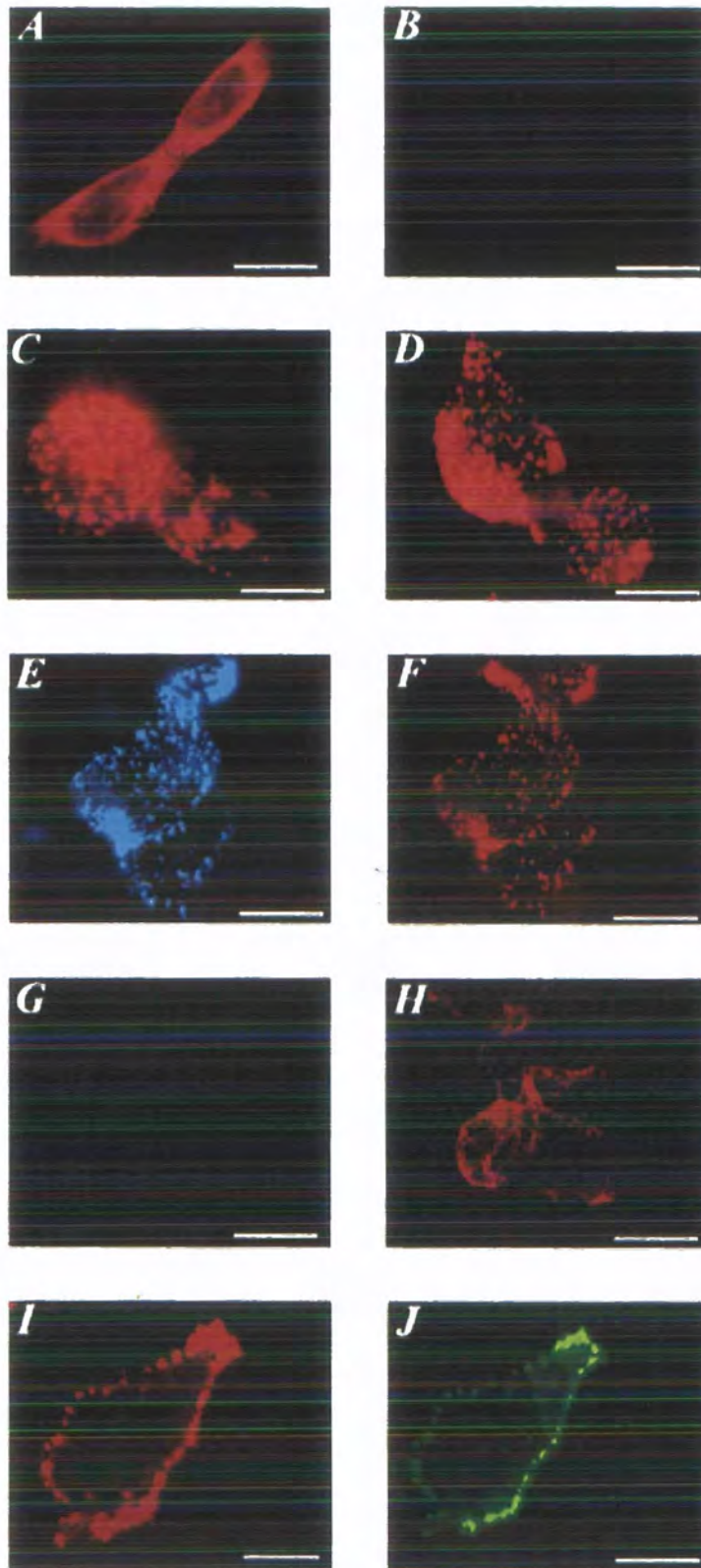


Figure 3.2. Localisation of GABA_A R subunits expressed in HEK293 cells

3.2.3. Expression of GABA_AR subunits in PC12 cells

The PC12 cell-line was derived from the neural crest. It is a well-characterized neuronal model system, expressing many of the cytoskeletal proteins found in neurons (Greene and Carpenter, 1982; Kelly and Grote, 1993; Smith *et al.*, 1995). PC12 cells were differentiated by treatment with NGF as described in methods (2.2.1.2.). NGF-promoted differentiation of PC12 cells results in neurite extension and is accompanied by the expression of microtubules, MAP-2, actin, α - and β -spectrin, ankyrin, and neurofilaments in the neurite and B-50/GAP on the neuronal growth cone (San Erajt *et al.*, 1995). PC12 cells were used, therefore to explore whether the acquisition of its polarized morphology and the formation of different membrane domains (neuronal differentiation) led to the confinement of GABA_A receptors to cell bodies and/or neurites and/or to restriction in receptor mobility. Neuronal-like cells are notoriously difficult to transfect. Transfection efficiency using lipofectin or calcium phosphate precipitation methods were very low. However, electroporation (methods, 2.2.3.3.) of GABA_AR subunits cDNA into PC12 cells was significantly more efficient (around 20%). Therefore, this was the method of transfection used throughout this study.

Immunocytochemical analysis of differentiated, GABA_AR α 1 subunit cDNA-transfected, PC12 cells showed that the GABA_AR α 1 subunit was localised intracellularly within the cell body (Figure 3.3. Panel A). When GABA_AR α 1 subunit expression was examined on live cells no immunostaining was observed (Figure 3.3. Panel B).

In contrast, when the GABA_AR β 3 subunit was transiently expressed in PC12 cells it was observed that the GABA_AR β 3 subunit was sorted to the plasma

membrane (Figure 3.3. Panel C). The GABA_AR β 3 subunit was distributed on the cell body and along the entire length of the neurites in a punctate rather than a homogeneous pattern indicating that, as in the other cells, the GABA_AR β 3 subunit alone forms clusters. Double immunolabelling with an anti-spectrin antibody highlights the highly differentiated morphology of the transfected PC12 cells (Figure 3.3. Panel D). The same clustered pattern was also observed in live cells (Figure 3.3. Panel E) confirming that the observed expression pattern was not a consequence of the process of either fixation or permeabilisation. PC12 cell differentiation and process extension are not a prerequisite for sorting of the GABA_AR β 3 to the plasma membrane since undifferentiated PC12 cells also expressed the GABA_AR β 3 subunit at the cell surface with a similar distribution pattern (Figure 3.3. Panel F and G). Panel G shows a higher magnification image of the clustered distribution of β 3-complexes expressed in the cell shown in Panel F.

Co-transfection of PC12 cells with the GABA_AR α 1 and GABA_AR β 3 subunits showed that when co-expressed with the GABA_AR β 3 subunit, the GABA_AR α 1 subunit was also sorted to the cell surface where the GABA_AR α 1 subunit also assumed a clustered distribution on both the cell soma and neurites in a pattern similar to GABA_AR β 3 subunit itself. GABA_AR α 1 β 3 subunit complexes were double immunolabelled on live cells using fluorescently-tagged subunit-specific antibodies (Figure 3.3. Panels H and I, clusters indicated by arrows). Both subunits are clearly co-localise on the surface. Co-expression of the GABA_AR γ 2s subunit with GABA_AR α 1 and β 3 subunits neither altered the relative cell surface

distribution of the receptor complex (e.g. cell bodies vs. neurites) nor the pattern of cell surface clustering (Figure 3.3. Panels J and K).

Figure 3.3. Localisation of GABA_AR subunits expressed in PC12 cells.

Panels A and B: Localisation of the recombinant GABA_AR α 1 subunit homo-oligomeric complexes expressed in differentiated PC12 cells. **Panel B:** live cells immunolabelled with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody. **Panel A:** Fixed and permeabilised cells visualised with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody.

Panels C and D: Differentiated PC12 cells transfected with the GABA_AR β 3 subunit cDNA. **Panel C:** Fixed and permeabilised cells were immunolabelled with the GABA_AR β 3 subunit-specific monoclonal antibody, bd-17, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody. **Panel D:** same cell as in Panel C except cells were immunolabelled with anti-spectrin antibody and visualised with FITC-conjugated anti-rabbit IgG specific-antibody.

Panels E, F and G: PC12 cells transfected with the GABA_AR β 3 subunit cDNA and immunostained live with the GABA_AR β 3 subunit-specific monoclonal antibody, bd-17, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody. **Panel E** shows a differentiated cell while **Panel F** shows an undifferentiated PC12 cell, **Panel G** shows an enhanced section of the cell shown in Panel F.

Panels H and I: Cell surface expression of GABA_AR α 1 β 3 complexes immunolabelled with the GABA_AR α 1 subunit-specific monoclonal antibody, bd24, and with the rabbit polyclonal GABA_AR β subunit-specific antibody, anti- β -102/103, which recognises all three GABA_AR β subunit isoforms, GABA_AR β 1, β 2 and β 3 subunits, for the β 3 subunit. **Panel H:** GABA_AR α 1 β 3 receptors expressed in PC12 cells immunostained, live, for the GABA_AR α 1 subunit and visualised with TRITC-conjugated anti-mouse IgG specific-antibody. **Panel I,** same cell as Panel F except cells were immunostained, live, for the GABA_AR β 3 subunit and visualised with FITC-conjugated anti-rabbit IgG specific-antibody.

Panels J and K: Differentiated PC12 cells coexpressing recombinant GABA_AR α 1, β 3, and γ 2s subunits, immunolabelled live for the GABA_AR α 1 subunit with the antibody, bd24, and visualised with TRITC-conjugated anti-mouse IgG specific-antibody (**Panel J**) and with Bodipy-Ro-1986 for the GABA_AR α 1 β 3 γ 2s complex (**Panel K**).

Scale bars for all panels: 10 μ m except Panel G: 2 μ m.

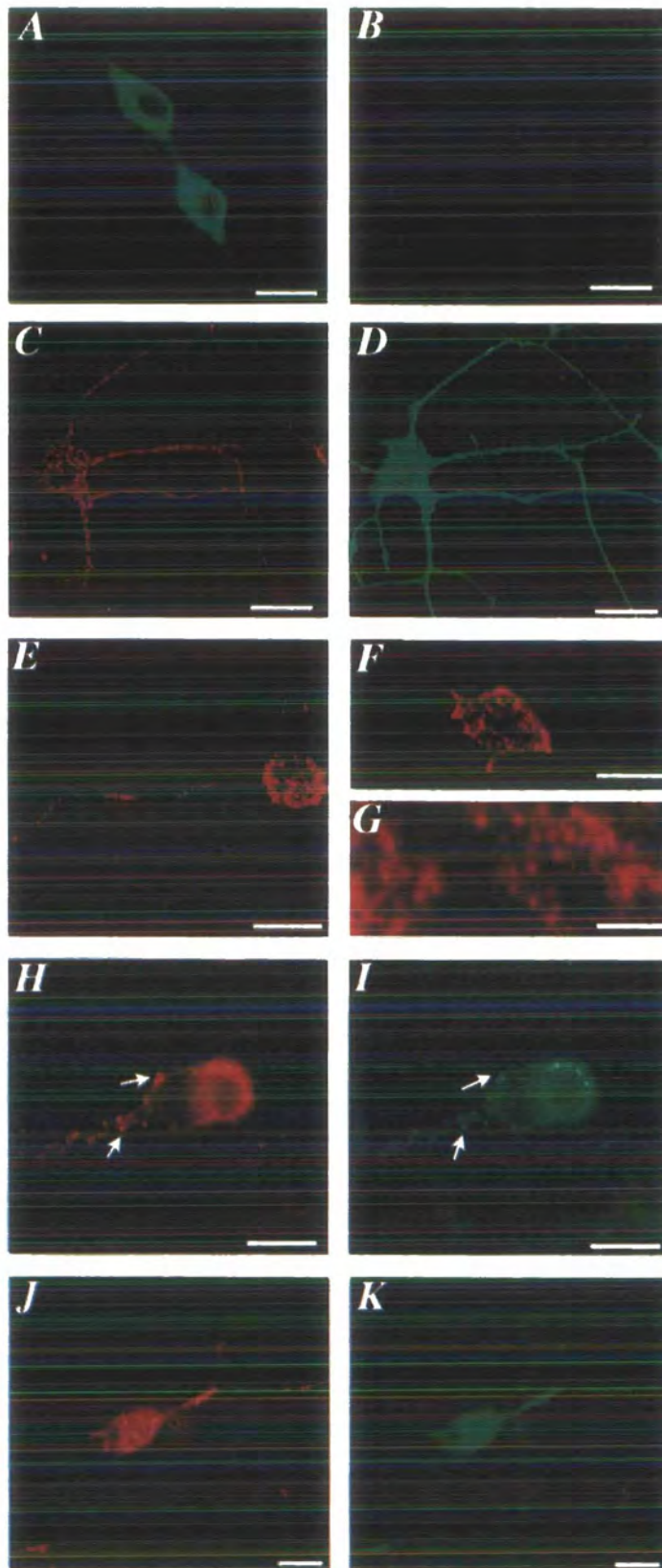


Figure 3.3. Localisation of GABA_AR subunits expressed in PC12 cells

3.2.4. Rescue of $\alpha 1$ from the endoplasmic reticulum by β subunits anchors receptors on the cell surface

To gain insight into the mechanisms that might govern GABA_AR distribution, the lateral mobility of receptors expressed on the cell surface of transfected COS7, HEK293 and PC12 cells was measured by FPR as described in methods (2.2.7.). Table 3.1., shows the results of experiments performed to calculate the average fraction of recombinant GABA_ARs that were mobile on the time scale of the experiment (% Recovery). Representative FPR recovery curves are shown in Figure 3.4.

Table 3.1. A summary of the measured lateral mobilities of recombinant GABA_ARs expressed in PC12, COS7, and HEK293 cells.

<i>GABA_AR-subunits transfected</i>	<i>Cell type</i>	<i>Mobile Fraction (% Recovery)</i>	<i>n</i>
$\alpha 1\beta 3\gamma 2s$	COS7	17+/-4	24
$\alpha 1\beta 3\gamma 2s$	HEK293	74+/-12	10
$\alpha 1\beta 3\gamma 2s$	PC12	27+/-11	13

FPR analysis of recombinant GABA_A $\alpha 1\beta 3\gamma 2s$ subunit-containing receptors transiently expressed in three different types of cells, revealed that the rates of receptor lateral mobility were not the same for each cell line. In COS7 and PC12 cells almost 80% of expressed GABA_A $\alpha 1\beta 3\gamma 2s$ receptors had a restricted mobility, however, when the lateral mobilities of GABA_A $\alpha 1\beta 3\gamma 2s$ receptors expressed in HEK293 cells were examined, receptors had free and unrestricted mobility, as defined by the $74 \pm 12\%$ recovery of fluorescence following photobleach.

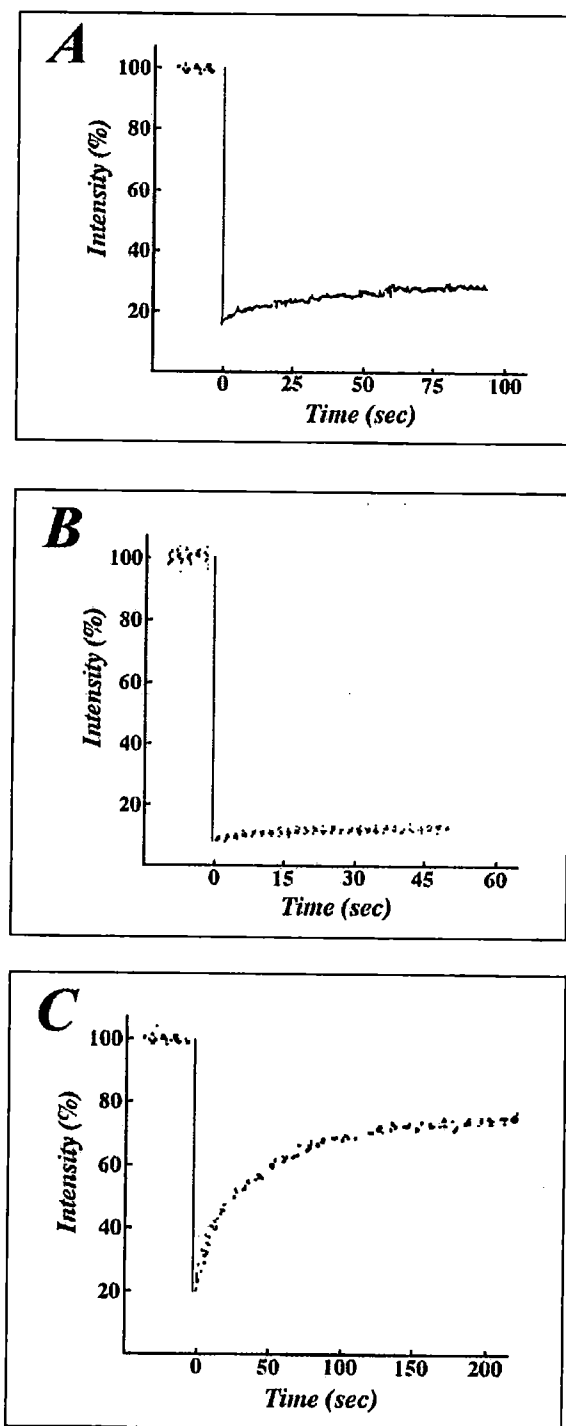


Figure 3.4. Typical FPR recovery curves obtained from $\alpha 1\beta 3\gamma 2s$ recombinant GABA_AR expressed in COS7 cells (A), PC12 cells (B) and HEK293 cells (D)

3.3. Discussion

This study demonstrates that when the GABA_AR α 1 subunit is expressed alone in HEK293, COS7, and PC12 cells it is retained in an intracellular compartment, unable to be ferried to the cell surface. This retention is consistent with the abundance of intracellular GABA_AR α 1 subunits seen in cerebellar granule neurons (Somogyi *et al.*, 1989). However, it has been reported that GABA_AR subunits have the potential to assemble as both homo- and heteromeric receptors (Blair *et al.*, 1988; Pritchett *et al.*, 1988 and 1989; Verdoorn *et al.*, 1990). Although, homomeric expression of receptor subunit has proven controversial. In the case of β 1 and β 3 subunit functional Cl⁻ channel have been reported on expression in a range of heterologous systems (Sigel *et al.*, 1989; Connolly *et al.*, 1996b; Krishek *et al.*, 1996), in agreement with the cell surface expression of homomeric β 1 and β 3 subunit receptors presented in this chapter. In contrast, homomeric expression of the α 1 or β 2 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 cDNA used may be of significance. Another explanation is that some expression systems may produce trace levels of GABA_AR subunits, which may complicate the interpretation of homomeric expression.

In this chapter a typical ER distribution of recombinantly expressed GABA_AR α 1 subunit is shown in all cells tested, suggesting that the GABA_AR α 1 subunit does not acquire the necessary structural conformation required to exit the ER. Intracellular retention of the GABA_AR α 1 subunit in neurons and in transfected cells have been reported also by Connolly *et al.* (1996a) and Gorrie *et al.* (1997),

and likely reflects a mechanism that ensures that GABA_AR α 1 subunit homomers are not expressed on the cell surface. One possible explanation for these results is that GABA_AR α 1 subunit could have a specific ER retention signal. However, inspection of the GABA_AR α 1 subunit sequence shows no classical C-terminal KDEL ER retention signal (Pelham, 1990). Presumably single GABA_AR α 1 subunits are retained in the ER via the interaction with chaperone proteins such as calnexin and BiP (Connolly *et al.*, 1996a).

The results obtained with transfected COS7, HEK293 and PC12 cells show that the GABA_AR β 3 subunit is able to transport the GABA_AR α 1 subunit from its intracellular localisation to the cell surface. How does the GABA_AR β 3 subunit aid in cell surface expression of the GABA_AR α 1 subunit? One possibility is that assembly of GABA_AR β 3 subunits with GABA_AR α 1 subunits masks an ER retention signal on the GABA_AR α 1 subunits, thus permitting its exit from the ER and allowing it to be transported to the cell surface using the cell surface transport signal of the GABA_AR β 3 subunit. Alternatively, a conformational change induced by the coassembly of both subunits might expose a targeting signal present in the GABA_AR α 1 subunit sequence allowing it to exit from the ER. Chaperone-like effects of channel subunits have been reported for K⁺ channels (Shi *et al.*, 1996). Further, despite the potential of all subunits to assemble, it is possible that neurons have an additional level of control that architecturally edit certain GABA_AR complexes prior to their exit from the ER (Klausner, 1989) and/or Golgi. This interpretation is consistent with the results obtained in this chapter, and with recent experiments showing that, despite consistently high GABA_AR α 1 subunit mRNA levels, GABA_AR α 1 subunit polypeptide is only

expressed on the cerebellar granule cell surface late in development, coincident with the expression of both GABA_AR $\beta 2/\beta 3$ and $\gamma 2$ subunit mRNAs and proteins (Nadler *et al.*, 1996).

The role of β subunit in targeting GABA_ARs has been previously reported. The $\beta 1$ subunits were proven to re-route the $\alpha 1$ subunits from the basolateral to the apical membrane of transfected MDCK cells (Velazquez and Angelides, 1993). Similar experiments using again MDCK cells drew the same conclusions (although with some divergences regarding $\alpha 1$ subunit localisation) that the β subunit is the one which controls the subcellular distribution of GABA_ARs, and that β subunit can selectively target GABA_ARs to distinct cellular locations (Connolly *et al.*, 1996b). Connor *et al.* (1998) also proposed that a β subunit was necessary for GABA receptor assembly and expression on the cell surface. Following the injection of a GABA_AR $\alpha 1$ subunit tagged at the C-terminus with a green fluorescent protein into *Xenopus* oocytes either alone, or in combination with other subunits, they observed that the fluorescence was associated with the plasmalemma only when the $\beta 2$ subunit was co-injected with the $\alpha 1$.

Recently, Taylor *et al.* (1999), using a chimeric approach, have identified four amino acids in the N-terminal domain of the $\beta 3$ subunit that mediate subunit homo-oligomerization and cell surface expression. Presumably this assembly signal within the $\beta 3$ subunit will interact in concert with other as yet undefined assembly signals to ensure the fidelity of GABA_AR assembly.

In COS7, HEK293 and PC12 cells, GABA_AR $\beta 3$, $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2s$ complexes are clustered on the cell surface. The clustering of recombinant

GABA_ARs in non-neuronal cells suggests that clustering might be encoded, in part, by the subunits contained within the receptor complex. Homo-oligomeric GABA_AR β 1 and β 3 subunit-containing receptors demonstrate clustering, suggesting that the information orchestrating receptor clustering is encoded in the GABA_AR β subunit sequence. However, GABA_AR β 3 subunits do not appear to specify cell surface distribution, because no differences were observed between the expression patterns of GABA_AR β 3 subunit on cell bodies versus neurites in PC12 cells, despite their highly differentiated morphology.

In addition, the GABA_AR γ 2s subunit was not able to transport the GABA_AR α 1 subunit to the cell surface. These data are in agreement with the observations of Connolly *et al.* (1996a) and Gorrie *et al.* (1997) and indicate that the GABA_AR γ 2s subunit is not required for translocation of functional GABA_ARs to the cell membrane.

Despite their ability to direct GABA_AR surface expression and receptor clustering, GABA_AR β 3 subunit homo-oligomeric complexes remain mobile either over the cell surface shown by FPR or within a 1.4-2.0 μm^2 domain as revealed by Single Particle Tracking (SPT) (Peran *et al.*, submitted). Anchoring of receptors appears to be a property that is endowed by the GABA_AR α 1 subunit, since its inclusion in the receptor complex confers immobility at least in COS7 and PC12 cells (Peran *et al.*, submitted). This suggestion is consistent with the finding that GABA_ARs are clustered and immobile on hippocampal and cortical neurons (Velazquez *et al.*, 1989). Furthermore, the immobility of the GABA_AR α 1 β 3 γ 2 complex is also consistent with "Type I" receptors, to which both GABA_AR α 1 β 1 γ 2 and α 1 β 3 γ 2 belong (Lo *et al.*, 1982), which by

“operational” criteria have been found in Triton X-100 insoluble fractions associated with the cytoskeleton.

The molecular elements, however, that interact with the GABA_AR α 1 subunit and mediate clustering and restrict receptor lateral mobility are not known. The precise intermediate subplasmalemmal element(s) that interact with GABA_AR complexes are likely to be unique because, while other ion channels and receptors interact with the ankyrin-based cytoskeleton, GABA_ARs do not (Srinivasan *et al.*, 1988). FPR experiments on COS7 and PC12 cells suggest that a cytoskeletal linking protein other than ankyrin, found within both COS7 and PC12 cells, serves to link GABA_ARs. Furthermore, the finding that receptors are freely mobile in HEK293 cells demonstrates that these cells likely lack those proteins.

Another possible explanation for the differences in mobility found between expressed GABA_ARs in COS7, PC12 and HEK293 cells could be different levels of expression between these expression systems. A large expression level of GABA_AR subunits transfected in HEK293 cells in comparison with COS7 and PC12 cells could determine the unrestricted mobility found in these cells. If the number of expressed receptors exceed the number of native possible anchoring proteins, then the transfected receptors will not “find enough proteins” to bind and consequently these receptors will diffuse freely. But differences in expression levels, based on differences in fluorescence emission of the transfected cells labelled with specific antibodies, were not detected. This result was not unexpected taking into account that the expression of the transfected subunit, in every expression system used, was under the control of the eukaryotic cytomegalovirus (CMV) promoter. Thus, the expression of foreign proteins was

in every case much enhanced in comparison with expression of endogenous proteins.

In addition, the free mobility of recombinantly expressed GABA_AR $\alpha 1\beta 3\gamma 2s$ receptors found in HEK293 cells and their clustered distribution, suggest that clustering *per se*, is not the mechanism by which receptors are immobilized.

This study shows that constraints on the cell surface lateral mobility of GABA_ARs are specified by the GABA_AR $\alpha 1$ subunit. GABA_AR $\alpha 1$ subunits are, however, sequestered in an internal cellular compartment prior to their coassembly with GABA_AR β subunits. This is a novel function for a GABA_AR subunit and shows that in addition to their role in specifying receptor function, α subunit codes for receptor anchoring. In analogy with other ion channels concentrated at distinct neuronal membranes domains, cytoskeleton associated proteins may anchor GABA_ARs by linking to specific receptor subunits. In fact, the interaction of the GABA_AR $\alpha 1$ subunit with two main cytoskeletal components, tubulin and actin, has been proven by Kannenberg *et al.* (1997). They used the specific $\alpha 1$ subunit monoclonal antibody bd24, (Ewert *et al.*, 1990) to isolate proteins associated with GABA_ARs via coprecipitation with the receptors from solubilised calf brain membranes. In addition to the above mentioned proteins, tubulin and actin, at least three other intracellular GABA_ARs associated proteins were found.

There is emerging evidence that in spite of the high levels of specific GABA_AR subunit mRNAs expressed by individual neurons at specific times during development, the corresponding GABA_AR subunit polypeptide is not always expressed on the cell surface (Killisch *et al.*, 1991; Jones *et al.*, 1997). The

intracellular rescuing and sorting of the GABA_AR α 1 subunit by the GABA_AR β subunits suggest that the ability of neurons to control assembly and incorporate specific subunits from an internally sequestered pool might give rise to a population of GABA_ARs that are spatially segregated and immobilized on the cell surface. Thus, the composition and location of GABA_ARs expressed on the cell surface during development or plasticity-induced changes might depend on the temporal availability of a specific subunit required for assembly and may be controlled at the level of translation and/or subunit assembly, rather than at the transcriptional level. Indeed, studies have shown that cerebellar granule cells are able to modulate their expression of GABA_AR α 1 and α 6 subunit-containing receptors in response to cAMP-mediated signalling (Thompson *et al.*, 1996). The recruitment of specific subunits into complexes and their immobilisation at specific domains provides a mechanism by which receptors can be anchored at discrete sites on the neuron's cell-surface.

Chapter 4

Anchoring of GABA_AR: subunit specificity

Chapter Four

Anchoring of GABA_A receptors: subunit specificity***4.1. Introduction***

The vast array of GABA_AR α , β , and γ subunit isoforms, the biochemical identification of receptor subunit composition (McKernan and Whiting, 1996), allied with differential distribution of the GABA_AR α 1 (Zimprich *et al.*, 1991), α 3 (Turner *et al.*, 1993), α 5 (Richards *et al.*, 1987), and β 2/3 subunits (Nusser *et al.*, 1996; Richards *et al.*, 1987; Houser *et al.*, 1988) has raised the question of whether, in addition to creating physiological and pharmacological differences, the composition of the receptor might determine its distribution and maintenance on the nerve cell surface. Recent studies on cerebellar granule cells have shown that GABA_AR α 6 and α 1 subunit-containing receptors are co-localized at many GABAergic Golgi synapses (Somogyi *et al.*, 1989; Baude, *et al.*, 1992). However α 6-, but not α 1-containing receptors, are concentrated at glutamatergic mossy fiber synapses (Nusser *et al.*, 1996). These studies suggest that GABA_AR subunits can be differentially targeted to specific domains on the surface of the same neuron. In addition to differential targeting, GABA_ARs are also found to form clusters. As described in Chapter 3, recombinant GABA_ARs expressed in COS7, HEK293 and PC12 cells are found clustered at the cell surface. Likewise, GABA_ARs are found in clusters on cultured neurons, even in regions with no apparent synaptic contact (Caruncho *et al.*, 1993; Craig *et al.*, 1994). The clustering of recombinant GABA_ARs in non-neuronal cells suggests that this property might be encoded by the subunit composition.

It was shown in Chapter 3 that when $\alpha 1$ homo-oligomeric recombinant GABA_ARs were expressed in COS7, HEK293 or PC12 cells they were retained in the endoplasmic reticulum. GABA_AR $\beta 1$ and $\beta 3$ subunits, however, were sorted to the plasma membrane where they formed clusters. When GABA_AR $\alpha 1$ and $\beta 3$ subunits were co-expressed in these cells, the $\beta 3$ subunit rescued the intracellularly restrained GABA_AR $\alpha 1$ subunits allowing the transport of the GABA_AR $\alpha 1\beta 3$ complex to the cell surface where they formed co-localized clusters. Inclusion of $\alpha 1$ in $\beta 3$ or $\beta 3\gamma 2s$ complexes, however, dramatically reduces the receptor's lateral mobility and anchors GABA_ARs on the cell surface suggesting the formation of a direct link to a component of the cytoskeleton (Peran *et al.*, submitted). These results imply that the differences in mobility might be related to the GABA_AR's composition possibly encoded by the α -subunit included in the complex. And raise the question as to whether the $\beta 3$ subunit might serve to cluster or aggregate receptors while α -subunits provide those links for anchoring.

The work described in this chapter aimed to address these issues. Thus, a range of recombinant GABA_ARs of the form $\alpha x\beta 3\gamma 2s$, where $x = 1, 2, 3, 4, 5$ or 6 , were expressed in COS7 and HEK293 cells. The lateral mobilities of these recombinant receptors were subsequently analysed by FPR. In addition, the lateral mobility of endogenous GABA_ARs was measured in cultured rat cerebellar granule cells. These studies enabled a direct comparison between defined recombinant receptors in transfected cells and native receptors in neurons, to reveal whether differences in mobility of the receptor are linked to its subunit

composition and whether the neuron imposes additional restraints on their mobility.

4.2. Results

In order to examine the role that each of the GABA_AR α subunits plays in GABA_AR receptor dynamics, the lateral mobility of recombinant GABA_ARs, in which each of the six different GABA_AR α subunits were co-expressed with GABA_AR β 3 and γ 2s subunits in HEK293 and COS7 cells was measured by FPR (methods, 2.2.7.). For cell transfection see methods 2.2.3. Experiments performed on recombinant receptors expressed in non-neuronal cells were followed by studies on native GABA_AR α 1 and α 6 subunit-containing receptors expressed in rat cerebellar granule cells. Ten to twenty recordings were made on each cell preparation. Furthermore, experiments were repeated at different days post-transfection with little variation in the receptor mobility as shown in the following figures. Results presented in Figure 4.1. are for HEK293 cells, in Figure 4.2. for COS7 cells and in Figure 4.3. for cerebellar granule cells.

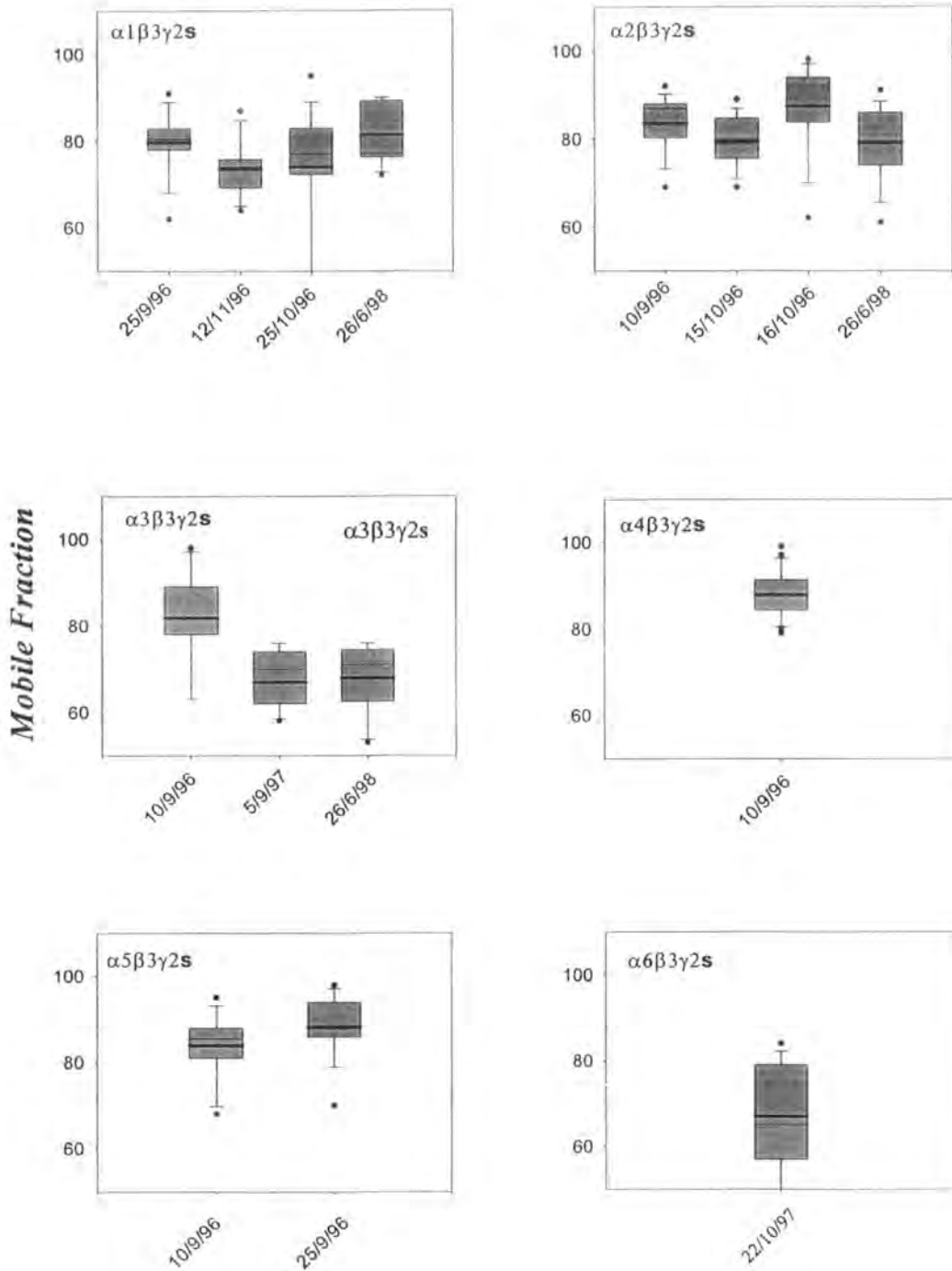


Figure 4.1. The mobile fraction (% Recovery) of recombinant GABA_A α(1–6)β3γ2s receptors expressed in HEK293 cells was determined, at different days, by FPR as described in methods, 2.2.7. The boundaries of the boxes closest to, and furthest from, zero indicate the 25th and 75th percentiles. The thin and thick lines with the box mark the median and mean of the data, respectively. Bars above and below the box indicate the 90th and 10th percentiles. Outlying points are also indicated.

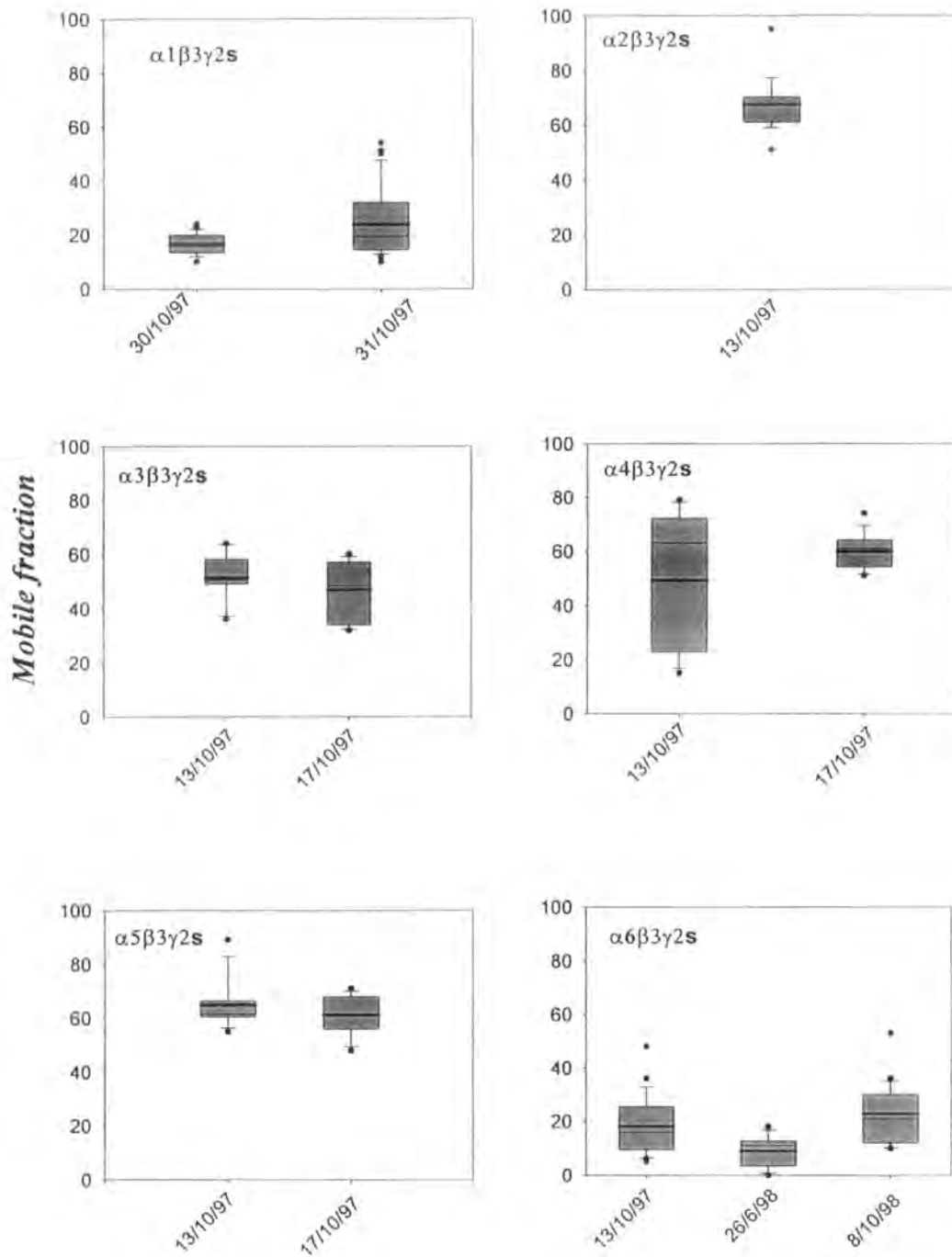


Figure 4.2. The mobile fraction (% Recovery) of recombinant GABA_A $\alpha(1-6)\beta 3\gamma 2s$ receptors expressed in COS7 cells was determined, at different days, by FPR as described in methods, 2.2.7. The boundaries of the boxes closest to, and furthest from, zero indicate the 25th and 75th percentiles. The thin and thick lines with the box mark the median and mean of the data, respectively. Bars above and below the box indicate the 90th and 10th percentiles. Outlying points are also indicated.

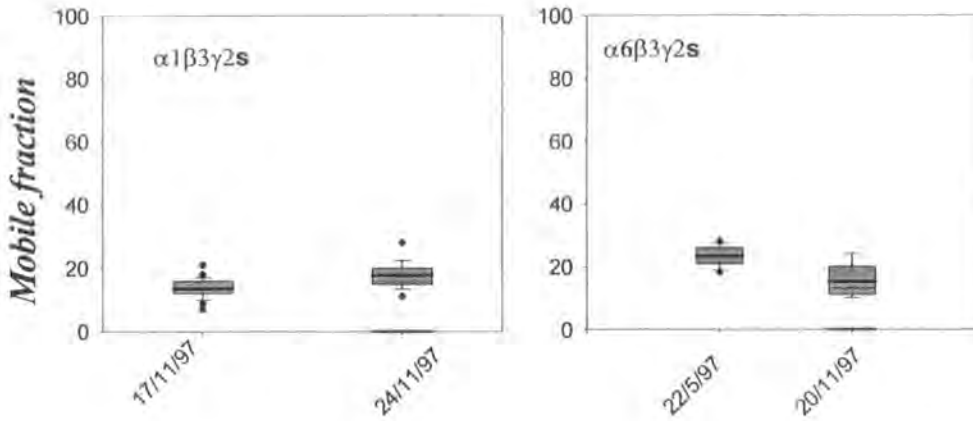


Figure 4.3. The mobile fraction (% Recovery) of native GABA_AR $\alpha 1$ subunit-containing and GABA_AR $\alpha 6$ subunit-containing receptors expressed in primary cultured rat cerebellar granule cells was measured by FPR as described in methods, 2.2.7. Four different cultures of cerebellar granule cells assayed at 7-9 days in vitro are presented. 10-20 cells were analysed per culture. The boundaries of the boxes closest to, and furthest from zero indicate the 25th and 75th percentiles. The thin and thick lines with the box mark the median and mean of the data, respectively. Bars above and below the box indicate the 90th and 10th percentiles. Outlying points are also indicated.

4.2.1. Lateral mobility of recombinant GABA_AR complexes containing different α subunits expressed in COS7 and HEK293

The lateral mobility coefficients of recombinant GABA_ARs, of the form of $\alpha x \beta 3 \gamma 2s$, where $x = 1, 2, 3, 4, 5$ or 6 , expressed in HEK293 cells and COS7 cells were determined by FPR. The results are summarised in Tables 4.1. and 4.2. respectively. Recombinant GABA_A receptors comprising the $\alpha(1, 2, 3$ or $5) \beta 3 \gamma 2s$ combinations are benzodiazepine agonist sensitive and therefore were amenable to labelling with the fluorescent benzodiazepine-derivative, Bodipy-Ro-1986. GABA_A receptor $\alpha 6$ subunit-containing receptors, which are insensitive to benzodiazepine-agonists, were labelled with Bodipy-Fab' fragments of the $\alpha 6$ subunit-specific antibody (methods 2.2.6.). In addition, GABA_A receptor $\alpha 1$ subunit-containing receptors were also studied with Fab' fragments of the $\alpha 1$ subunit-specific antibody, the monoclonal antibody, bd24. This antibody which specifically recognizes the bovine and human GABA_AR $\alpha 1$ subunit was digested with papain generating the Fab' fragments (methods 2.2.5). This was required to avoid crosslinking of $\alpha 1$ subunit-containing receptors by the bifunctional, intact antibody, in the FPR experiments. The principal sites of papain cleavage are found on the amino-terminal side of the disulfide bonds that hold the two heavy chains of the antibody molecule together. Therefore, digestion with papain releases two antigen binding domains and one Fc fragment.

The results obtained when GABA_A receptors were expressed in HEK293 cells (Table 4.1.), showed that all GABA_A receptor complexes, irrespective of the α subunit isoform that was transiently co-expressed with $\beta 3$ and $\gamma 2s$ subunits, were mobile and encountered little, if any, restriction to their cell surface mobility. The

recovery curves obtained for every cell preparation analysed demonstrated that approximately 80% of the labelled receptors were diffusely distributed and freely mobile with diffusion coefficients of the order of 10^{-10} cm²/s.

Table 4.1. FPR measurements of recombinant GABA_ARs expressed in HEK293 cells

<i>GABA_AR</i> - subunits transfected	<i>Label</i>	<i>Mobile Fraction (%)</i>	<i>Diffusion Coef. ($\times 10^{-10}$ cm²/sec)</i>	<i>n</i>
$\alpha 1\beta 3\gamma 2s$	Bodipy-Ro-1986	74+/-15	2.6+/-0.9	15
$\alpha 1\beta 3\gamma 2s$	Fab'- $\alpha 1$	74+/-12	2.6+/-0.5	11
$\alpha 2\beta 3\gamma 2s$	Bodipy-Ro-1986	79+/-6	5+/-1.5	15
$\alpha 3\beta 3\gamma 2s$	Bodipy-Ro-1986	82+/-10	5.84+/-1.9	14
$\alpha 4\beta 3\gamma 2s$	Bodipy-Ro-1986	88+/-6	5+/-1	17
$\alpha 5\beta 3\gamma 2s$	Bodipy-Ro-1986	84+/-8	4+/-1.8	14
$\alpha 6\beta 3\gamma 2s$	Fab'- $\alpha 6$	67+/-12	0.8+/-0.2	14

However, when GABA_A receptors were expressed in COS7 cells, they showed differential lateral mobilities depending upon which GABA_AR α subunit isoform was expressed in the complex (Table 4.2.). Approximately 80% of both $\alpha 1$ and $\alpha 6$ subunit-containing receptors were immobile in COS7 cells. In contrast, $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\alpha 5$ subunit-containing receptors were relatively mobile. The lateral mobility coefficients of these receptors paralleled those found when they were expressed in HEK293 cells. The relative immobility of GABA_AR $\alpha 1$ subunit-containing receptors in COS7 cells was confirmed by labelling with Fab' fragments of the GABA_AR $\alpha 1$ subunit-specific antibody.

Table 4.2. FPR measurements of recombinant GABA_AR_s expressed in COS7 cells

<i>GABA_AR</i> - <i>subunits</i> <i>transfected</i>	<i>Label</i>	<i>Mobile</i> <i>Fraction</i> <i>(%)</i>	<i>Diffusion Coef.</i> <i>(x10⁻¹⁰ cm²/sec)</i>	<i>n</i>
α1β3γ2s	Bodipy-Ro-1986	17+/-4	0	24
α1β3γ2s	Fab'-α1	17+/-5	0	10
α2β3γ2s	Bodipy-Ro-1986	67+/-10	1.4+/-0.9	13
α3β3γ2s	Bodipy-Ro-1986	49+/-10	1+/-0.6	16
α4β3γ2s	Bodipy-Ro-1986	60+/-7	0.5+/-0.1	10
α5β3γ2s	Bodipy-Ro-1986	63+/-9	0.6+/-0.2	16
α6β3γ2s	Fab'-α6	18+/-11	0	19

Mobile fractions or percentage of recovery of the order of 20% can be considered to reflect total receptor immobility, see rationale in Methods (2.2.7.3.).

The lateral mobility coefficients of GABA_AR α2, α3, α4 and α5 subunit-containing receptors are typical of most membrane glycoproteins, which have diffusion coefficients in the range of 10⁻¹⁰ - 10⁻¹¹ cm²/s but is different from the free and rapid mobility of membrane lipids (x10⁻⁹ cm²/s). The reduced rate of lateral movement of the α4 and α5 subunit-containing receptors (in the range of 10⁻¹¹ cm²/s) relative to other α subunit-containing receptors, might be a consequence of the long cytoplasmic loops between transmembrane domains 3 and 4 of the α4 and α5 subunits, that might encounter cytoplasmic barriers limiting the rate of diffusion.

To illustrate the differences between the membrane dynamics of the recombinantly expressed receptors in HEK293 and COS7 cells, the mobile fraction (% Recovery) of expressed receptors are compared (Figure 4.4.). It is

clear that in every experiment performed the fraction of expressed receptor able to move in HEK293 was always higher than in COS7 cells.

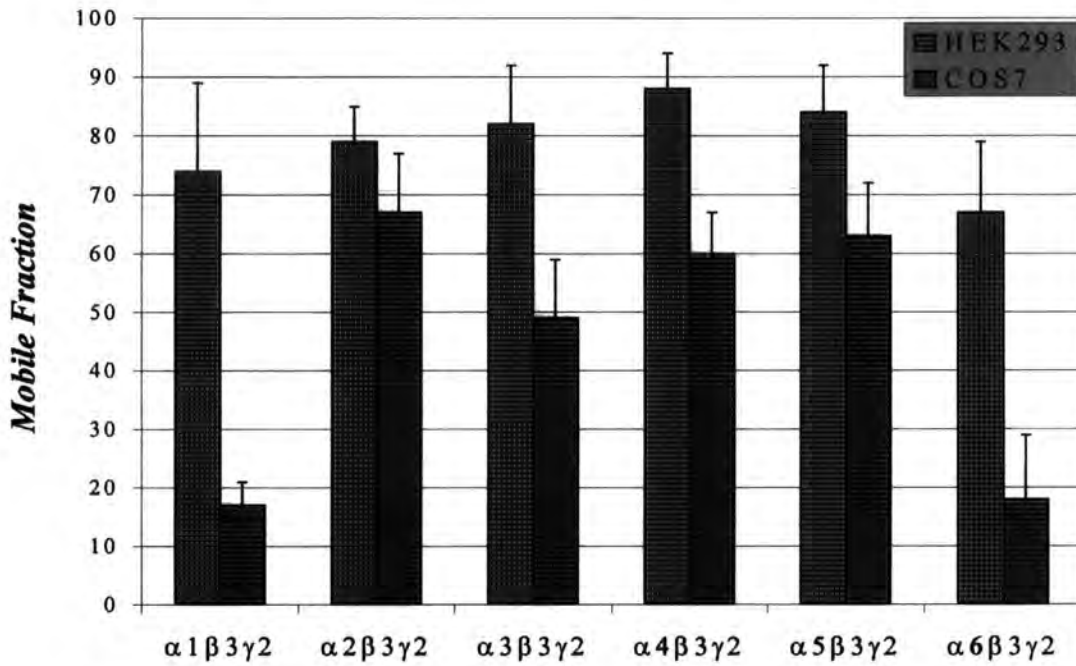


Figure 4.4. Histogram comparing the mobile fraction (% Recovery) of recombinant GABA_ARs in HEK293 and COS7 cells.



4.2.2. Mobility of native GABA_ARs

FPR measurements of recombinant GABA_A receptors expressed in both HEK293 and COS7 cells provide insights into the mechanisms, intrinsic to the α subunit isoform introduced into the receptor complex, that contribute to the modulation of the receptor's cell surface dynamics. However, in addition to the intrinsic dynamic properties that are endowed by the subunit's amino-acid sequence, revealed by expressing different α subunit-containing receptors in non-neuronal cells, additional neuron specific mechanisms that exploit the molecular differences between the subunits are likely. Measurements of the lateral mobility of native GABA_A $\alpha 1$ and $\alpha 6$ subunit-containing receptors expressed in cultured cerebellar granule cells showed that nearly all of these receptors were immobile (see rationale 2.2.7.3.) (Table 4.3.). Cerebellar granule cells express $\alpha 1$, $\alpha 6$, and $\alpha 1\alpha 6$ subunit-containing receptors (Pollard *et al.*, 1995; Khan *et al.*, 1996, Jechlinger *et al.*, 1998). The use of Bodipy-Ro-1986 to label the cells do not report the individual contributions of the $\alpha 1$ or $\alpha 6$ subunits to the receptor mobilities. A recent report based upon the direct binding of flunitrazepam to recombinant $\alpha 1$ and $\alpha 6$ subunit-containing receptors showed that both receptor types bind flunitrazepam (a benzodiazepine full-agonist) with similar affinity (Hauser *et al.*, 1997). At the concentration of the fluorescent benzodiazepine probe, Bodipy-Ro-1986, used (20 nM) in these experiments, it would be expected that the mobilities of both $\alpha 1$ and $\alpha 6$ subunit-containing receptors in cerebellar granule cells were measured. To learn more about the contribution of each of these subunits to the mobility of the complexes, the mobility of $\alpha 6$ subunit-containing receptors was examined further, using $\alpha 6$ subunit-specific antibody

Fab' fragments. The results of these FPR experiments showed no recovery of the fluorescence following photobleach (Table 4.3.), indicating that $\alpha 6$ subunit-containing receptors are immobile in cerebellar granule cells. Studies have reported that 45% of GABA_ARs in cerebellar extracts contain $\alpha 6$ subunits (Jechlinger *et al.*, 1998) in agreement with Pollard *et al.* (1995), Khan *et al.* (1996) and Jones *et al.* (1997). Thus, by using $\alpha 6$ subunit-specific Fab' fragments as a probe, almost half of the total number of GABA_ARs expressed by the cerebellar granule cells were tested and these receptors were immobile on the cell surface. By inference from the results obtained when cells were labelled with Bodipy-Ro-1986, $\alpha 1$ subunit-containing receptors expressed by cerebellar granule cells must be also immobile.

Table 4.3. FPR measurements of native GABA_ARs in cerebellar granule cells

<i>Label</i>	<i>Mobile Fraction (%)</i>	<i>n</i>
Bodipy-Ro-1986	18+/-4	13
Fab'- $\alpha 6$	23+/-3	13

4.2.3. Is receptor clustering responsible for differences in receptor dynamics?

It was considered that the reported differences in the lateral mobilities of GABA_ARs comprising different α subunits might be a result of varying degrees of receptor clustering. A Pearson's correlation analysis (2.2.8.) was carried out to study the relationship between the fluorescent intensity (number of counts recorded) immediately prior to the bleach (PC) with the mobile fraction (MF) and diffusion coefficients (DC) for each experiments. No significant correlation ($\alpha=0.05$) was found for any of the measurements performed on cerebellar granule

cells (Table 4.4.), HEK293 cells (Table 4.5.) nor for COS7 cells (Table 4.6.).

Therefore the above possibility was rejected.

Table 4.4. Pearson's correlation analysis for FPR measurements of GABA_A receptors expressed in cultured cerebellar granule cells

<i>Native GABA_AR labelling</i>	<i>PC/MF</i>	<i>PC/DC</i>	<i>n</i>
Bodipy-Ro-1986	0.045	–	24
Fab'-α6	-0.48	–	19

Table 4.5. Pearson's correlation analysis for FPR measurements in recombinant GABA_A receptors expressed in HEK293 cells

<i>GABA_AR-subunit transfected</i>	<i>PC/MF</i>	<i>PC/DC</i>	<i>n</i>
α1β3γ2s	0.045	-0.25	24
α2β3γ2s	0.38	-0.18	13
α3β3γ2s	-0.004	-0.2	16
α4β3γ2s	-0.1	-0.15	10
α5β3γ2s	-0.25	-0.42	16
α6β3γ2s	-0.48	-0.38	19

Table 4.6. Pearson's correlation analysis for FPR measurements in recombinant GABA_A receptors expressed in COS7 cells

<i>GABA_AR-subunit transfected</i>	<i>PC/MF</i>	<i>PC/DC</i>	<i>n</i>
α1β3γ2s	-0.07	–	24
α2β3γ2s	-0.31	-0.23	13
α3β3γ2s	-0.37	-0.07	16
α4β3γ2s	0.03	-0.5	10
α5β3γ2s	-0.4	-0.58	16
α6β3γ2s	-0.53	–	19

4.2.4. Comparison of the predicted secondary structures

Network protein sequence @analysis at IBCP, France (methods 2.2.9.), was used to compare the predicted secondary structures of the cytoplasmic domains of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ subunits. No common feature attributable to $\alpha 1$ and $\alpha 6$ subunits that discriminated them from the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ subunits was found. Each of the cytoplasmic domains are characterised by short regions of α -helical structure as the cytoplasmic loop emerges from M3 and a high degree of coil structure toward the C-terminal region of the domain as it enters into M4.

4.3. Discussion

The FPR results presented here on recombinant and native GABA_A receptors reveal that GABA_AR mobility at the cell surface is partly an intrinsic property of the α -subunit isoform expressed in the receptor complex. While the global and cellular distribution of α subunit-containing receptors have been reported (reviewed in Stephenson, 1995; Darlinson and Albrecht, 1995), little information has emerged regarding the mechanisms that contribute to the differential localisation of α subunit-containing receptors on neurons. It is known that some neurons express several α subunit isoforms which in some cases co-exist within the same receptor complex, e.g. $\alpha 1\alpha 6$ subunit-containing receptors in the cerebellar granule cells (Pollard *et al.*, 1995; Khan *et al.*, 1996 and Jechlinger *et al.*, 1998 also reviewed in Darlinson and Albrecht, 1995). Furthermore, when $\beta 3\gamma 2s$ subunit-containing complexes were coexpressed with the $\alpha 1$ subunit in COS7 and PC12 cells, the receptors were immobile (see Chapter 3). In this chapter, it has been shown that the $\alpha 6$ subunit also confers receptor immobility when expressed with $\beta 3\gamma 2$ subunits in COS7 and when analysed in primary cultured cerebellar granule cells. In contrast, recombinant GABA_A receptors comprising $\alpha 2-5$ subunits expressed with $\beta 3$ and $\gamma 2s$ subunits in COS7 cells were shown to be freely mobile on the cell surface. These results suggest that the $\beta 3$ subunit serves to cluster or aggregate receptors while in recombinant receptors expressed in COS7 cells and native receptors in cerebellar granule cells, the $\alpha 1$ and $\alpha 6$ subunits provide links, presumably to cytoskeletal elements, to anchor the receptors. HEK293 cells, on the other hand, seem to lack the essential elements required to immobilize GABA_AR $\alpha 1$ or $\alpha 6$ subunit-containing receptors, even

though the $\alpha 1$ and $\alpha 6$ subunit-containing receptors still form clusters on the cell surface (see Chapter 3 for $\alpha 1$ -containing receptors). Although certain subunit-combinations are freely mobile when expressed in HEK293 and COS7 cells this is not proof positive that these subunits are mobile when they are expressed in specific neurons. However, what these results do indicate is that the mechanism by which the $\alpha 1$ and $\alpha 6$ subunit-containing receptors are immobilised is distinct from that which might regulate the cell surface dynamics of $\alpha 2$ - $\alpha 5$ subunit-containing GABA_AR, and extends to those neurons, cerebellar granule cells, which express $\alpha 1$ and $\alpha 6$ subunit-containing receptors.

The immobility of recombinant $\alpha 1\beta 3\gamma 2s$ (Type I) receptors and the relative mobility of $\alpha(2-5)\beta 3\gamma 2s$ (Type II) receptors in these experiments correlates with the reported differences in Triton-X-100 extractability of Type I and Type II benzodiazepine receptors (Lo *et al.*, 1982). The α subunit variants provide the structural basis for Type I and Type II benzodiazepine receptors (Caruncho *et al.*, 1993; Pritchett *et al.*, 1989b). Type II receptors, to which $\alpha 2\beta(1/2/3)\gamma 2$, $\alpha 3\beta(1/2/3)\gamma 2$, and $\alpha 5\beta(1/2/3)\gamma 2$ belong (Pritchett *et al.*, 1989b; Hadingham *et al.*, 1993), are Triton X-100-soluble and are thought, on this basis, not to be attached to the underlying cytoskeleton. It is conceivable that mechanisms other than direct coupling to cytoskeletal elements, such as a subplasmalemmal cytoskeletal barrier, may serve to compartmentalize $\alpha 2$ - $\alpha 5$ subunit-containing receptors. On the other hand, Type I receptors, to which $\alpha 1\beta(1/2/3)\gamma 2$ belongs are found in Triton X-100 insoluble fractions suggesting that they are associated with the cytoskeleton, which are often insoluble in nondenaturing detergents.

One of the striking features of GABA_AR α subunit isoforms is their high level of primary amino acid sequence homology. What features of the α -subunit, therefore, might contribute to their differential mobilities when measured by FPR? Is it possible to extract any information about the putative mechanisms by which the $\alpha 1$ and $\alpha 6$ subunit-containing receptors might be anchored based upon similarities and differences between α -subunit sequences? Comparison of the primary amino acid sequence of the receptor subunits shows that they are highly homologous in the transmembrane and N- and C-terminal regions but differ most in the cytoplasmic loop between transmembrane domains 3 and 4, or M3/M4. Comparison of the primary sequences in this region of the sequence of the $\alpha 1$ and $\alpha 6$ subunits, did not reveal any common element that might be responsible for receptor tethering at the cell surface. Similarly, there are no distinguishing features that differentiate the $\alpha 1$ or $\alpha 6$ subunit cytoplasmic loop domains from those of the $\alpha 2$, $\alpha 3$, $\alpha 4$ or $\alpha 5$ subunits, other than the obvious differences in length and charge. The primary sequence, however, is unlikely to reveal subunit-specific elements that might link receptors to the cytoskeleton. Interactions with the cytoskeleton have been shown to be complex (Sheets *et al.*, 1995; Kusumi and Sako, 1996) and involve several interactions between regions of defined secondary, tertiary, or even quaternary structure. Again, comparison of the predicted secondary structures showed no common feature attributable to $\alpha 1$ and $\alpha 6$ subunits that discriminated them from the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ subunits that might account for their differential mobilities. The failure to find any common motif in the secondary structure, however, is not unexpected since it is known that the binding of cytoskeletal proteins to membrane proteins involves interactions

between many specific motifs, for instance the binding of dystrophin to the dystrophin related glycoproteins (Suzuki *et al.*, 1994, Suzuki *et al.*, 1995), rapsyn to the AchR through the Zn⁺² finger domains (Scotland *et al.*, 1993), gephyrin to the glycine receptor (Kirsch *et al.*, 1991; Kirsch and Betz, 1993) and the concentration of voltage-gated K⁺ channels by the PSD-95 family of membrane-associated putative guanylate kinases (Kim *et al.*, 1995). Each of these protein-protein interactions involves several regions of the protein. The GABA_A receptor is probably not an exception, interaction of the $\alpha 1$ subunit, for instance, with its associating protein(s) is likely to be conferred by the acquisition of both tertiary structure and quaternary structure following assembly with β and/or γ subunits. It is not known whether, for those receptors that contain more than one type of α subunit, one of the α subunits dominates in determining the mechanism of compartmentalisation of the receptor. In large part the immobilisation of a receptor is likely to be determined by those cytoskeletal structures that are found in different parts of the neuron as well as the specific subunit composition of the receptor. In this sense the differential localisation of the $\alpha 1$ and $\alpha 6$ subunit-containing receptors in cerebellar granule cells might result from the differential targeting of specific cytoskeletal elements that dictates the placement of the subunit rather than differential targeting of the subunit itself.

In neurons that express two or more α subunits, are the same or different mechanisms used to cluster and segregate receptors? Immunocytochemistry of cerebellar granule cells, *in situ*, shows that the GABA_AR $\alpha 1$ and $\alpha 6$ subunits can be found at the same synapse (Nusser *et al.*, 1996). In cerebellar granule cells *in vitro* the clustered $\alpha 1$ and $\alpha 6$ subunit-containing receptors are co-localized in the

same domain at synaptophysin positive sites (Gao and Fritschy, 1995). There is biochemical evidence from immunoprecipitation studies that support and argue against the coassembly of both $\alpha 1$ and $\alpha 6$ subunits within the same GABA_AR complex (Quirk *et al.*, 1994, Pollard *et al.*, 1995; Khan *et al.*, 1996 and Jechlinger *et al.*, 1998). Double-immunolabelling studies of clustered receptors on cultured cerebellar granule cell surfaces suggests that these subunits do not co-assemble in the same receptor complex (Caruncho *et al.*, 1994). In this chapter, it has been shown, that these different receptors containing either the $\alpha 1$ or $\alpha 6$ subunits have no lateral mobility when measured by FPR.

There has been considerable interest in trying to identify those cytoskeletal proteins that interact with GABA receptors and specify their localisation in neurons. Gephyrin has been reported to be involved in the clustering and postsynaptic positioning of GABA_ARs (Koulen *et al.*, 1996), with the γ subunit specifying gephyrin-induced clustering (Essrich *et al.*, 1998). Recently, Rapsyn, the 43kD protein associated with the nicotinic acetylcholine receptor, has been reported to anchor recombinant GABA_ARs that are composed of $\alpha 1$, $\beta 1$, and $\gamma 2s$ subunits in transfected cells (Yang *et al.*, 1997). However, it is known that these proteins, like all cytoskeletal proteins, associate non-specifically. It is therefore not known whether these proteins, although associating, contribute to the immobilisation of the α subunit-containing receptors *in vivo*. It was reported that erythrocyte ankyrin did not bind to purified GABA_ARs (Srinivasan *et al.*, 1988). Although there are several ankyrin genes and spliced isoforms each of which have different membrane protein specificity. Thus, it is possible that there are ankyrin isoforms, ankyrin-like proteins, or new undiscovered proteins that associate with GABA_ARs with specific subunit compositions. Recently, a new

cellular protein has been identified, the GABA_A receptor-associated protein (GABARAP) which interact with the $\gamma 2$ subunit of the GABA_AR and links the complex to the cytoskeleton (Wang *et al.*, 1999)

The studies reported here reveal important differences in the dynamic behaviour of $\alpha 1$ and $\alpha 6$ subunit-containing receptors in non-neuronal and neuronal cells. Examination of the mobilities of recombinant receptors by FPR is important because, in contrast to *in vitro* reconstitution methods that report on the association of proteins but do not establish that these interactions contribute to the cellular localisation of the receptor, FPR allow the direct reconstitution of the cellular function by measuring the immobilisation of the receptor when the specific subunit containing receptors are expressed in neuronal cells or when co-expressed with specific cytoskeletal elements

Chapter 5

*The M3/M4 cytoplasmic loop of $\alpha 1$
subunits are linked to immobilisation of*

GABA_ARs

Chapter Five

***The M3/M4 cytoplasmic loop of $\alpha 1$ subunits are linked to
immobilisation of GABA_ARS***

5.1. Introduction

Receptors to glycine, acetylcholine, γ -aminobutyric acid (GABA) and glutamate have all been shown to be clustered on neuronal postsynaptic membranes, although they are also present at extrasynaptic plasma membrane sites (Triller *et al.*, 1985; Somogyi *et al.*, 1989; Petralia and Wenthold, 1992; Baude *et al.*, 1993; Craig *et al.*, 1993). Their precise targeting to domains opposing appropriate presynaptic terminals implies a great degree of organisation and specificity. It remains to be established whether the neurotransmitter receptors are edited and routed to their final postsynaptic domains or whether a freely mobile pool of receptors is maintained on the cell surface that is recruited to a specific locality in response to neuronal demands.

The previous chapters have provided evidence that there are differences in the lateral mobilities of GABA_ARs containing specific α subunit isoforms. The mobilities of recombinant $\alpha 1$ and $\alpha 6$ subunit-containing $\alpha\beta 3\gamma 2$ receptors differ from those comprising $\alpha 2$, $\alpha 3$, $\alpha 4$ or $\alpha 5$ subunit-containing $\alpha\beta 3\gamma 2$ receptors when expressed in COS7 cells. The signals that specify mobility might be encoded in the primary sequence of the α subunit, although comparison of the primary sequences reveals no common motif in the $\alpha 1$ and $\alpha 6$ subunits that would account for their relative immobility. Recently, the intracellular domains of some of the subunits of glycine and NMDA receptors have been implicated in the spatially distinct clustering of the complex (Kirsch *et al.*, 1993; Kornau *et al.*, 1995;

Meyer *et al.*, 1995; Ehlers *et al.*, 1996). Because the major amino acid sequence divergence between α subunit isoforms is found in the M3/M4 cytoplasmic domain, it was hypothesised that this region might potentially mediate α -subunit specific associations with the cytoskeleton and/or confine receptors to specific neuronal domains. Furthermore, the M3/M4 cytoplasmic loop of the $\alpha 1$ subunit might be responsible for the retention of recombinant $\alpha 1$ subunit homo-oligomers in an intracellular compartment of HEK293 and COS7 cells, as reported in Chapter 3.

In this chapter the role that the M3/M4 cytoplasmic loop of the α subunit plays in controlling receptor mobility, assembly and localisation has been explored. To do so a series of chimeric $\alpha 1$ subunits were constructed.

The first approach to determine whether the M3/M4 cytoplasmic loop of the $\alpha 1$ subunit regulates receptor mobility was to transiently express $\alpha 1$ subunits, which had been engineered such that the M3/M4 loop was deleted ($\alpha 1_{CD}$, see methods 2.2.2.10.), together with the $\beta 3$ and $\gamma 2s$ subunits in COS7 cells. The lateral mobility of these $\alpha 1_{CD}\beta 3\gamma 2s$ receptors was determined by FPR (2.2.7.), and compared to the lateral mobility coefficient of $\alpha 1\beta 3\gamma 2s$ receptors. Furthermore, the mobilities of recombinant receptors containing $\beta 3\gamma 2s$ subunits coexpressed with chimeric $\alpha 1$ subunits, where the M3/M4 cytoplasmic loop was replaced or exchanged with the M3/M4 cytoplasmic loops of $\alpha 2$, $\alpha 3$ and $\alpha 6$ subunits ($\alpha_{CH-\alpha(x=2,3,6)}$, see methods 2.2.2.10.) were also analysed. The lateral mobilities of these recombinant constructs would reveal whether the α subunit-specific M3/M4 cytoplasmic loops were involved in the conferring of their unique differential mobilities to what were otherwise essentially $\alpha 1\beta 3\gamma 2s$

receptors. The final aim of this chapter was to attempt to gain insight into the role that the α subunit M3/M4 cytoplasmic loop domain plays in the process of receptor trafficking. Thus, chimeric $\alpha 1$ subunits were transiently transfected into COS7 and HEK293 cells. The final localisation of the expressed receptors was subsequently determined by conventional immunocytochemical approaches (methods, 2.2.4.)

5.2. Results

5.2.1. Removal of the M3/M4 cytoplasmic domain of the $\alpha 1$ subunit releases the lateral constraints imposed on GABA_AR mobility

In the previous chapter recombinant $\alpha 1\beta 3\gamma 2s$ and $\alpha 6\beta 3\gamma 2s$ receptors were transiently expressed in COS7 cells and using Bodipy-Ro-1989 as an FPR probe, shown to have restricted lateral mobilities relative to those receptors comprising the combinations $\alpha 2$, $\alpha 3$, $\alpha 4$ or $\alpha 5\beta 3\gamma 2s$. If the M3/M4 cytoplasmic loop of the $\alpha 1$ subunit played a part in the interaction of the subunit with a component of the cytoskeleton, then expression of an $\alpha 1$ subunit in which this domain had been excised might be expected to be freely mobile in the membrane. To examine the role that the M3/M4 cytoplasmic loop of the $\alpha 1$ subunit plays in controlling receptor tethering, the rate of mobility of receptors comprising the truncated $\alpha 1$ subunits, that lacked the M3/M4 loop domain, $\alpha 1_{CD}$, was measured and compared with normal and also chimeric $\alpha 1$ subunit, $\alpha 1_{CH}$ -containing receptors. The chimeric $\alpha 1$ subunit was engineered such that the rat $\alpha 1$ subunit cDNA, used in the previous studies, now consisted of the 5' end of the bovine $\alpha 1$ subunit cDNA ligated to the 3' end of the rat $\alpha 1$ subunit cDNA. This construct results in a translated $\alpha 1$ subunit that has an amino acid sequence that is identical to that of the wild-type rat $\alpha 1$ subunit with the sole exception of a single amino acid at the N-terminus that is an essential component of the epitope for the monoclonal antibody bd 24 (Ewert *et al.*, 1990, see 2.2.2.10.). This enabled analysis of $\alpha 1$ subunit-mobility by FPR using the Bodipy-labelled anti- $\alpha 1$ subunit-specific antibody (2.2.5. and 2.2.6.). All subsequent experiments were performed using this construct. The $\alpha 1_{CH}$ was also used to construct the excision construct, $\alpha 1_{CD}$

and the cytoplasmic loop domain exchange constructs, $\alpha_{CH-\alpha x(x=2,3,6)}$. (2.2.2.10.). COS7 and HEK293 cells were transfected (methods, 2.2.3.) with $\beta 3$ and $\gamma 2s$ subunit cDNAs together with either the chimeric $\alpha 1_{CH}$ subunit cDNA construct or the truncated $\alpha 1_{CD}$ subunit construct. The mobility of the expressed receptors was measured by FPR (see methods 2.2.7.). Transfected cells were labelled live with the fluorescent benzodiazepine Bodipy-Ro-1986 (Figure 5.1.) and with a Bodipy-labelled Fab' fragment of the monoclonal antibody, bd24 (Figure 5.2.).

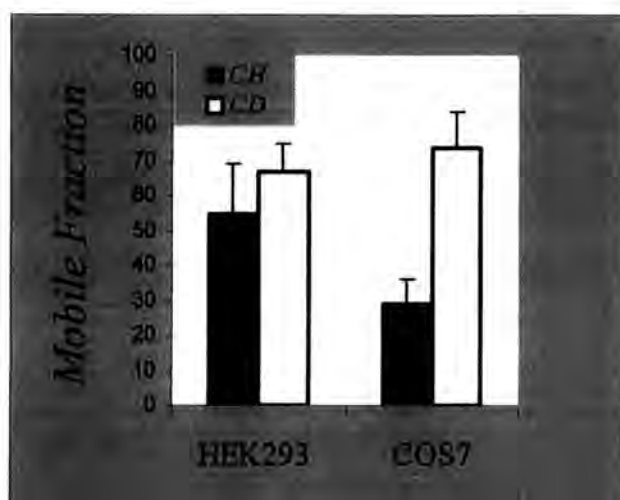


Figure 5.1. Histogram showing the relative mobile fraction (% Recovery) of recombinant $GABA_A$ R $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ expressed in HEK293 and COS7 cells. Receptors were probed with Bodipy-Ro-1986. Recombinant $GABA_A$ receptors, $\alpha 1_{CH}\beta 3\gamma 2s$ (CH) and $\alpha 1_{CD}\beta 3\gamma 2s$ (CD) were transiently expressed in HEK293 and COS7 cells and subsequently labelled with Bodipy-Ro-1986 (40 nM). The mobile fraction (% Recovery) was determined by FPR as described in methods (2.2.7.) (n=15-20)

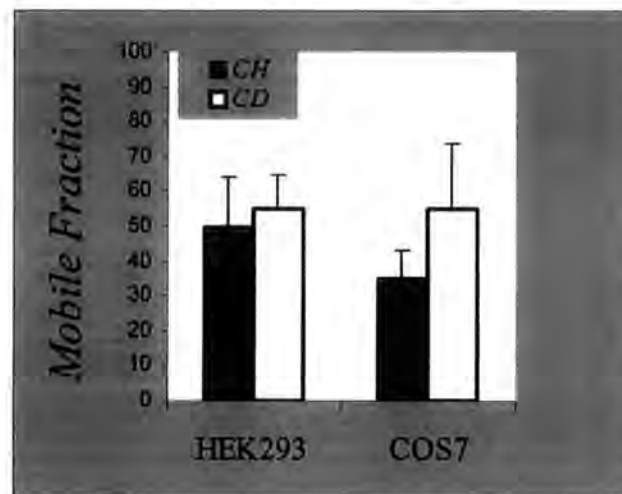


Figure 5.2. Histogram showing the relative mobile fraction (% Recovery) of recombinant GABA_AR $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ expressed in HEK293 and COS7 cells. Receptors were probed with Bodipy-Fab'-bd24. Recombinant GABA_A receptors, $\alpha 1_{CH}\beta 3\gamma 2s$ (CH) and $\alpha 1_{CD}\beta 3\gamma 2s$ (CD) were transiently expressed in HEK293 and COS7 cells and subsequently labelled with Bodipy-tagged Fab' fragments of the monoclonal antibody, bd24. The mobile fraction (% Recovery) was determined by FPR as described in methods (2.2.7.)(n=15-20)

Recombinant GABA_ARs, $\alpha 1_{CH}\beta 3\gamma 2s$ (CH, control) and $\alpha 1_{CD}\beta 3\gamma 2s$ (CD, $\alpha 1$ subunit with the M3/M4 cytoplasmic loop excised) were expressed in HEK293 cells and analysed by FPR following labelling with either Bodipy-Ro1989 (labels assembled receptors, Figure 5.1.) or Bodipy-Fab' bd 24 (labels $\alpha 1$ subunit, Figure 5.2.). No statistically significant difference in the mobile fraction (% Recovery) of control (CH) and truncated (CD) receptors was observed using either probes (2.2.8.). Using Bodipy-Ro1989, the mobile fraction for CH was 55 (± 14)% and for CD 67 (± 7)%, when these receptors were probed with Bodipy-Fab' bd24 these values were 50 (± 14)% for CH and 55 (± 8)% for CD (Figure 5.1.).

These values are similar to those obtained with the wild type $\alpha 1$ subunit (see Chapter 3 and 4).

In contrast, when the same receptors were expressed in COS7 cells, a statistically significant difference in the mobilities of CH and CD were observed (2.2.8.). Using the 'assembled receptor' probe, Bodipy-Ro1989, only 29 (± 8)% of CH receptors were freely mobile, whilst 75 (± 10)% of CD receptors were untethered (Fig. 5.1.). These values were 35 (± 10)% and 55(± 19)% when probed with Bodipy-Fab' bd24 (Fig. 5.2.).

In addition to the FPR measurements, the cellular distribution of recombinant $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ receptors was studied by immunocytochemistry (2.2.4.) using the Bodipy-Fab' bd24 antibody ($\alpha 1$) and bd17 antibody ($\beta 3$) in HEK293 cells (Figure 5.3) and COS7 cells (Figure 5.4.). The figures show that both $\alpha 1_{CH}\beta 3\gamma 2s$ (Panels A, $\alpha 1$ and B, $\beta 3$) and $\alpha 1_{CD}\beta 3\gamma 2s$ (Panels C, $\alpha 1$ and D, $\beta 3$) were expressed in clusters on the surface of live HEK293 and COS7 cells. Despite the clear difference in the percentage of relative mobile fractions of $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ receptors expressed in COS7 cells, both receptor types are clustered at the cell surface. This implies that receptor aggregation does not govern receptor mobility.

Figure 5.3. Cell surface expression of $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ GABA_ARs in HEK293 cells.

Panels A and B: HEK293 cells were cotransfected with the $\alpha 1_{CH}$ chimera cDNA, together with $\beta 3$ and $\gamma 2s$ subunit cDNAs. Cells were labelled live for the $\alpha 1_{CH}$ subunit with Bodipy-labelled-Fab' fragments of the antibody bd24 (**Panel A**) and for the $\beta 3$ subunit with the $\beta 2/3$ subunit-specific monoclonal antibody (bd-17) and visualised with TRITC-conjugated anti-mouse secondary antibody (**Panel B**).

Panels C and D: HEK293 cells were cotransfected with cDNA encoding the $\alpha 1$ subunit lacking the cytoplasmic loop, $\alpha 1_{CD}$, together with $\beta 3$ and $\gamma 2s$ subunit cDNAs. Cells were labelled live for the $\alpha 1_{CD}$ subunit with Bodipy-labelled-Fab' fragments of the antibody bd24 (**Panel C**) and for the $\beta 3$ subunit with the $\beta 2/3$ subunit-specific monoclonal antibody (bd-17) and visualised with TRITC-conjugated anti-mouse secondary antibody (**Panel D**).

Scale bars for all panels: 10 μ m.

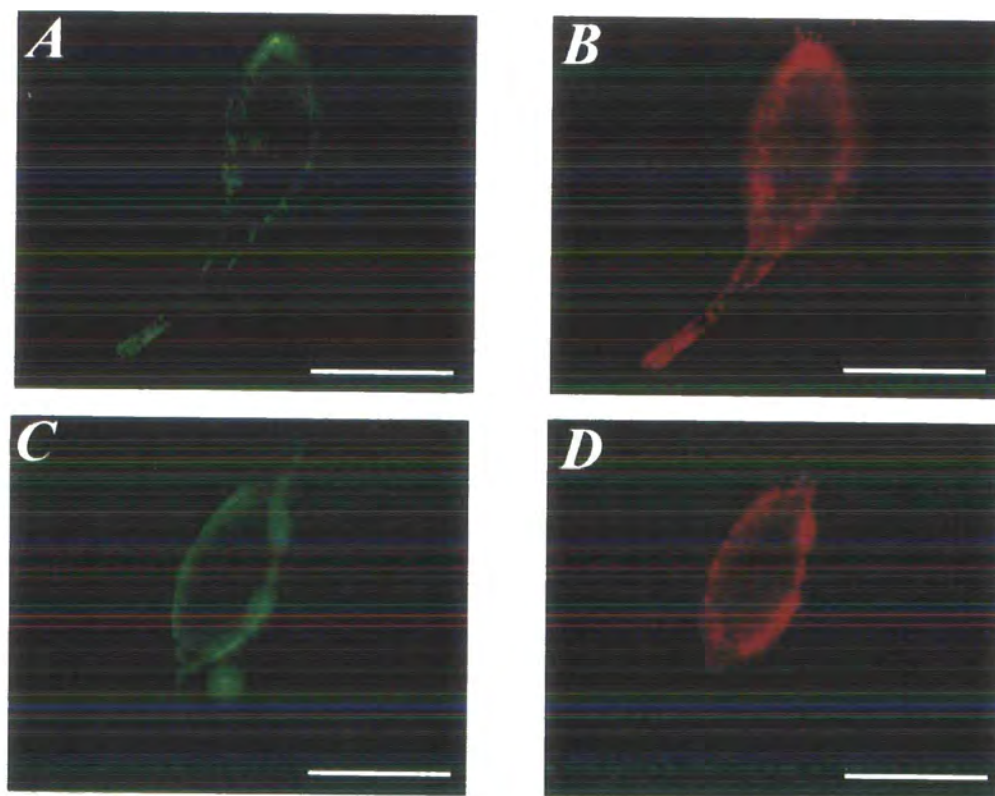


Figure 5.3. Cell surface expression of $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ GABA_ARs in HEK293 cells.

Figure 5.4. Cell surface expression of $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ GABA_ARs expressed in COS7 cells.

Panels A and B: COS7 cells cotransfected with the chimeric $\alpha 1$ subunit, $\alpha 1_{CH}$, together with $\beta 3$ and $\gamma 2s$ subunits. Live cells were labelled for the $\alpha 1_{CH}$ subunit with Bodipy-labelled-Fab' fragments of the antibody bd24 (**Panel A**) and for the $\beta 3$ subunit with the specific monoclonal antibody (bd-17) visualised with TRITC-conjugated anti-mouse secondary antibody (**Panel B**).

Panels C and D: COS7 cells were cotransfected with the cDNA encoding the $\alpha 1$ subunit lacking the cytoplasmic loop, $\alpha 1_{CD}$, together with $\beta 3$ and $\gamma 2s$ subunit cDNAs. Cells were labelled live for the $\alpha 1_{CD}$ subunit with Bodipy labelled-Fab' fragments of the $\alpha 1$ subunit-specific antibody bd24 (**Panel C**) and for the $\beta 3$ subunit with the $\beta 2/3$ subunit-specific monoclonal antibody (bd-17) and visualised with TRITC-conjugated anti-mouse secondary antibody (**Panel D**).

Scale bars for all panels: 20 μ m.

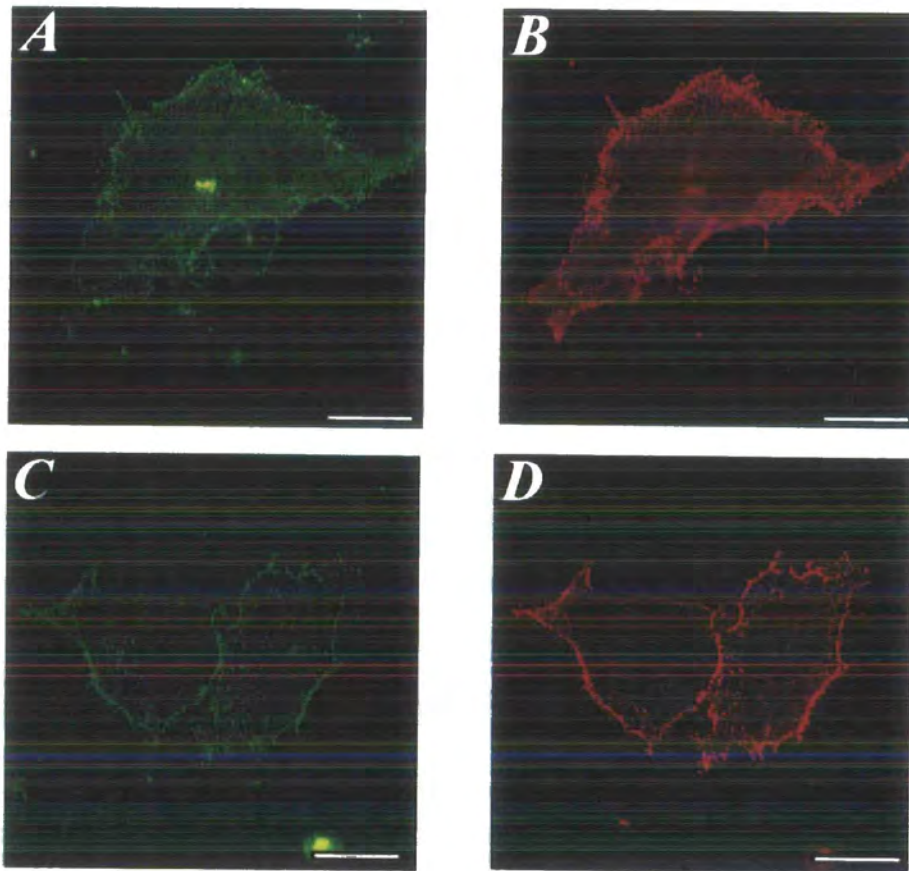


Figure 5.4. Cell surface expression of $\alpha 1_{CH}\beta 3\gamma 2s$ and $\alpha 1_{CD}\beta 3\gamma 2s$ GABA_ARs expressed in COS7 cells.

5.2.2. *Comparative mobility of recombinant GABA_A $\alpha 1\beta 3\gamma 2$ receptors: Effects of transposing the cytoplasmic loop domain of the $\alpha 1$ subunit with that of the $\alpha 2$ ($\alpha 1_{CH-\alpha 2}$), $\alpha 3$ ($\alpha 1_{CH-\alpha 3}$) and $\alpha 6$ ($\alpha 1_{CH-\alpha 6}$) subunits*

If a unique segment of the $\alpha 1$ subunit is required for controlling lateral mobility of the receptors, then replacing it by a corresponding segment from another α subunit might be expected to change the mobility pattern of the receptors. To test this hypothesis, the M3/M4 cytoplasmic loop of the $\alpha 1$ subunit was replaced by the loops of the $\alpha 2$ subunit ($\alpha 1_{CH-\alpha 2}$), the $\alpha 3$ subunit ($\alpha 1_{CH-\alpha 3}$) and the $\alpha 6$ subunits ($\alpha 1_{CH-\alpha 6}$). Since the primary sequences of these loop domains are poorly conserved between GABA_A receptor α subunits the changes made by the exchange were, in each case, significant (see methods 2.2.2.10.).

COS7 and HEK293 cells were transfected with $\beta 3$ and $\gamma 2s$ subunit cDNAs together with each of the $\alpha 1$ chimeras, $\alpha 1_{CH-\alpha 2}$, $\alpha 1_{CH-\alpha 3}$ and $\alpha 1_{CH-\alpha 6}$, and the mobility of the expressed receptors was measured by FPR (2.2.7.) and compared to unmodified $\alpha 1_{CH}\beta 3\gamma 2s$ receptors. Transfected cells were labelled live with the fluorescent benzodiazepine Bodipy-Ro-1986.

Table 5.1. and Table 5.2. show the differential mobility patterns of the GABA_AR receptors expressed in HEK293 and COS7 cells, respectively.

With HEK293 cells (Table 5.1.) the results obtained are concordant with previous studies (Chapter 3 and 4). These cells seems to lack those elements necessary to anchor GABA_ARs since the receptors were mobile whichever subunit combination was expressed. The mobile fraction of receptors comprising the $\alpha 1$ subunit chimeras ranged from 55-71%.

Table 5.1. FPR measurements of recombinant $GABA_A$ Rs expressed in HEK293 cells

<i>GABA_AR-</i> <i>subunits</i> <i>transfected</i>	<i>Mobile Fraction</i> <i>(%Recovery)</i>	<i>Diffusion Coef.</i> <i>($\times 10^{-10} \text{ cm}^2/\text{sec}$)</i>	<i>n</i>
$\alpha 1_{\text{CH}}\beta 3\gamma 2\text{s}$	55+/-14	3+/-1	17
$\alpha 1_{\text{CH-}\alpha 2}\beta 3\gamma 2\text{s}$	69+/-13	2.8+/-1.1	13
$\alpha 1_{\text{CH-}\alpha 3}\beta 3\gamma 2\text{s}$	71+/-11	2.3+/-0.6	14
$\alpha 1_{\text{CH-}\alpha 6}\beta 3\gamma 2\text{s}$	65+/-14	2.3+/-0.6	19

Table 5.2. FPR measurements of recombinant $GABA_A$ Rs expressed in COS7 cells

<i>GABA_AR-</i> <i>subunits</i> <i>transfected</i>	<i>Mobile Fraction</i> <i>(%Recovery)</i>	<i>Diffusion Coef.</i> <i>($\times 10^{-10} \text{ cm}^2/\text{sec}$)</i>	<i>n</i>
$\alpha 1_{\text{CH}}\beta 3\gamma 2\text{s}$	29+/-8	4+/-2	12
$\alpha 1_{\text{CH-}\alpha 2}\beta 3\gamma 2\text{s}$	66+/-12	2+/-0.1	16
$\alpha 1_{\text{CH-}\alpha 3}\beta 3\gamma 2\text{s}$	61+/-14	2+/-1.015	18
$\alpha 1_{\text{CH-}\alpha 6}\beta 3\gamma 2\text{s}$	53+/-14	1.81+/-0.49	15

For COS7 cells (Table 5.2.) the recovery of the fluorescence signal was 66 (\pm 12) % for $\alpha 1_{\text{CH-}\alpha 2}\beta 3\gamma 2\text{s}$ and 61 (\pm 14)% for $\alpha 1_{\text{CH-}\alpha 3}\beta 3\gamma 2\text{s}$ receptors, compared to 29 (\pm 8) % for $\alpha 1_{\text{CH}}\beta 3\gamma 2\text{s}$. These results suggest that exchanging the cytoplasmic loop of the $\alpha 1$ subunit with the corresponding domain of the $\alpha 2$ or $\alpha 3$ subunits released the receptor complex from the constraints that tethered the receptor on the cell surface. Thus, although $\alpha 1_{\text{CH-}\alpha 2}$ and $\alpha 1_{\text{CH-}\alpha 3}$ chimeras have an $\alpha 1$ subunit amino-acid backbone the replacement of the cytoplasmic loop transformed the mobility pattern of wild type $\alpha 1$ subunits.

The observation that 53 (\pm 14)% of receptors comprising the chimeric $\alpha 1$ with the loop of the $\alpha 6$ subunit, $\alpha 1_{\text{CH-}\alpha 6}$, were mobile was unexpected. This contrasts

with the observation that only 18 (\pm 11)% of $\alpha 6\beta 3\gamma 2s$ receptors expressed in COS7 cells were mobile (Table 4.2).

The rate of movement of $\alpha 1_{CH-\alpha 2}$, $\alpha 1_{CH-\alpha 3}$ and $\alpha 1_{CH-\alpha 6}$ -containing receptors was typical of most membrane glycoproteins with diffusion coefficients of the range of 10^{-10} cm²/s.

5.2.3. Localisation of the $\alpha 1$ subunit chimeras in transfected COS7 and HEK293 cells

Chapter 3 provides evidence that $\alpha 1$ subunit homo-oligomers expressed in HEK293 or COS7 cells are not directed to the cell surface but remain intracellular, unable to exit the ER. To test whether an ER-retention signal is contained within the M3/M4 cytoplasmic loop the truncated $\alpha 1$ subunit construct lacking this domain, $\alpha 1_{CD}$, was transiently transfected into COS7 and HEK293 cells. Immunocytochemistry was performed to determine where in these cells this subunit was expressed and compared with the distribution of the wild type $\alpha 1$ subunit (Chapter 3), and with the bovine/rat chimera, $\alpha 1_{CH}$. When COS7 and HEK293 cells transiently transfected with the $\alpha 1_{CD}$ subunit were stained live with the monoclonal antibody bd24, no surface fluorescence was observed. The same results were obtained with the chimeric subunit, $\alpha 1_{CH}$ and with the wild type subunit (see Chapter 3). Immunoreactive product was only visible if the cells were fixed and permeabilised. Hence, $\alpha 1_{CH}$ and $\alpha 1_{CD}$, were retained in an intracellular compartment. Figure 5.5., Panel A shows that the intracellular distribution of $\alpha 1_{CH}$ -containing receptors in HEK293 cells, is similar to the pattern shown by $\alpha 1_{CD}$ -containing receptors (Panel B) and to the one obtained for

$\alpha 1$ subunit homo-oligomers (see Chapter 3). Matching results were obtained in transfected COS7 cells (Figure 5.5.), the intracellular retention of expressed $\alpha 1_{CH}$ and $\alpha 1_{CD}$ -containing receptors are shown in Panel D and Panel E, respectively.

To test whether the M3/M4 cytoplasmic loops of $\alpha 2$, $\alpha 3$ or $\alpha 6$ subunits could direct $\alpha 1$ subunit homo-oligomers to the cell surface, the distribution of transiently expressed $\alpha 1_{CH-\alpha 2}$, $\alpha 1_{CH-\alpha 3}$ and $\alpha 1_{CH-\alpha 6}$ subunit constructs, in which the cytoplasmic loops of the $\alpha 2$, $\alpha 3$ and $\alpha 6$ subunits replaced the loop domain of the $\alpha 1$ subunit, were analysed by immunocytochemistry. All the $\alpha 1$ subunit constructs were found retained intracellularly as shown in Figure 5.5. Panels F, G and H, for COS7 cells. In HEK293 cells only $\alpha 1_{CH-\alpha 6}$ subunit homo-oligomers were tested (Figure 5.5. Panel C).

Figure 5.5. *Intracellular localisation of the $\alpha 1$ subunits chimeras expressed in HEK293 and COS7 cells.*

HEK293 cells were transfected with cDNAs encoding the panel of $\alpha 1$ subunit chimeras: $\alpha 1_{CH}$ (Panel A), $\alpha 1_{CD}$ (Panel B) and $\alpha 1_{CH-\alpha 6}$ (Panel C). Cells were fixed and permeabilised in order to visualise the intracellularly expressed homo-oligomers. Cells were labelled with the anti- $\alpha 1$ subunit-specific monoclonal antibody (bd24) and visualised with TRITC-conjugated anti-mouse secondary antibody

COS7 cells were transfected with the cDNAs encoding the $\alpha 1$ subunit chimeras: $\alpha 1_{CH}$ (Panel D), $\alpha 1_{CD}$ (Panel E), $\alpha 1_{CH-\alpha 2}$ (Panel F), $\alpha 1_{CH-\alpha 3}$ (Panel G) and $\alpha 1_{CH-\alpha 6}$ (Panel H). Cells were fixed and permeabilised in order to visualise the intracellular homo-oligomers. Cells were labelled with the anti- $\alpha 1$ subunit-specific monoclonal antibody (bd24) and visualised with TRITC-conjugated anti-mouse secondary antibody.

Scale bars for panels A, B and C: 10 μ m.

Scale bars for panels D, E, F, G and H: 20 μ m.

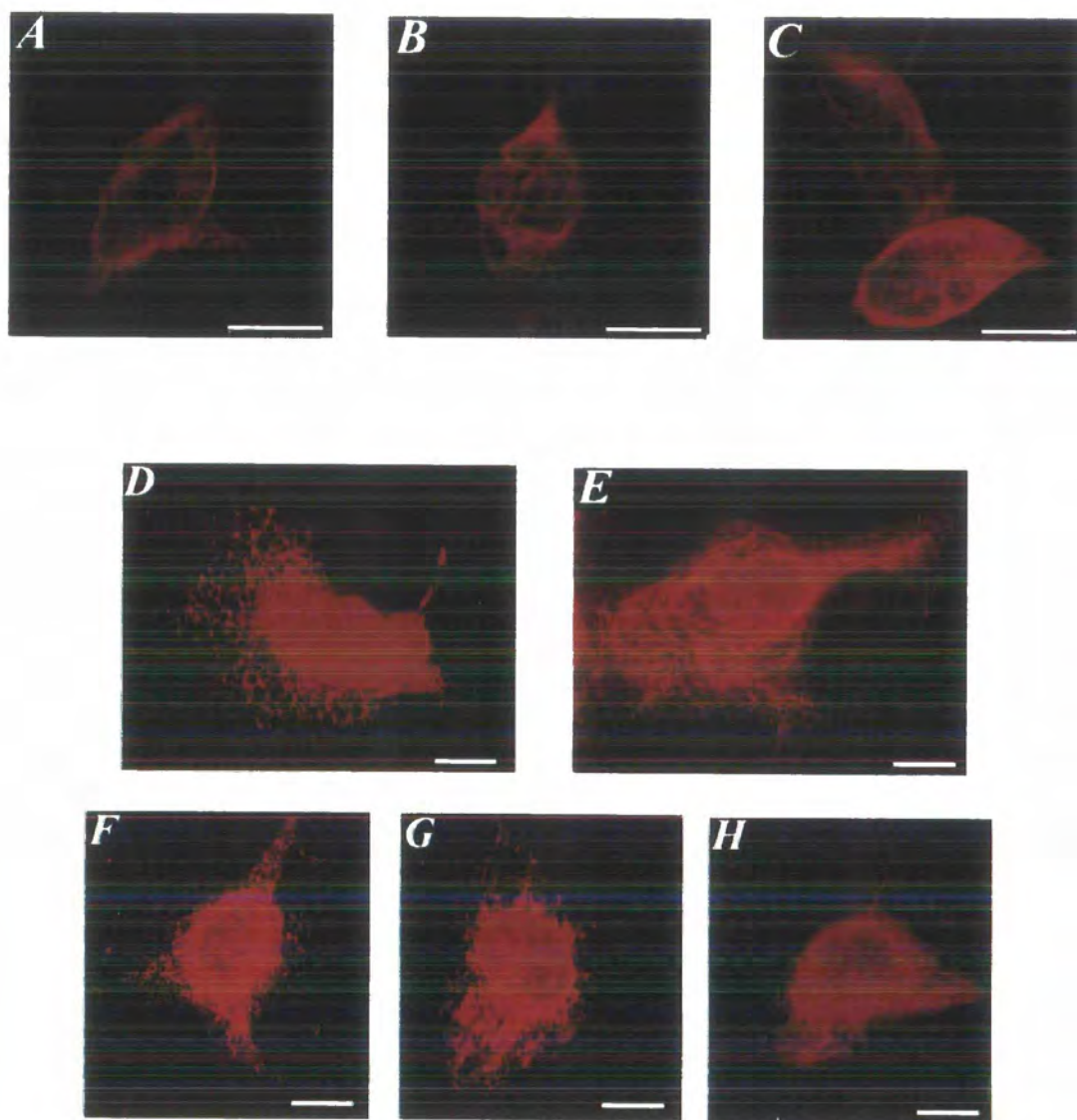


Figure 5.5. Intracellular localisation of the $\alpha 1$ subunits chimeras expressed in HEK293 and COS7 cells

5.2.4. *$\beta 3$ subunits re-route $\alpha 1$ subunits chimeras to the cell surface*

It was shown in Chapter 3 that the $\beta 3$ subunits rescued $\alpha 1$ subunits from their intracellular retention site and re-routed them to the cell surface. To determine if the M3/M4 cytoplasmic loop is necessary for $\alpha 1$ - $\beta 3$ subunits assembly, the $\alpha 1$ subunit constructs used in the previous section were transfected into COS7 cells together with the $\beta 3$ subunit. The lack of a cytoplasmic loop ($\alpha 1_{CD}$) did not prevent $\alpha 1$ subunits from being rescued by $\beta 3$ subunits. When $\alpha 1_{CD}$ subunit cDNA and $\beta 3$ subunit cDNA were cotransfected into COS7 cells (Figure 5.6. Panel C and D), these subunits were found clustered at the cell surface in a pattern that paralleled that found for $\alpha 1\beta 3$ (Chapter 3) and $\alpha 1_{CH}\beta 3$ (Figure 5.6. Panels A and B) complexes expressed in these cells. Identical results were obtained with the $\alpha 1$ subunit constructs $\alpha 1_{CH-\alpha 2}$, $\alpha 1_{CH-\alpha 3}$ and $\alpha 1_{CH-\alpha 6}$ when they were cotransfected into COS7 cells with the $\beta 3$ subunit (Figure 5.6. Panels E-F; G-H and I-J, respectively).

Together these results demonstrate that the $\alpha 1$ subunit constructs ($\alpha 1_{CD}$, $\alpha 1_{CH-\alpha 2}$, $\alpha 1_{CH-\alpha 3}$ and $\alpha 1_{CH-\alpha 6}$) are restricted to the ER when expressed alone as for the wild-type $\alpha 1$ subunit. Only when these subunits are co-expressed with the $\beta 3$ subunit, did the $\alpha 1_{CH-\alpha x}\beta 3$ combinations acquire the signal to leave the ER and form co-localised clusters on the cell membrane. Thus, the M3/M4 cytoplasmic loop of the $\alpha 1$ subunit appears not to be involved in subunit targeting to the cell surface, subunit-subunit assembly nor receptor aggregation.

Figure 5.6. Cell surface expression of $\alpha 1_{CH-x}\beta 3$ receptors in COS7 cells

COS7 cells were cotransfected with cDNAs of the $\alpha 1$ subunit chimeras together with the $\beta 3$ subunit cDNA. $\alpha 1_{CH-x}\beta 3$ complexes were labelled live with the $\alpha 1$ subunit-specific monoclonal antibody, bd24 and visualised with cascade blue conjugated anti-mouse secondary antibody and with the polyclonal antibody anti- $\beta 102/103$ for the $\beta 3$ subunit and visualised with TRITC-conjugated anti-rabbit secondary antibody. **Panel A and B:** $\alpha 1_{CH}\beta 3$ complexes, **Panel C and D:** $\alpha 1_{CD}\beta 3$ complexes, **Panel E and F:** $\alpha 1_{CH-\alpha 2}\beta 3$ complexes; **Panel G and H:** $\alpha 1_{CH-\alpha 3}\beta 3$ complexes and **Panel I and J:** $\alpha 1_{CH-\alpha 6}\beta 3$ complexes.

Scale bars for all panels: 20 μ m.

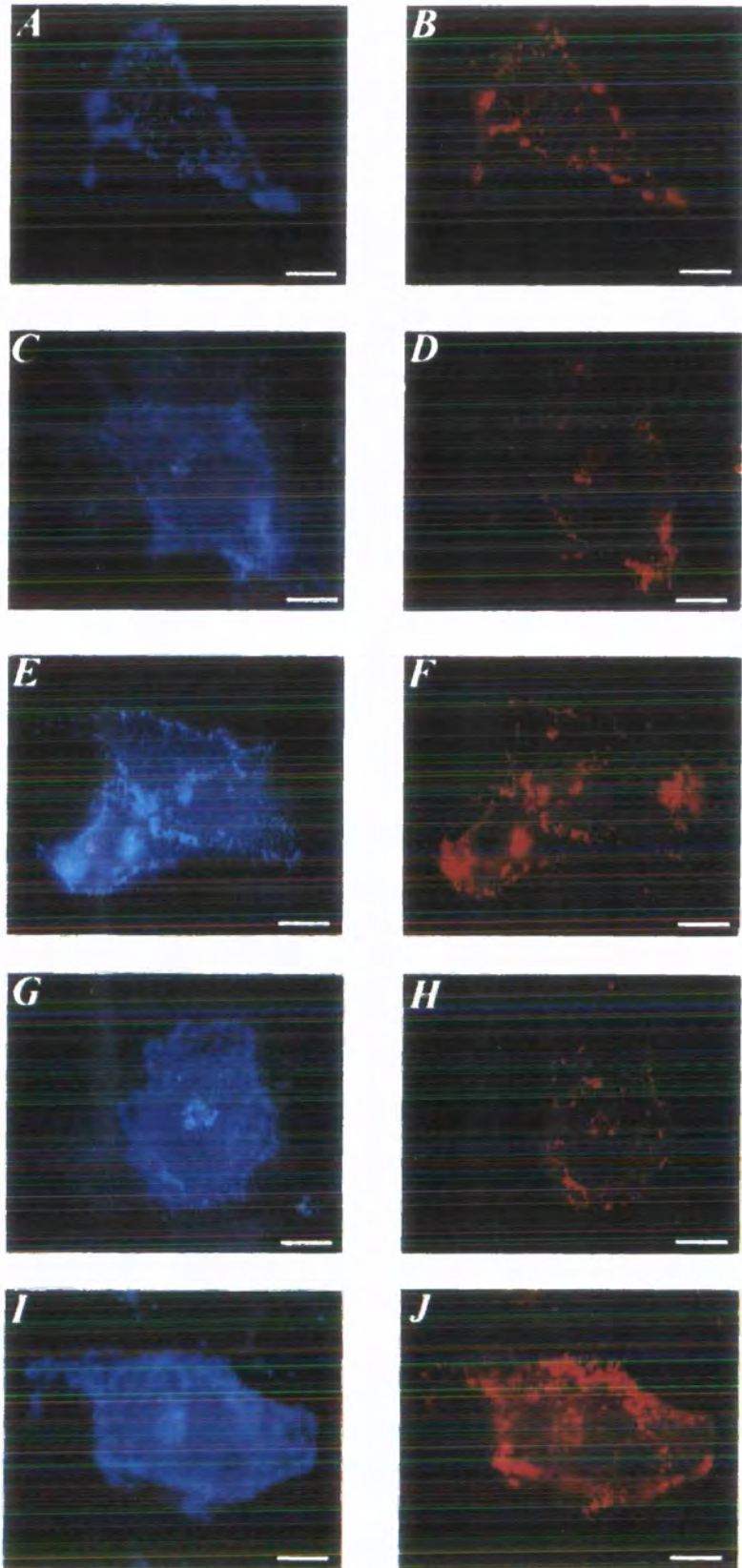


Figure 5.6. Cell surface expression of $\alpha 1_{CH3}\beta 3$ receptors in COS7 cells

5.3. Discussion

A crucial problem in neurobiology is how a neurone maintains its mosaic of membrane proteins. GABA_A receptors are routed and localised in specific neuronal domains (Velazquez *et al.*, 1989) possibly taking advantage of the diversity of subunit isoforms that form the receptor complexes. In Chapter 4 it was demonstrated that recombinant GABA_A receptors expressed in COS7 or PC12 cells had different surface lateral mobilities that were α subunit dependent. Thus, $\alpha x\beta 3\gamma 2s$ receptors were generally immobile when the alpha subunit was either $\alpha 1$ or $\alpha 6$ subunits. In contrast GABA_A $\alpha x\beta 3\gamma 2s$ receptors which comprised $\alpha 2$, $\alpha 3$, $\alpha 4$ or $\alpha 5$ subunits were generally mobile. The primary amino-acid sequence of the intracellular M3/M4 loop of α subunit isoforms is poorly conserved and might be a determining factor in these differences in receptor anchoring. In this chapter new insights have been presented into the possible role that the $\alpha 1$ subunit M3/M4 cytoplasmic loop plays in controlling lateral mobility of the receptor.

The experimental approach described in this chapter was based on the hypothesis that the intracellular M3/M4 loop of $\alpha 1$ and $\alpha 6$ subunits may interact with some region of the cytoskeleton or a protein that restricts the mobility of the whole complex. Thus, removal or alteration of these sequences would be expected to prevent the formation of links with the cytoskeletal elements that tether the receptors at the cell surface. Under these conditions it was anticipated that the GABA_AR would be freely mobile. To test this hypothesis, a panel of $\alpha 1$ subunit chimeras were engineered. Alpha 1 subunits that lacked the M3/M4 loop ($\alpha 1_{CD}$) or had the intracellular loop domain of the $\alpha 1$ subunit exchanged for the

loops of $\alpha 2$, $\alpha 3$ and $\alpha 6$ subunits ($\alpha 1_{CH-\alpha 2}$, $\alpha 1_{CH-\alpha 3}$ and $\alpha 1_{CH-\alpha 6}$), could then be transiently expressed.

Based upon the FPR analyses on receptors containing these mutated $\alpha 1$ subunits described here, it can be concluded that the M3/M4 domain of the $\alpha 1$ subunit does effect the mobility of GABA_AR complexes. Removal of the cytoplasmic loop from the $\alpha 1$ subunit transformed $\alpha 1\beta 3\gamma 2s$ receptors from being essentially immobile to being freely mobile when expressed in COS7 cells. In addition, when either the cytoplasmic loops of $\alpha 2$ and $\alpha 3$ subunits were inserted into $\alpha 1_{CD}$ ($\alpha 1_{CH-\alpha 2}$ and $\alpha 1_{CH-\alpha 3}$), the receptors containing the modified $\alpha 1$ subunit showed an increased mobility that was comparable with the mobility found in receptors containing the intact $\alpha 2$ and $\alpha 3$ subunits (Chapter 4). These results imply that the cytoplasmic loop region of the $\alpha 1$ subunit, but not the M3/M4 loops of the $\alpha 2$ and $\alpha 3$ subunits, might interact with some cellular element that restricts the mobility of the complex. The final configuration of the $\alpha 2$ and $\alpha 3$ M3/M4 cytoplasmic loops do not demonstrate obvious structural motifs that are known to bind cytoskeletal elements nor do they show homologies with other membrane proteins whose cytoskeletal binding domains have been mapped (Srinivasan *et al.*, 1993; Srinivasan *et al.*, 1988; Lambert and Bennett, 1993). On the other hand, the results obtained with the $\alpha 1_{CH-\alpha 6}$ chimera, in which the intracellular loop domain of the $\alpha 6$ subunit replaced that for the $\alpha 1$ subunit were unexpected. In contrast to previous studies (Chapter 4) these receptors were freely mobile and did not show the restriction in mobility found for $\alpha 6\beta 3\gamma 2s$ or $\alpha 1\beta 3\gamma 2s$ receptors. The cytoplasmic loop of the $\alpha 6$ subunit seems not to be uniquely responsible for the restricted mobility of GABA_A $\alpha 6\beta 3\gamma 2s$ receptors. It

is possible that interaction with a restricting element requires more than a single domain of the $\alpha 6$ subunit protein to anchor these complexes. Furthermore, the inclusion of the $\alpha 6$ loop into the $\alpha 1$ subunit seems to have masked an effect over the restriction of receptor mobility that the $\alpha 1$ subunit confers.

In addition to estimates of receptor dynamics using FPR, the steady-state cellular distribution of receptors containing the $\alpha 1$ subunit chimeras was also analysed. It was found that homo-oligomers of GABA_AR $\alpha 1$ subunits lacking the M3/M4 cytoplasmic domain were retained in the ER and only expressed at the cell surface if coexpressed with the $\beta 3$ subunit. Similar results were obtained with the wild-type $\alpha 1$ subunit (Chapter 3) and with the other $\alpha 1$ subunit chimeras, $\alpha 1_{CH}$, $\alpha 1_{CH-\alpha 2}$ and $\alpha 1_{CH-\alpha 6}$. It appears that this region of the protein does not contain the information that dictates whether the subunit is retained or programmed to leave the ER. In addition, expressed receptors containing all the $\alpha 1$ subunit chimeras described, together with the $\beta 3$ subunits, were visualised on the cell surface in a clustered pattern. Thus, the cytoplasmic loop of the $\alpha 1$ subunit is not required for receptor clustering. The information contained in the M3/M4 domains of the $\alpha 2$, $\alpha 3$ and $\alpha 6$ subunits also failed to modify the ability of receptors to aggregate.

Chapter 6

*Cell surface immobilisation of GABA_ARs in
cerebellar granule cells*

Chapter Six

***Cell surface immobilisation of GABA_AR α 1 subunits in
cultured rat cerebellar granule cells depends on the M3/M4
cytoplasmic loop***

6.1. Introduction

In the previous chapters the localisation and relative mobilities of recombinant GABA_ARs were studied in HEK293, COS7 and the neuronal model system, NGF-treated PC12 cells. It was concluded from observations on COS7 that the M3/M4 cytoplasmic domain of the α 1 subunit contributes to the restricted mobility of the receptor complex. The information acquired through these approaches can be useful to elucidate the molecular mechanisms underlying neurotransmitter receptor anchoring. While these experiments are indicative, it is necessary to extend the study to cells of the central nervous system to have a more realistic understanding of the processes involved in directing the distribution and cell surface maintenance of GABA_AR in neurons. In this chapter the mobility of recombinantly expressed GABA_ARs were analysed in cultured rat cerebellar granule cells. The α 1 subunit constructs, α 1_{CH} (bovine/rat chimera) and α 1_{CD} (α 1_{CH} with the M3/M4 cytoplasmic loop excised) were transfected into cultured rat cerebellar granule cells to investigate the role the cytoplasmic loop domain plays in receptor tethering in a well-characterised neuronal preparation, *in vitro*.

6.2. Results

6.2.1. Efficiency of transfecting cultured rat cerebellar granule cells with GABA_AR subunit cDNAs

Transfecting neurons is known to be notoriously difficult. Traditional methods of transfecting cells were tested in rat cerebellar granule cells immediately following dissociation of these cells from the cerebellum of neonatal rats ('After dispersion', Table 6.1.), and in cells seeded in culture media ('In culture', Table 6.1.). Several traditional transfection procedures were employed such as calcium phosphate precipitation (CPP), electroporation (E.poration), lipofectamine (Lp) and TfxTM-50 Reagent (as described in methods, 2.2.3.). The efficiency of transfection for each of these methods was estimated by counting the number of transfection positive cells by immunocytochemistry using the $\alpha 1$ -subunit specific monoclonal antibody, bd24.

Table 6.1. A summary of the gene transfer efficiencies obtained.

	<i>After dispersion</i>	<i>In culture</i>
CPP	0%	0%
E.poration	0.1%	Not tested
Lp	0.01%	0.2%
TfxTM-50	Not tested	2%

The efficiency of the methods tested was low compared to transfection efficiencies obtained with HEK293 (~30%) and COS7 (~30%) cells. However, the transfection efficiency was deemed sufficient for the experiments to be performed. Because the transfection efficiency was higher in granule cells in culture than in cells just after the dispersion, FPR measurements and immunocytochemical analyses were conducted in cells cultured for 4-5 days *in vitro* (4-5 DIV). The transfection strategies involving lipofectamine and TfxTM-50 were adopted

hereafter since these were the most efficient systems in cultured granule cells (Table 6.1.)

6.2.2. Immunochemical localisation of the recombinant $\alpha 1$ subunits, $\alpha 1_{CH}$ and $\alpha 1_{CD}$, in cultured rat cerebellar granule cells

The GABA_AR $\alpha 1$ subunit chimeras, $\alpha 1_{CH}$ (control) and $\alpha 1_{CD}$ (in which the M3/M4 cytoplasmic loop had been deleted), were constructed using as the "parent" the 5' end of the bovine $\alpha 1$ subunit cDNA and the 3' end of the rat $\alpha 1$ subunit cDNA. In this way, the monoclonal antibody bd24, that is specific for three amino acids located at the extracellular N-terminus of the bovine and human $\alpha 1$ sequences (Ewert *et al.*, 1990) but not for the rat $\alpha 1$ N-terminal sequence, could be used to specifically label these constructs, see methods 2.2.2.10. This enabled the chimera transfected $\alpha 1$ subunits to be distinguished from the native rat $\alpha 1$ subunits that are expressed in these neurons (Thompson and Stephenson, 1994).

The extracellular and/or intracellular distribution of recombinantly expressed GABA_AR $\alpha 1$ subunits (homo-oligomers) and $\alpha 1$ subunit-containing receptors ($\alpha 1\beta 3\gamma 2$) was investigated previously in recombinant HEK293 and COS7 cells (Chapter 3). In this chapter the same approaches were performed in cultured cerebellar granule cells. The first aim was to determine whether the recombinant $\alpha 1$ subunit constructs, $\alpha 1_{CH}$ and $\alpha 1_{CD}$, transfected into these neurons were edited, assembled and expressed at the neuronal cell surface. Cultured cerebellar granule cells were transfected with either the $\alpha 1_{CH}$ or the $\alpha 1_{CD}$ constructs (methods 2.2.3.) and labelled with Bodipy-Fab' fragments of the $\alpha 1$ subunit-specific antibody, bd24 (2.2.4.). The fluorescence patterns obtained revealed that both $\alpha 1_{CH}$ and $\alpha 1_{CD}$

subunit proteins were expressed in clusters on the cell surface. In previous chapters it was shown that homo-oligomers of both wild-type $\alpha 1$ and $\alpha 1$ subunit chimeras were retained in an intracellular compartment (Chapters 3 and 5). The cell surface fluorescence pattern observed in cerebellar granule cells transfected with $\alpha 1$ subunit cDNA alone is strong evidence that the 'foreign' protein had been properly edited and assembled with native subunits into receptors. The complexes formed were routed and localized on the cell surface in a clustered pattern. Differences between the localisation of GABA_ARs containing the $\alpha 1_{CH}$ subunits and the $\alpha 1_{CD}$ subunits were noted. $\alpha 1_{CH}$ subunit-containing receptors were found located at the soma, dendrites and axon of cultured cerebellar granule cells (Fig. 6.1. Panel C). On the other hand, receptors containing the $\alpha 1_{CD}$ subunit, which lacked the M3/M4 cytoplasmic loop were expressed mainly at the cell somas (Fig. 6.1. Panels A and B).

Figure 6.1. *Cell surface localisation of the $\alpha 1_{CH}$ and $\alpha 1_{CD}$ subunits expressed in cultured cerebellar granule cells*

Cerebellar granule cells were transfected with the cDNAs encoding $\alpha 1_{CH}$ and the $\alpha 1_{CD}$ constructs. Cells were labelled live with the anti- $\alpha 1$ subunit-specific monoclonal antibody, bd24. Expressed receptors containing the $\alpha 1_{CD}$ subunit were distributed in a clustered pattern (**Panel A**), and mainly retained at the cell soma (**Panel B**). Expressed receptors containing the $\alpha 1_{CH}$ subunit were also distributed in clusters but over the entire cell surface (**Panel C**).

Scale bar: Panel A: 10 μm ; Panel B: 5 μm ; Panel C: 20 μm

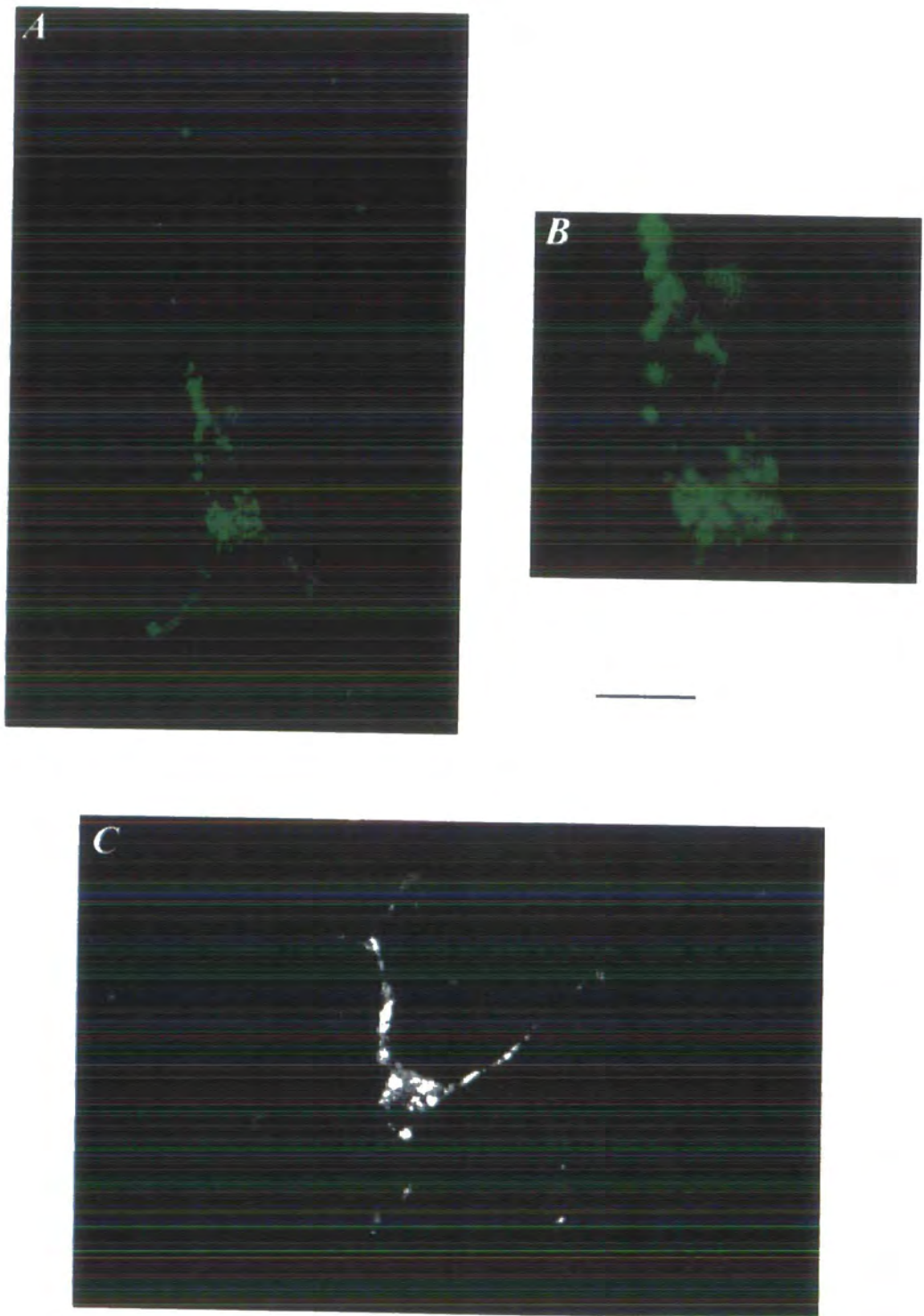


Figure 6.1. Cell surface localisation of the $\alpha 1_{CH}$ and $\alpha 1_{CD}$ subunits expressed in cultured cerebellar granule cells

6.2.3. Comparative mobility of GABA_AR containing the $\alpha 1_{CH}$ or the $\alpha 1_{CD}$ subunits

In Chapter 3, native GABA_A receptors in cerebellar granule cells were shown to have restricted lateral mobilities. In non-neuronal cells, the M3/M4 cytoplasmic loop of recombinantly expressed $\alpha 1$ subunit was shown to play a role in the immobilizing of receptors, presumably by providing a substrate for interactions with component(s) of the cytoskeleton (Chapter 5). To gain a better understanding of the properties of this $\alpha 1$ subunit domain in neurons, and its possible role in controlling the lateral mobility of native receptors, cerebellar granule cells were transfected with $\alpha 1_{CH}$ and $\alpha 1_{CD}$, see methods (2.2.3.). The lateral mobility of receptors containing the $\alpha 1$ subunits lacking the M3/M4 cytoplasmic loop ($\alpha 1_{CD}$) was measured by FPR, see methods (2.2.7.) and compared with the lateral mobility of receptors containing the 'parent' $\alpha 1$ subunit ($\alpha 1_{CH}$). FPR measurements showed marked differences in the lateral mobilities of these receptors when expressed in cerebellar granule cells (Figure 6.2.). The recombinant receptors were selectively labelled with the Bodipy-Fab' fragment of the $\alpha 1$ subunit-specific antibody, bd24 since the antibody directed against the bovine and human GABA_AR N-terminal sequence fails to recognize or label those endogenous $\alpha 1$ subunits present in the rat cerebellar granule cells.

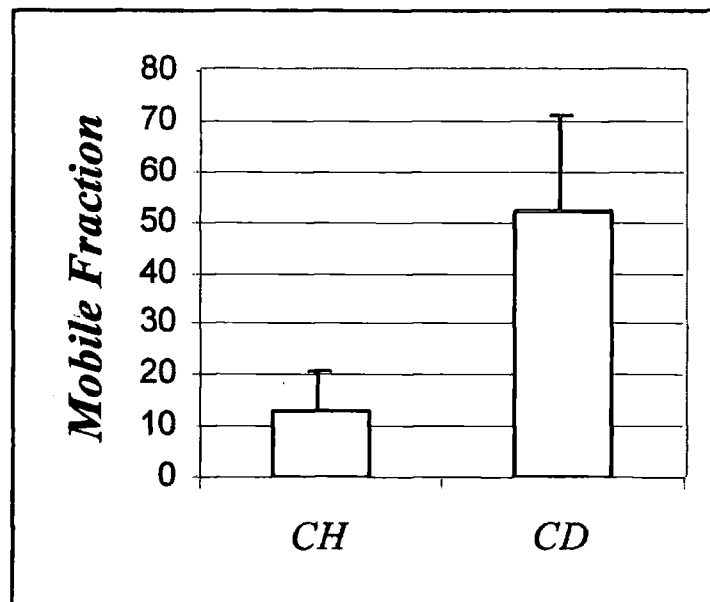


Figure 6.2. Histogram showing the relative mobile fraction (% Recovery) of recombinant GABA_AR $\alpha_{1CH}\beta_3\gamma_2s$ (CH) and $\alpha_{1CD}\beta_3\gamma_2s$ (CD) expressed in cerebellar granule cells. Cells were probed with Bodipy-Fab' fragments of the antibody, bd24.

Because of the low efficiency of the transfection, only a few positive cells of each cell set were localized and subsequently examined by FPR. The term 'cell set' refers to a cerebellar granule cell-coated dish that has been transfected with the $\alpha 1$ subunit constructs. Cells transfected with the $\alpha 1_{CH}$ subunits or with the $\alpha 1_{CD}$ subunits were tested and the FPR experiments were repeated in four different cell preparations (named as first, second, third and fourth set in Tables 6.2 and 6.3.). Tables 6.2. and 6.3. show the mobile fraction (% Recovery) of receptors measured for every positive cell analysed.

Receptors containing the chimeric $\alpha 1$ subunit ($\alpha 1_{CH}$) were used as control. These receptors were generally immobile on the time scale of the experiments, recovery from photobleach ranged from 3-20%. Due to the limitations of the FPR detection system photobleached receptors that show percentage recoveries below 20% are considered to be totally immobile (see rationale in methods, 2.2.7.3.). When the cytoplasmic loop of the $\alpha 1$ subunit was deleted the mobility patterns of the expressed receptors containing this truncated $\alpha 1$ subunit changed dramatically. Generally, these receptors were converted from being immobile or tethered to being mobile. Close inspection of the data in Table 6.2. however, reveals that two populations of cells can be clearly distinguished (Figure 6.3.). One population (Population I) whose receptors appear to be freely mobile and another (Population II) in which the receptors have a more restricted mobility but never the less are more mobile than in control cells ($\alpha 1_{CH}$). Seven cells (Population I) were found to express receptors which were freely mobile on the cell surface, recoveries ranged from 63 to 93% with a mean grouping of 75-85 % recovery. The excision of the cytoplasmic loop from the $\alpha 1$ subunit appears to

have released these receptors from interactions with the mobility restraining elements e.g. cytoskeleton. The receptors expressed in the remainder of the immunopositive cells (Population II) had mobile fractions (% Recovery) that centered around the 30-39% recovery grouping

Table 6.2. Mobile fraction (% Recovery) of $\alpha 1_{CH}$ -containing GABA_ARs expressed in cerebellar granule cells

<i>First set</i>	<i>Second set</i>	<i>Third set</i>	<i>Fourth set</i>
17%	20%	14%	4%
16%	13%	4%	4%
6%	10%	10%	10%
19%		8%	4%
18%		3%	5%
		13%	

Table 6.3. Mobile fraction (% Recovery) $\alpha 1_{CD}$ -containing GABA_ARs expressed in cerebellar granule cells

<i>First set</i>	<i>Second set</i>	<i>Third set</i>	<i>Fourth set</i>
37%	44%	48%	25%
34%	48%	45%	45%
93%	47%	48%	38%
65%	83%	63%	39%
87%	70%	77%	36%
			46%
			30%

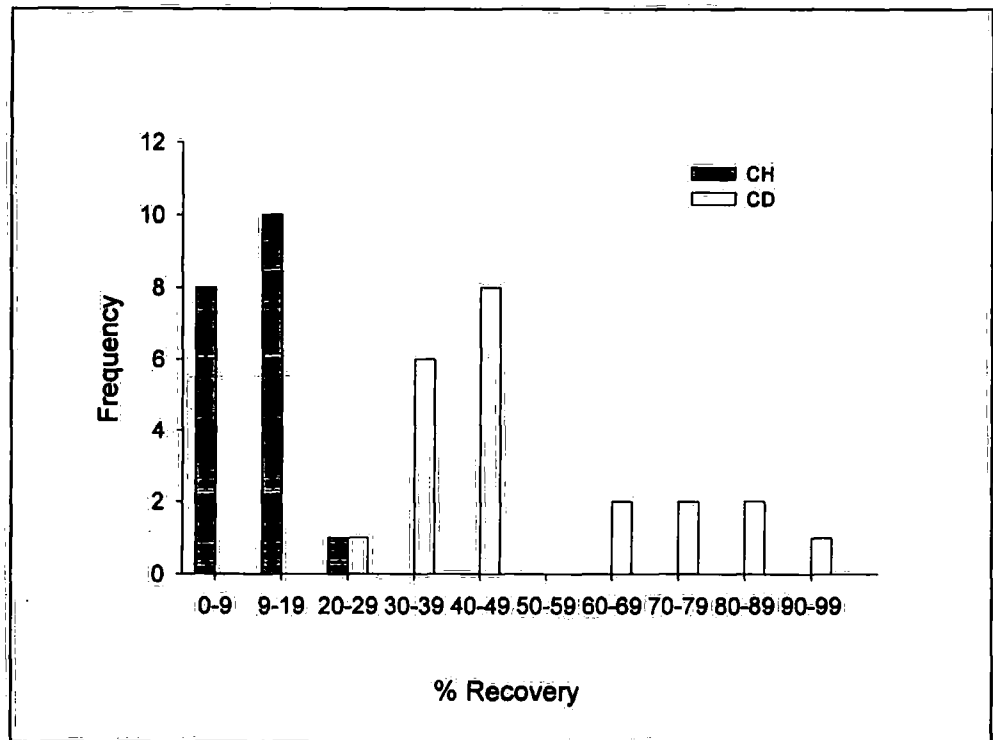


Figure 6.3. Histogram showing the frequency of the relative mobile fractions (% Recovery) measured in recombinant GABA_AR $\alpha 1_{CH}\beta 3\gamma 2s$ (CH) and $\alpha 1_{CD}\beta 3\gamma 2s$ (CD) expressed in cerebellar granule cells. Cells were probed with Bodipy-Fab' fragments of the antibody, bd24.

6.3. Discussion

Several models have been advanced to describe the mechanisms by which receptors are tethered to specific domains of the plasma membrane. The forces that restrict GABA_AR mobility and regulate distribution could arise from several possible kinds of interactions. It is possible that an impermeable barrier, like a corral, that defines domains within the cell could prevent movement between these compartments. Second, the receptor may be linked to specialised cytoskeletal elements (Kannenberg *et al.*, 1997). The identification of a series of associating proteins or “anchoring proteins” have recently been described (Wang *et al.*, 1999). The consensus of opinion suggest that association of the neurotransmitter receptor to its specific anchoring protein is critical to receptor immobilisation/ targetting (Philip *et al.*, 1991). It has been demonstrated that the interaction between these anchoring proteins and the receptors is achieved by the binding of some domains of the proteins that couple the receptor complexes with the anchoring protein. For example gephyrin binds to the cytoplasmic loop between transmembrane segments M3 and M4 of the β subunit of glycine receptors (Meyer *et al.*, 1995).

In the previous chapter, it was tested whether the $\alpha 1$ subunit cytoplasmic loop, M3/M4, played a role in controlling recombinant GABA_AR lateral mobility in transfected COS7 cells. The results indicated that this domain may play a role in receptor anchoring. In this chapter the same experiments were performed in cerebellar granule cells and the results obtained are in agreement with those described in Chapter 5.

FPR experiments described in this chapter demonstrate that recombinant complexes containing an $\alpha 1$ subunit lacking the cytoplasmic loop M3/M4 ($\alpha 1_{CD}$) are freely mobile in comparison to complexes that contain an intact $\alpha 1$ subunit ($\alpha 1_{CH}$). The FPR results imply that the elimination of this domain prevents the formation of a link between the receptor and an anchoring protein or indeed, the cytoskeleton itself. In addition, because both $\alpha 1_{CH}$ and $\alpha 1_{CD}$ were detected at the cell surface it implies that they were able to assemble with native GABA_AR subunits that are required for transport of the $\alpha 1$ subunit to the cell surface (see Chapter 3).

Interestingly, close inspection of the FPR data from cells expressing complexes containing the $\alpha 1_{CD}$ subunit revealed that there were two distinct cell populations, in terms of the lateral mobilities of receptor complexes, that were both distinct from the complexes that contained the $\alpha 1_{CH}$ subunit. One population in which the receptors were freely mobile (Population I) and another (Population II) with complexes displaying an intermediate mobility. One possible explanation might be that depending on the transfection efficiency, some cells would express complexes that contained only truncated $\alpha 1_{CD}$ subunits whereas other cells, which had incorporated less foreign $\alpha 1_{CD}$ cDNA, would express complexes containing a combination of the native type $\alpha 1$ with the foreign $\alpha 1_{CD}$. Receptors containing two α subunits have been previously described (Backs *et al.*, 1993; Khan *et al.*, 1994; Pollard *et al.*, 1995). Expressed complexes containing only the truncated $\alpha 1_{CD}$ subunit (with no cytoplasmic loop) would be predicted to be freely mobile in contrast, complexes containing the native or “wild type” $\alpha 1$ subunit and the

foreign type $\alpha 1_{CD}$ subunit would be tethered by the intact cytoplasmic loop M3/M4 of the wild $\alpha 1$ subunit included in the complex.

Immunocytochemical localisation of the expressed receptors containing the truncated $\alpha 1$ subunits showed a differential distribution pattern. When the $\alpha 1_{CD}$ expressing cells were analysed, the fluorescence was found to be restricted mainly to the cell soma. It seemed that the receptors were not able to leave this central location and migrate to the dendrites, although they still formed large receptor clusters. On the other hand, control receptors ($\alpha 1_{CH}$ -containing), were distributed clustered along both the soma and the dendrites of the neuronal cell. These results indicate that the deletion of the intracellular loop of the $\alpha 1$ subunit dictates that the receptor is retained in the soma, but none-the-less is freely mobile. The $\alpha 1_{CH}$ -containing receptors that are representative of native receptors are anchored throughout the entire cell surface. Cerebellar granule cells show a relatively simple synaptic organisation, they are activated by glutaminergic mossy fibers and receive GABA from local Golgi cells only on their distal dendrites (Ottersen *et al.*, 1988), thus the data presented here may indicate that receptors containing the truncated $\alpha 1_{CD}$ subunit are "in some way" not allowed to reach a synaptic location but are retained in the cell soma.

The constructs used in this chapter represent a first attempt to elucidate, *in vivo*, the functional importance of the $\alpha 1$ M3/M4 cytoplasmic loop in cytoskeletal anchoring and GABA_AR clustering. The present results indicate an involvement of this domain in controlling receptor lateral mobility. Although the information obtained is novel and of relative significance, it will be necessary to determine which are the cytoskeletal proteins involved.

Recently, Wang *et al.* (1999) reported the identification of a novel protein, GABARAP, which has sequence similarity with the light chain-3 of microtubule-associated proteins 1A and 1B (MAP-1A, MAP-1B), and interacts with the γ subunit of the GABA_AR. In addition, it has been reported that a ubiquitin-like protein interacts with the intracellular loop domain of GABA_AR α 1 and α 2 subunits (Bedford *et al.*, 1998), based on studies using the yeast two-hybrid screen system. While these proteins may interact with the receptor subunits, only in the case of the MAP-1B protein has a functional study been performed. Here, the MAP-1B protein was shown to be able to induce clustering of GABA_C receptors (Hanley *et al.*, 1999). However, it is not clear whether the clustering in the transfected cells is related to their immobilisation. The work presented in this thesis has clearly demonstrated the clustering of receptors in HEK293 cells without any cell surface immobilisation providing strong evidence that the two processes are mutually exclusive. The function of these “interacting” proteins in segregating and immobilising the receptors can only be ascertained by direct observation of the mobility of the receptors by techniques such as FPR or SPT.

Rapsyn and gephyrin have been identified as the proteins responsible for anchoring of other important neurotransmitter receptors, such as nicotinic acetylcholine and glycine receptors, respectively (Froehner, 1989; Kirsch *et al.*, 1991; Kirsch and Betz, 1995). This led to the hypothesis that these proteins may be involved in the anchoring of GABA_ARs. Although recent studies support the hypothesis that gephyrin (Graig *et al.*, 1996) and rapsyn (Yang *et al.*, 1997) might be involved in postsynaptic positioning of GABA_AR, there is as yet no clear “*in vivo*” evidence. Acetylcholine, glycine and glutamate receptors interact with submembraneous molecules which have no common peptide sequence. This

molecular heterogeneity of the postsynaptic somato-dendritic membrane agrees with the idea that the GABA_AR anchoring proteins may not have been identified yet.

Summary and Significance

The main objective of the study presented here was to gain a better understanding of how neurons are able to maintain neurotransmitter receptors at specific membrane domains. The large structural heterogeneity of GABA_AR led to the hypothesis that there could be a link between GABA_AR gene diversity and the targeting properties of the receptor complex. To investigate the mechanisms responsible for the localisation of GABA_AR, immunocytochemical approaches and FPR experiments were carried out in cells expressing recombinant GABA_ARs. The results obtained showed that the subunit composition of the receptors determines not only different targeting characteristics but also differences in the lateral mobility of the complexes. Thus, the different ways in which these receptors are routed to specific synaptic locations could be determined by the types of subunit isoform that compose the protein complex, which represents a form of synaptic plasticity. To elucidate which domain of the subunit proteins was responsible for the receptor anchoring, a series of truncated $\alpha 1$ subunits were engineered.

Some important conclusions were drawn as a result of the expression of these constructs in non-neuronal and neuronal cells, and the consecutive experiments performed in order to measure the lateral mobility and the final localisation of the expressed complexes containing the array of $\alpha 1$ subunit constructs. Receptor clustering does not imply receptor immobility. The M3/M4 cytoplasmic domain of the $\alpha 1$ subunit restricts the mobility of the receptor complexes but is not necessary for the aggregation between receptors. Finally, the inclusion of at least one $\alpha 1$ subunit, with an intact cytoplasmic loop, in the receptor complex seems to be necessary for immobilisation of the GABA_A receptors.

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