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IMMUNOLOCALIZATION AND TRANSMEMBRANE TOPOLOGY OF LIGAND-GATED ION CHANNELS BY FUNCTIONAL EPITOPE TAGGING

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July, 1995

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Science.

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ABSTRACT

Fast chemical neurotransmission is mediated by specialized receptors known as neurotransmitter-gated ion channels. The transmembrane topology of ion channel subunits provides insight about the structure and mechanism of activation of the receptors.

The transmembrane topology of a membrane protein is predicted by hydropathy information giving an indication of the orientation of domains of the protein with respect to the plasma membrane. Functional C-terminal epitope-tagged receptors of neurotransmitter-gated ion channels were constructed in order to investigate the orientation of the C-terminal domain.

This thesis will provide original experimental evidence demonstrating the transmembrane topology of the 5-HT_3 subunit of the serotonin-gated channel as well as the $P_{2X}3$ subunit of the ATP-gated channel.

In addition, for the first time, polyclonal antisera have been raised against the putative intracellular loop of the 5-HT_3 subunit and are used for the immunolocalization of the wild-type and tagged receptors in transfected mammalian cells and western blots.

RESUME

La neurotransmission chimique rapide est effectuée par des récepteurs spécialisés connus sous le nom de canaux ioniques activés par ligands. La topologie transmembranaire des sous-unités du canal ionique donne un aperçu de la structure et du mécanisme d'activation du récepteur.

La topologie transmembranaire d'une protéine intégrale peut être prédite par l'hydropathie de la chaîne suggérant l'orientation des domaines de la protéine en relation avec la membrane cytoplasmique. Des récepteurs fonctionnels étiquettés d'un épitope sur l'extrémité C terminale des sous-unités ont été construits dans le but de découvrir l'orientation du domaine comprenant l'extrémité C terminale.

Cette thèse exposera des résultats expérimentaux originaux démontrant la topologie transmembranaire de la sous-unité 5-HT_3 du canal ionique activé par la sérotonine, de même que celle de la sous-unité $P_{2X}3$ du canal ionique activé par l'ATP.

De plus, pour la première fois, un antiserum polyclonal a été élaboré contre la boucle intracellulaire de la sous-unité 5-HT_3 et a été utilisé pour l'immunolocalisation des récepteurs endogènes et étiquettés sur des cellules mammifères transfectées et des western blot.

PREFACE

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examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

In order to conform to the above-stated regulations, I would like to acknowledge the technical assistance of the following people in performing the stated experiments: Mr. Ali Haghighi for electrophysiological recordings from injected *Xenopus laevis* oocytes presented in Chapter 2 and Mr. Michel Paquet for electrophysiological recordings from injected *Xenopus laevis* oocytes presented in Chapter 3. I would also like to acknowledge the kind help of the following technician and students in the lab: Éric Chicoine, Éric Dumont, and Sean Le.

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And last, but most certainly not the least, I would like to thank my colleagues at the Montreal Neurological Institute and all of my other good friends, especially Rosanne.

ABBREVIATIONS

$5\text{-}HT_3R$	serotonin-gated $5\text{-}HT_3$ receptors
5-HT	5-hydroxytryptamine (serotonin)
ACh	acetylcholine
cDNA	complementary DNA
CM	c-myc
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
FITC	fluorescein isothiocyanate
FL	flag
$GABA_AR$	γ-aminobutyric acid receptors
GluR	ionotropic glutamate receptors
GlyR	glycine receptors
GST	glutathione-s-transferase
HEPES	N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid
HRP	horseradish peroxidase
IPTG	isopropylthio-β-D-galactoside
mAChR	muscarinic acetylcholine receptors
mAb	monoclonal antibody
MI-MIV	membrane spanning domain one through four
nAChR	nicotinic acetylcholine-gated receptors
NMDA	N-methyl-D-aspartate
$P_{2X}R$	ATP-gated P_{2X} receptors
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis

(Abbreviations cont'd)

PBS	phosphate buffer saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RT	reverse transcribe
SDS	sodium dodecyl sulfate
TBC	d-tubocurarine
TMD_I-TMD_{IV}	transmembrane domains one through four
wt	wild type

Immunolocalization and Transmembrane Topology of Ligand-Gated Ion Channels By Functional Epitope Tagging

CHAPTER ONE: Introduction.

1.1 OBJECTIVES.

From the simplest multicellular organisms to human beings, the nervous system regulates all aspects of bodily function. Neurons are able to communicate with one another and with other cell types not only by electrical means but also by chemical means. As early as 1904 it was suggested that an electrical impulse traveling in a pre-synaptic cell could cause the release of neurotransmitters into the synaptic cleft, affecting the membrane potential of a post-synaptic cell, thus giving rise to a new impulse, propagating the original message. It has since then been demonstrated that neurotransmitter receptors mediate the conversion of extracellular chemical signals to electrical impulses (Unwin, 1993). The transmission of information between neurons occurs in two basic ways: electrical and chemical. Fast chemical neurotransmission is mediated by ionotropic neurotransmitter-gated ion channels and slow neurotransmission is mediated by metabotropic neurotransmitter receptors.

By studying the transmembrane topology of a membrane protein such as a neurotransmitter-gated ion channel receptor subunit, the structure and mechanism of activation of the protein can be inferred. Membrane-spanning domains are usually critical regions of channel function which may be involved in the formation of the channel pore and ion selectivity. By identifying the regions which span the membrane, extra-membranal regions can be deduced and their involvement in agonist binding, subunit associations, channel regulation, and cytoskeleton anchoring can be tested experimentally.

Based on the hypothetical predictions given by hydropathy plots of the deduced amino acid sequence, the 5-HT_3 serotonin-gated ion channel possesses the classical four peaks of hydrophobicity found in nicotinic receptors [FIG. 1.1]. Predictions of the P2X ATP-gated ion channel global structure from hydropathy plots [FIG. 1.2] seem to indicate a two membrane-spanning domain topology, which would define a new topological arrangement for neurotransmitter-gated ion channels (Brake et al., 1994).

After reviewing the basic principles of neurotransmission, the structure of receptor subunits of neurotransmitter-gated ion channels and their transmembrane topology, this thesis will present original data about how the transmembrane topology of two ion channels belonging to different gene families, the 5-HT_3 serotonin-gated channel and the P_{2X} ATP-gated channel, was investigated.

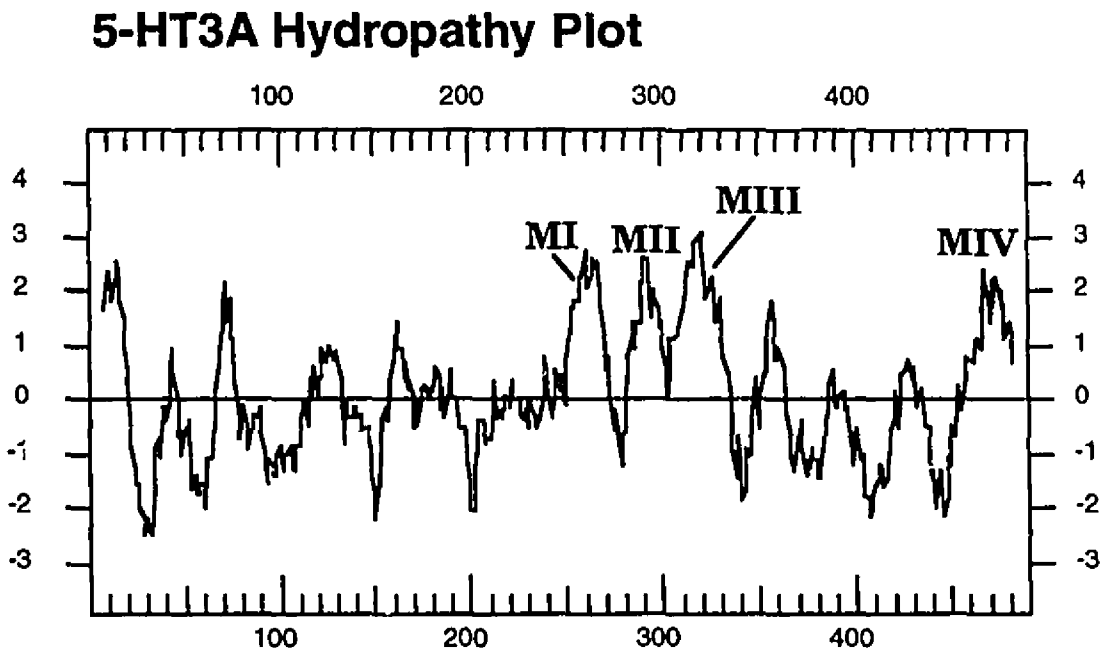

Figure 1.1: Kyte-Doolittle hydropathy profile of the wild-type 5-HT3 receptor.
Putative membrane spanning sequences of the 5-HT3A receptor subunit are indicated in capital letters (MI-MIV).

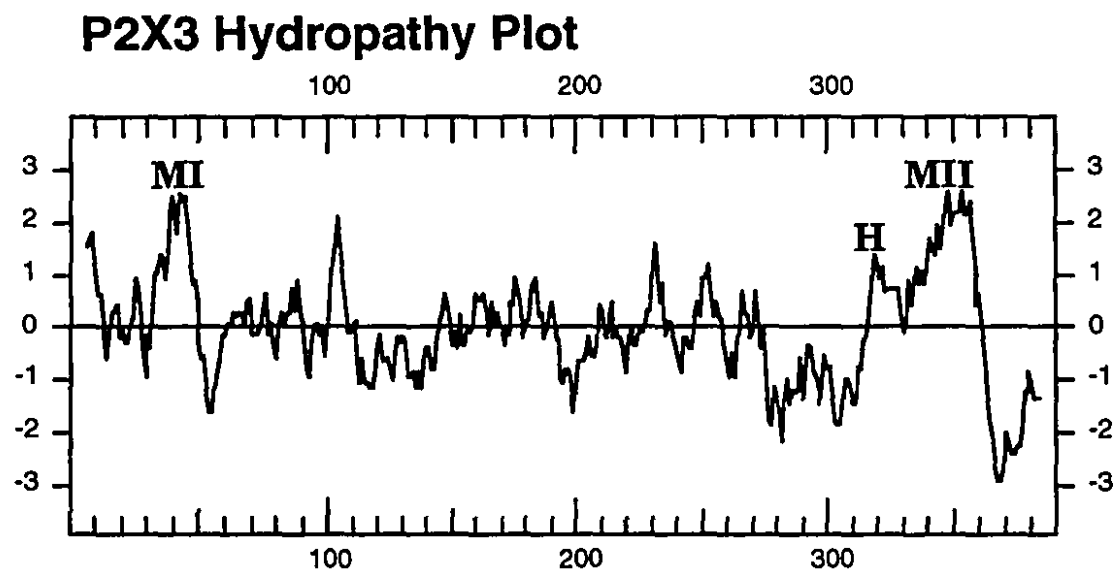

Figure 1.2: Kyte-Doolittle Hydropathy profile of the wild-type P2X receptor.
Putative membrane-spanning sequences and H loop of the P2X3 receptor channel subunit are indicated in capital letters (H and MI-MII)

1.2 SYNAPTIC TRANSMISSION.

Neurons make specific contacts with other cells at sites called synapses. The pre-synaptic axon terminal of a neuron sends a signal that is received by a post-synaptic cell. This signaling is mediated by changes in the electrical potential across the cell membrane at the synapse.

At electrical synapses, neurons are connected by gap junctions. A bio-electrical impulse (ions) can pass directly through this junction to the post-synaptic cell. The membrane depolarization associated with an action potential passes through the gap junction to the post-synaptic cell, causing a depolarization of the membrane in this cell which may result in an action potential. This action potential carries the signal. Electrical synapses have the advantage of speed (instantaneous) over chemical synapses (delayed), but the chemical synapse is far more predominant.

The axon terminal of the pre-synaptic cell in a chemical synapse possesses vesicles containing neurotransmitters. Upon the arrival of a nerve impulse to the pre-synaptic cell, these vesicles are exocytosed in a calcium-dependent manner and neurotransmitter is released into the synaptic cleft, the space between the pre- and post-synaptic cell. These neurotransmitter molecules traverse the synaptic cleft and bind to specific receptors on the post-synaptic cell.

The binding of neurotransmitter results in a change in ion permeability of the cell membrane, causing a membrane depolarization or hyperpolarization which may result in an action potential (or a muscle contraction, or the release

of hormone), thereby carrying the signal of the pre-synaptic cell to the post-synaptic cell.

Chemical synapses can be either excitatory or inhibitory in nature whereas electrical synapses are solely excitatory. Another advantage of the chemical synapse is that several types of neurotransmitters can affect several different receptors causing a diversity of biochemical and electrical responses. This complexity of function likely led to the predominancy of this type of synapse.

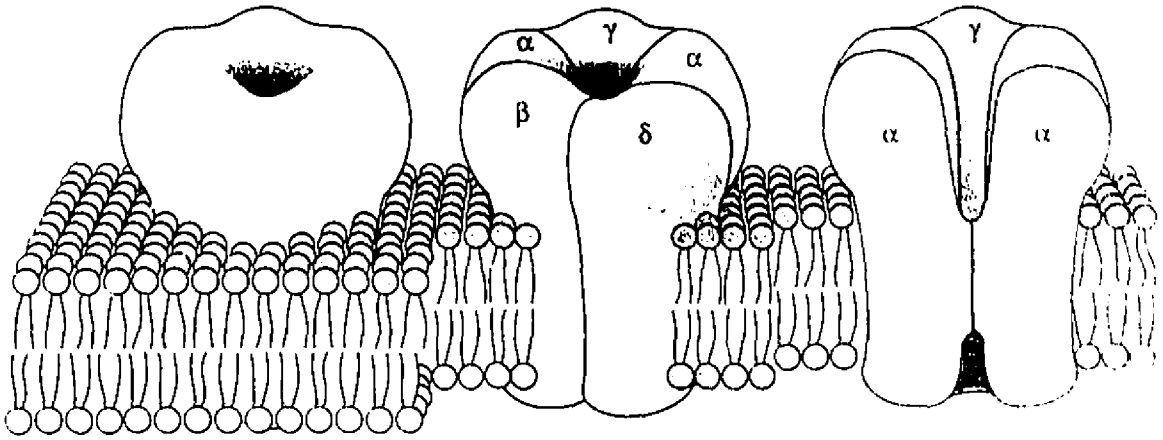

Figure 1.3: The model ACh activated receptor-channel shown integrated within the phosphlipid bilayer. The pore of the channel forms the link between the extracellular environment and the cytoplasm of the cell. (From Kandel et al., 1991).

1.3 NEUROTRANSMITTER-GATED ION CHANNELS.

The cell membrane of a neuron is a phospholipid bilayer. Ion channels are specialized electrotonic proteins integrated within this membrane [FIG. 1.3]. These channels form a link between the extracellular environment and the cytoplasm of the cell. Different ion channels are opened in response to specific stimuli, either 1) changes in the cell membrane potential (which may be caused by many events) or 2) the binding of a ligand on receptors.

Receptors are surface proteins which interact with extracellular signals and convert them into intracellular effects. The receptors of neurotransmitters are integral membrane proteins that belong to two major groups, G protein-coupled receptors and ligand-gated ion channels.

G-protein coupled receptors (Type II receptors) can function in several ways, two of which include: 1) Neurotransmitter binds to the receptor, activating a G-protein which, in turn, can activate adenylate cyclase, phospholipase C, or other effector enzymes that cause a change in cytoplasmic levels of second messengers which may affect ion channels; 2) The activated G-protein can directly bind to an ion channel to modify its activity, as in the case of the muscarinic acetylcholine receptor which is coupled to a K^+ channel.

The neurotransmitter receptor can also be directly coupled to the ion channel protein (Type I receptors). Therefore, when the neurotransmitter binds to the receptor, a conformational change occurs in the receptor/channel protein, resulting in opening of the channel and the flow of ions across the cell

membrane of the post-synaptic cell. Examples of these ligand-gated ion channels include: nicotinic acetylcholine-gated receptors (nAChR), serotonin-gated 5-HT_3 receptors (5-HT_3R), ionotropic glutamate receptors (GluR), γ-aminobutyric acid receptors ($GABA_A$R), glycine receptors (GlyR), and ATP-gated P_{2X} receptors (P_{2X}R).

By cDNA cloning, a number of ligand-gated ion channels have been discovered. There are two known types of acetylcholine receptors, 1) nAChR and 2) muscarinic acetylcholine receptors (mAChR). The nAChR is a ligand-gated channel receptor whereas the mAChR is a G-protein coupled receptor. The nAChR found at the neuromuscular junction has been the "model" receptor for ligand-gated ion channels both in terms of structure and function. The nAChR derived from the neuromuscular junction consists of five subunits (α_2,β,γ,δ) [FIG. 1.4]. Opening of the channel occurs with the cooperative binding of two ACh molecules on the two α subunits of the channel. When quantally released acetylcholine molecules bind to nAChRs on the post-synaptic cells, the post-synaptic cell membrane shows a large increase in ion permeability within 0.1 milliseconds, a characteristic of receptor-channel mediated fast neurotransmission.

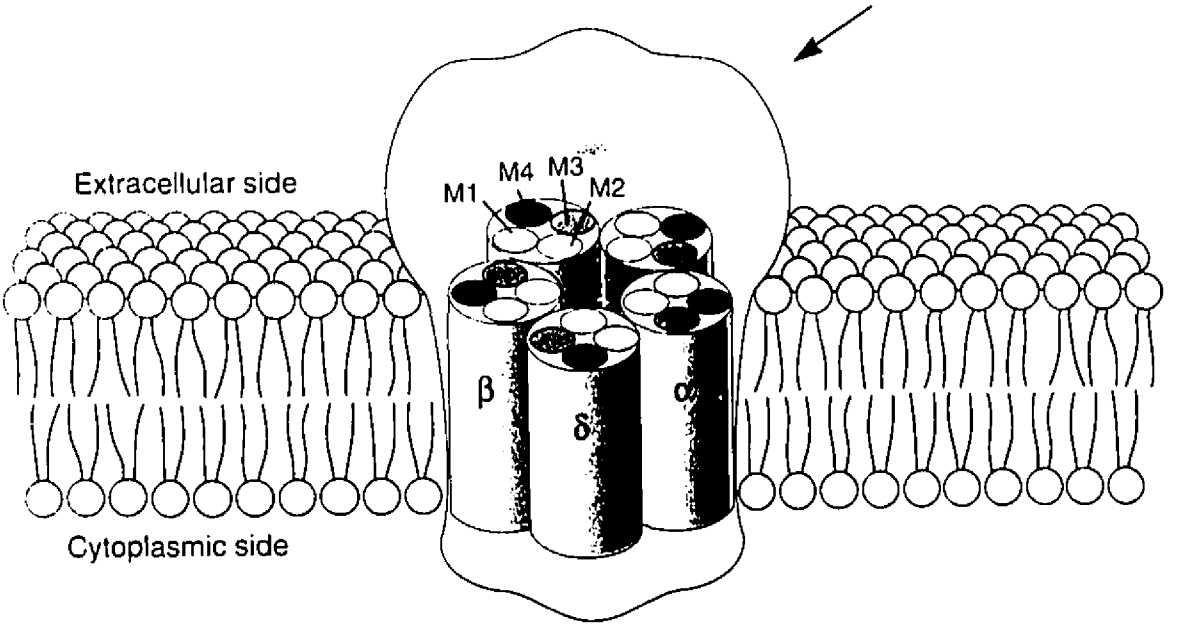

Figure 1.4: The nicotinic acetylcholine receptor-channel of the neuromuscular junction consists of five subunits ($\alpha_2,\beta,\gamma,\delta$). Each of the subunits is shown with four membrane-spanning domains (M1-M4). The M2 membrane-spanning domain is thought to line the channel pore. (From Kandel et al., 1991).

1.4 5-HT_3 SEROTONIN-GATED CHANNELS.

Serotonin (5-hydroxytryptamine or 5-HT) can function both as a neurotransmitter and as a hormone in the peripheral and central nervous systems (Peroutka, 1988; Richardson and Engel, 1986). 5-HT can exert its effects by binding to a number of cell-surface receptor subtypes, designated 5-HT_{1a}, 5-HT_{1b}, 5-HT_{1d}, 5-HT_{1e}, 5-HT_{1f}, 5-HT_{2a}, 5-HT_{2b}, 5-HT_{2c}, 5-HT_3, $5HT_4$, $5HT_{5a}$, $5HT_{5b}$, $5HT_{6,\ and}$ $5HT_7$.

Aside from synaptic transmission, 5-HT can also exert its effects by volume transmission. In this type of neurotransmission released molecules of transmitter can diffuse long distances in the extracellular space before affecting a number of cells sensitive to this transmitter, not only one post-synaptic cell.

Both Type I and Type II receptors are represented in the 5-HT receptor group. However, 5-HT_3 is the only known ligand-gated ion channel receptor in this group which mediates fast responses to 5-HT. The others are G protein-coupled receptor subtypes which mediate slower modulatory responses. By affecting both Type I and Type II receptors, this monoamine can bring about a diverse array of cellular and physiological responses.

5-HT_3 antagonists (such as ICS 205-930 and MDL 72222) have been shown to prevent cytotoxic drug-evoked emesis, a severe side effect of chemotherapy, as well as to have anxiolytic properties (Tyers et al., 1987).

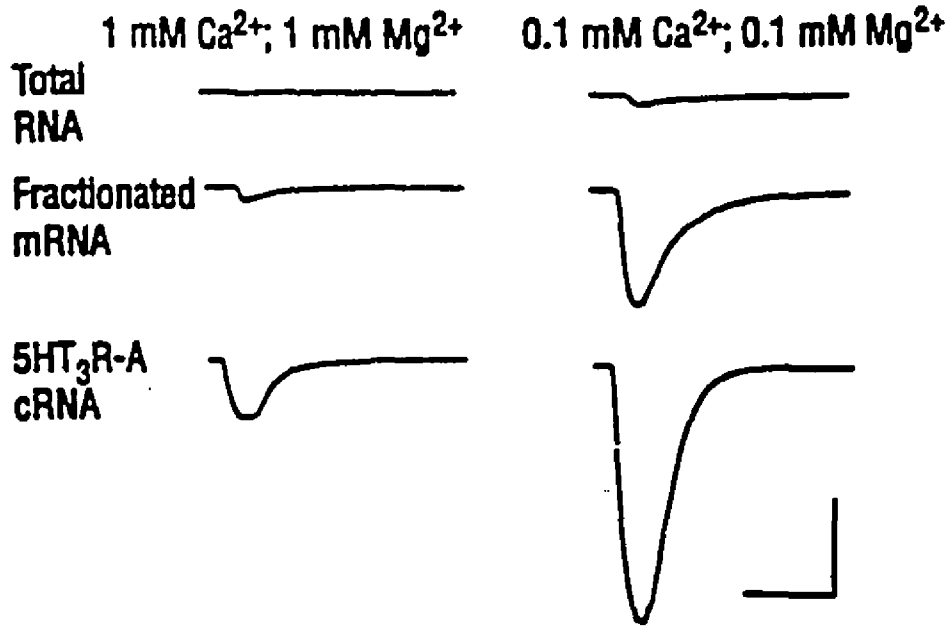

Figure 1.5: Serotonin dependent currents in *Xenopus* oocytes injected alone with mRNA from a mouse neuroblastoma cell line (NCB-20) or RNA transcribed *in vitro*.

A brief bath application of 10 μM 5-HT was given to evoke the rapid inward currents. Calibration bars: 75 nA; 15 s (top two rows), 25 s (bottom row) (from Maricq et al., 1991).

Through the distribution of 5-HT_3 ligands and by electrophysiological recordings, 5-HT_3 receptors have been localized in the 1) enteric nervous system, 2) on sympathetic, 3) parasympathetic and 4) sensory nerve fibers in the central nervous system, and 5) mouse neuroblastoma cell lines (Gehlert et al., 1991; Lummis and Martin, 1991). In the periphery, 5-HT_3 receptors have been shown to function in intestinal contraction and transmission of pain sensation by sensory nerve fibers (Peroutka, 1988). In the central nervous system, the function of 5-HT_3 receptors is still as yet unclear.

Disturbances in serotonergic pathways have been implicated in a wide variety of neuropsychiatric disorders such as depression, anxiety, migraine, and substance abuse (Murphy, 1990).

Using expression cloning, the 5-HT_3 clone was isolated from the NCB-20 mouse neuroblastoma cell line (Maricq et al., 1991). By elucidating the primary structure of this receptor it was shown that a new member of the ligand-gated ion channel family was discovered. When 5-HT_3 RNA transcripts from this clone were injected alone into *Xenopus* oocytes, typical ligand-gated ion channel activation was recorded [FIG. 1.5]. This suggests that the 5-HT_3 receptor may also function as a homo-oligomeric channel in its native conformation. The channel desensitized rapidly in the presence of agonist and upon application of selective antagonists, the current was blocked.

Hydropathy plots are constructed by examining the hydrophobicity of the deduced amino acid sequence. These plots have a predictive value by indicating regions of hydrophobic residues which may span the bilayer.

Analysis of the hydropathy plot of neurotransmitter-gated ion channels of the nicotinic receptor gene family reveals four stretches of hydrophobic amino acids suitable in length to span the lipid bilayer (MI-MIV). The classical transmembrane topology of these ligand-gated ion channels consists of an extracellular N-terminal domain (the signal peptide leader sequence suggests an extracellular N-terminal domain) followed by four membrane-spanning domains (MI-MIV) and an extracellular C-terminal domain. A large intracellular loop connects MIII and MIV. Five subunits are arranged in a "rosette" to form the ion channel. The MII membrane spanning domain of each subunit is thought to line the channel pore and to contain determinants of ion selectivity (Giraudat et al., 1986).

It should be emphasized that structural models based solely upon hydrophobicity analysis must be considered hypothetical in the absence of experimental evidence. Precise three-dimensional analysis of neurotransmitter receptors will be required, like the one offerred by crystallization and X-ray diffraction analysis, which has yet to be realized for transmitter receptors.

The four peaks of hydrophobicity motif is a characteristic attribute of the ligand-gated ion channel superfamily which is also seen in the hydropathy plot of the 5-HT_3 receptor protein [FIG. 1.1]. As mentioned above, this topological arrangement can only be considered hypothetical both due to the lack of supporting experimental evidence and due to the weak homology between members of this gene family in their C-terminal domain.

Another hallmark of this superfamily which is conserved in the 5-HT_3 receptor, are two cysteines, likely forming a Cys-Cys loop, positioned exactly 14 residues apart, which are located in the extracellular N-terminal domain.

1.5 P_{2X} ATP-GATED CHANNELS.

ATP (adenosine 5'-triphosphate) can act through a variety of receptors in nervous tissue, from G protein-coupled receptors to ligand-gated ion channels (Evans et al., 1992). ADP, UTP, diadenosine polyphosphates, and other nucleotides can also act as neural signaling substances (Barnard et al., 1994). It has been demonstrated that ATP can be found within synaptic vesicles and that the release of ATP from the vesicles can be modulated by presynaptic action potentials in a calcium-dependent manner (Zimmerman, 1994).

ATP receptors include P_{2Y}, P_{2U}, P_{2X}, P_{2T}, and P_{2Z} receptor subtypes. P_{2X} is the only ATP-gated ion channel receptor, the others are G protein-coupled receptors, aside from P_{2T} and P_{2Z} for which no structural information is available. It has also been shown that ATP can act as a fast excitatory synaptic transmitter at central and peripheral synapses through P_{2X} receptors (Evans et al., 1992).

P_{2X} receptor subtypes were cloned from the rat vas deferens (Valera et al., 1994), from PC12 cells (Brake et al., 1994) and from rat brain (Séguéla et al., personal communication).

From their deduced amino acid sequence, these three clones have been shown to be members of the same gene family. They contain ten conserved cysteine residues in their putative extracellular loop as well as several conserved N-linked glycosylation sites. In addition, the region preceding the

putative second membrane-spanning domain of these receptors contains a sequence that resembles a motif also found in voltage-gated potassium channels, inward rectifier potassium channels, and cyclic-nucleotide-gated cation channels. This motif is thought to form a hemi loop (H loop) lining the channel pore (Li et al., 1994).

P_{2X} receptors resemble other members of the ligand-gated ion channel superfamily in several aspects involving channel function such as rapid kinetics, strong desensitization, and a selective ionic pore (Brake et al., 1994). Because of the functional similarities of the receptor with the nicotinic acetylcholine receptor and other members of the ligand-gated ion channel family, it was expected that the P_{2X} receptor would possess a similar structure. However, from the deduced amino acid sequence, hydropathy analysis [FIG. 1.2] shows the structure of this receptor to contain only two hydrophobic stretches of amino acids sufficiently long to be membrane-spanning domains. Due to the lack of a leader signal peptide sequence, it is expected that the N-terminal domain is intracellular which leads to the assumption that the C-terminal domain is located intracellularly as well. A large extracellular loop, containing N-linked glycosylation sites and several conserved cysteine residues would form the bulk of the protein. Although this model can only be considered hypothetical, it is clear that a new class of ligand-gated ion channels has been discovered.

In the *Xenopus* oocyte expression system, only a single channel subunit cDNA is needed for functional expression of an ATP-gated current, suggesting that P_{2X} may function as homomeric receptors (Valera et al., 1994). However, this result does not prove that the P_{2X} receptor functions as a homo-oligomeric

channel in the central nervous system.

It has also been suggested that the P_{2X} receptor may not only play a role in fast synaptic transmission, but also in programmed cell death (apoptosis) (Brake et al., 1994; Zambon et al., 1994). In much the same way that glutamate is involved in cytotoxic NMDA-mediated cell death, it may be possible that ATP acts at P_{2X} receptors to increase intracellular calcium levels to induce cell death.

1.6 TRANSMEMBRANE TOPOLOGY STUDIES OF NEUROTRANSMITTER-GATED ION CHANNELS

By studying the transmembrane topology of a membrane protein such as a ligand-gated ion channel receptor subunit, one gains insight about the structure and mechanism of activation (from ligand binding to channel opening) of the protein.

The goal of the study of the topology of a membrane protein is to determine the orientation of various stretches of amino acids with respect to the plasma membrane and also to determine which stretches are buried within the membrane itself.

Hydropathy analysis of the deduced amino acid sequence of membrane proteins can suggest a hypothetical transmembrane arrangement. A perpendicularly arranged α helix would require a stretch of approximately 20 hydrophobic amino acid residues to span the bilayer. However, it must again be emphasized that these studies must be interpreted carefully since a hydrophobic stretch of amino acids may merely be inserted into the membrane and loop back out on the side of insertion rather than span it. Topological profiles deduced using this form of analysis must therefore be considered hypothetical in the absence of supporting experimental evidence. For example, the hydropathy plot of glutamate receptor subunits reveals four stretches of hydrophobic residues which could span the bilayer. However, after extensive investigation (as described below) it was shown that these subunits possess only three membrane-spanning domains.

In order to provide a more precise model of topological arrangement, other experimental techniques must be used. The techniques described here provide two-dimensional information concerning membrane protein topology. A three-dimensional model would require high resolution crystallographic information, which has yet to be realized for most membrane proteins.

The nicotinic acetylcholine receptor (nAChR) has been used a model for topological studies concerning ligand-gated ion channels. Several methods of investigation have been used to probe the transmembrane topology of this receptor group including N-linked glycosylation, anti-peptide antibodies, and epitope insertion.

N-linked glycosylation only occurs on extracellular domains of plasma membrane proteins. Therefore, glycosylation at a site on a protein can be used as direct evidence of the extracellular location of that particular site. By examining native and engineered glycosylation sites in an *in vitro* translation system or in transfected mammalian cells (*in vivo* expression system), it is possible to determine the orientation of regions of subunit protein by verifying the shifts in molecular weight of the native and mutated translation products.

In order to study the orientation of the N-terminal of the mouse muscle α subunit of the nAChR, N-linked glycosylation sites were introduced (Chavez and Hall, 1991). Mutated constructs were examined for glycosylation both in transfected mammalian cells and in an *in vitro* translation system. It was found that all engineered sites were glycosylated and that the N-terminal domain up to at least amino acid 207 is located extracellulary.

The method of examining N-linked glycosylation sites has also been used in investigating the transmembrane topology of ionotropic glutamate receptors. By using site-specific mutagenesis, a native consensus glycosylation site of the GluR6 subunit located within the putative intracellular loop between MIII and MIV was removed (Roche et al,. 1994). This site was analyzed before and after mutagenesis. Following mutagenesis, glycosylated protein was not detected. These results lead to a new proposal for glutamate receptor topology. In this topological model, it is suggested that five membrane-spanning domains exist, the additional domain located between MIII and MIV.

In another study examining the topology of the GluR1 subunit, N-linked glycosylation consensus sequences were engineered at different sites along the entire length of the protein (Hollmann et al., 1994). In the *Xenopus laevis* oocyte expression system, the mutant receptors were analyzed for glycosylation. In this model, it is suggested that MII does not traverse the membrane, that only three membrane-spanning domains exist, and that the N- and C-terminal domains are on opposite sides of the membrane, the N-terminal domain being extracellular.

An independent study confirming the three membrane-spanning domain topology of glutamate receptors involves goldfish kainate receptors (Wo and Oswald, 1994). In this study, N-linked glycosylation sites were examined in an *in vitro* translation system to reveal an intracellular C-terminal domain with only MI, MIII and MIV spanning the membrane.

Site-directed antipeptide antibodies can be raised against specific epitopes of the receptor protein. In general, epitopes are selected on the basis of hydropathy plots for their putative intracellular or extracellular location. Using labeled secondary antibodies, the specificity of the antipeptide antibody for the epitope can be exploited to determine the sidedness of the particular epitope with respect to the plasma membrane

The topology of the GluR1 subunit of the glutamate receptor was studied using site-directed antipeptide antibodies (Molnár et al., 1994). These sequence-specific antibodies were used to examine the orientation of their respective specific epitopes with respect to the membrane by electron microscopic examination of the granule cells of the dentate gyrus after immunocytochemistry. It was concluded in this report that the N- and C-terminal domains are located on opposite sides of the membrane, the N-terminal domain being extracellular. This topological model would suggest that the subunit possessed an odd number of membrane-spanning domains, differing from the "classical" membrane topology of nicotinic receptor channels.

A new putative transmembrane topology has been identified for ligand-gated ion channels for the P_{2X} receptor subunit. This topology resembles that of the amiloride-sensitive sodium channel and the mechanosensitive channels found in *C. elegans* and differs from the classical four membrane-spanning domain topology.

From hydrophobicity analysis of the predicted P_{2X} channel subunit (Valera et al., 1994; Brake at al., 1994), it has been noted that only two hydrophobic stretches of amino acids are sufficiently long to span the

membrane. The intervening sequence between these two putative transmembrane domains contains several potential N-linked glycosylation sites suggesting that this region would be an extracellular loop. Therefore, the topological profile of this receptor would contain intracellular N- and C-terminal domains with the majority of the protein being extracellular.

1.7 REPORTER EPITOPES

Immunological techniques can also be used to probe the transmembrane topology of a membrane channel protein. These include site-directed antipeptide antibodies, prolactin epitope insertion followed by proteolysis and immunoprecipitation, and a unique method involving a reporter epitope and reporter antibody. Studies involving these tools exploit the specificity of the particular antibody for its respective epitope in order to determine the sidedness of that epitope with respect to the plasma membrane. In this section, immunological techniques involving the insertion of a specific epitope will be discussed.

The prolactin reporter epitope method involves inserting the epitope at specific sites within the membrane protein. After translation in microsomal vesicles the orientation of these epitopes is then assayed by verifying proteinase K sensitivity by immunoprecipitating the proteolysis product with anti-prolactin antibodies. Regions which are sensitive to digestion are deemed to be extracellular, whereas protected regions are intracellular and may be immunoprecipitated.

The prolactin reporter epitope was used in addition to examining N-linked glycosylation sites in order to investigate the topology of the GluR3 subunit of the glutamate receptor (Bennett and Dingledine, 1995). Again, in contrast to the "classical" membrane topology of ligand-gated channels, in this study it was found that this subunit contains an intracellular C-terminal domain, extracellular N-terminal domain, an extracellular loop between MIII and MIV, and that MII does not traverse the membrane, but merely enters the

plasma membrane and loops back intracellularly.

In support of the four transmembrane domain topological profile, it was shown by proteolysis protection that both the α and δ subunits of the mammalian muscle nAChR conform to the model (Chavez and Hall, 1992). This investigation involved inserting the prolactin epitope into specific positions downstream of all four putative membrane-spanning domains. The sensitivity of the reporter to proteinase K digestion was then analyzed. Epitopes located intracellularly following the membrane-spanning domain were protected from digestion.

A novel technique was used to study the transmembrane topology of the α1 subunit of the nicotinic acetylcholine receptor (Anand et al., 1993). In this study, by site-directed insertion of DNA fragments corresponding to the reporter epitope of a characterized reporter antibody into specific positions of the receptor subunit cDNA, it was possible to map the topological profile of the receptor. After expression in *Xenopus* oocytes, radioactively labeled reporter monoclonal antibodies were allowed to bind to the mutated receptors in the presence or absence of Triton X-100.

Using previously characterized antibodies rather than raising antibodies has the advantage of being less time-consuming and more specific (often antisera cross-react with closely related subunits). Also, using the reporter epitope technique, it is possible to retain the functionality of the channel whereas other insertions (such as glycosylation sites and prolactin epitopes) most often perturb function.

Using this reporter epitope technique, two regions of the α1 subunit of the nicotinic acetylcholine receptor were confirmed (in accordance with the hypothetical profile deduced from the amino acid sequence) in the study described above (Anand et al., 1993). The C-terminal domain was shown to be extracellular and the putative intracellular loop region between the third and fourth membrane-spanning domain was also confirmed.

In the following two chapters, original data is presented where the FLAG and c-myc epitopes and their specific antibodies (M2 and 9E10, respectively) [FIG. 1.6] are used to probe the transmembrane topology of the 5-HT_3 and P_{2X} ligand-gated ion channels. In addition, polyclonal sera have been raised for the first time against a non-conserved domain of the 5-HT_3 receptor and are also used as agents to explore the transmembrane topology of this receptor as well as for immunolocalization in transfected cells and in western blots.

Reporter Epitope	Amino Acid Sequence	Reporter Antibody
FLAG	LEQKLISEEDL	mAb M2
c-myc	LEDYKDDDDK	mAb 9E10

Figure 1.6: Small hydrophilic reporter epitopes used, their amino acid sequences, and their respective reporter antibodies.

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Immunolocalization and Transmembrane Topology of Ligand-Gated Ion Channels By Functional Epitope Tagging

CHAPTER TWO: Immunological characterization and transmembrane topology of 5-HT_3 receptors by functional epitope tagging.

2.1 ABSTRACT

5-HT_3 receptors are the only known monoamine receptors mediating fast excitatory responses in mammalian neurons. Their primary structure as well as their electrophysiological and pharmacological properties show a phylogenetic relation to nicotinic acetylcholine, $GABA_A$ and glycine receptors.

As a prototypical member of this gene superfamily, we investigated the membrane topology of functional homomeric 5-HT_3 receptors using epitope tagging of the channel subunits expressed in heterologous systems. Visualization of 5-HT_3 receptors in transfected COS-7 cells, either in Western blot (Mr 61.2 ± 0.8 K) or *in situ*, was performed with previously characterized antibodies recognizing artificial epitopes as well as with anti-fusion protein antibodies directed against a wild-type receptor intracellular domain.

The extracellular location of the distal C-terminal tagged domain demonstrates the presence of a fourth transmembrane domain in 5-HT_3 serotonin-gated channels. In this region, the significant homology between members of this class of neurotransmitter-gated channels strongly suggests that they have a common transmembrane organization basically different from glutamate-gated and ATP-gated channels.

2.2 INTRODUCTION

The 5-HT_3 receptor is the only known ligand-gated channel that mediates fast ionotropic monoaminergic neurotransmission in the brain and peripheral nervous system of mammals (Jackson and Yakel, 1995). The cloning of rodent 5-HT_3A receptor subunit cDNA by functional expression (Maricq et al., 1991) revealed that these ionotropic 5-HT receptors belong to a gene superfamily including central and peripheral excitatory (nicotinic acetylcholine) as well as inhibitory (GABA, glycine) neurotransmitter receptors (Ortells and Lunt, 1995).

From their predicted primary sequence, all these receptors have a similar profile of hydrophobicity suggesting a conserved transmembrane organization. Some aspects of the structure-function relationships of these integral membrane proteins have been thoroughly studied, using mainly the muscle-type acetylcholine nicotinic receptor as the prototypical member of this superfamily (Devillers-Thiéry et al., 1993, Unwin, 1993). A convergent view is emerging, from site-directed mutagenesis and chemical modification experiments, about critical determinants of the agonist-binding site located in the extracellular N-terminal domain (Devillers-Thiéry et al., 1993), about determinants of 5-HT_3 channels permeability and desensitization (Eisele et al., 1993, Yakel et al., 1993) in the second putative transmembrane domain (TMD). However, in the absence of high resolution topological data, any three-dimensional model of these channels remains predictive and subject to experimental evidence

The lack of unambiguous experimental data about the orientation of the poorly conserved C-terminal domain of the receptor-channel subunits does not allow us to conclude about the existence of a real fourth transmembrane-spanning region in these receptors.

In most cloned subunits of ligand-gated channels, potential phosphorylation sites for various protein kinases are present in a large hydrophilic domain that follows the third putative transmembrane domain (Levitan, 1994). Therefore, the assignment of different parts of the subunit to specific cellular compartments has important consequences on the physiology and post-translational modification of the receptors, as well as on the possible association with cytoskeleton-anchoring proteins (Froehner, 1991, Yu and Hall, 1994).

In this report, we take advantage of the homomeric nature of 5-HT_3 serotonin-gated channels to study the transmembrane topology of their functional epitope-tagged subunits when heterologously expressed in *Xenopus* oocytes and transfected mammalian cells. In addition, the sequence-specific approach used here allows us to report for the first time the immunological characterization and *in situ* localization of 5-HT_3 receptors in mammalian cells.

2.3 MATERIALS AND METHODS

Construction of C-terminally epitope-tagged 5-HT$_3$ receptors

Full-length mouse 5-HT$_3$A subunit cDNA (provided by A.V. Maricq, UCSF) was mutated using PCR amplification with Pfu DNA polymerase (Stratagene), a vector-specific primer and a mutagenic primer replacing the Stop codon with a unique XhoI site, then subcloned in pcDNAI (Invitrogen) previously digested with HindIII-XhoI. Two XhoI-XbaI cassettes containing either the in-frame human c-myc epitope GEQKLISEEDL (Evan et al., 1985) or the Flag epitope DYKDDDDK (IBI) followed by an artificial stop codon and a part of the natural 3' untranslated region of rat proton pump subunit SV-PP116 (Perin et al, 1991) were spliced alternatively to generate c-myc-tagged 5-HT$_3$ (5-HT$_3$-CM) or Flag-tagged 5-HT$_3$ (5-HT$_3$-FL). Subcloning of mutant receptors was checked by restriction pattern analysis and dideoxy sequencing with Sequenase (UBI).

Electrophysiological recording of 5-HT$_3$ currents in Xenopus oocytes

Functional integrity of mutant C-terminally tagged 5-HT$_3$ channels was verified using electrophysiological recording in two-electrode voltage-clamp configuration after expression in *Xenopus* oocytes. Collagenase-treated oocytes were incubated 3-5 days at 19-20 °C in Ringer solution after nuclear injection of 1-3 ng of wild-type 5-HT$_3$ (5-HT$_3$-wt), 5-HT$_3$-FL and 5-HT$_3$-CM supercoiled plasmids in injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES pH 7.4). During recording, cells were bathed in low Ca^{++} (0.1 mM $CaCl_2$) OR2 physiological salt solution containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 15 mM HEPES pH 7.4 (Bertrand et al., 1991). Channels were

challenged with application by perfusion of 10 μM of 5-HT creatinine sulfate (Sigma) alone or in combination with 1 μM d-tubocurarine (Sigma), a characteristic 5-HT_3 antagonist. Oocytes selected for resting potentials between -30 to -50 mV were clamped at Vh= -40 mV using a custom-made voltage clamp apparatus. The signals were digitized at 100 Hz then analyzed with the Patchkit program (Alembic Software).

Development of antiserum against wild-type 5-HT_3 receptors

A PCR product corresponding to nucleotides 1144-1413 or amino acids 362-451 of the mouse 5-HT_3A receptor subunit, a 90 amino acid domain in a putative intracellular loop, was amplified with sense primer SP1 (TGAGGATCCGAGCAGCCTATGGCCCAT) containing a BamHI site and reverse primer RP1 (TCGAATTCTAGAGCCAGTCCCTTGCCACCT) containing both EcoRI and XbaI sites. This insert was subcloned in-frame in the prokaryotic expression vector pGEX-2T (Pharmacia) to generate GST-5-HT_3 fusion protein (FP5). The IPTG-induced FP5 fusion protein of Mr 36.4 ± 1.6 K was purified on PAGE and mixed with Freund's complete adjuvant to use as an immunogen according to a standard immunization procedure in rabbits (Harlow and Lane, 1988). Non-specific binding of IgGs on GST carrier protein was eliminated in positive serum 1232 by adsorption. Specific reactivity against the insert from 5-HT_3 was checked by immunoblot with a homologous maltose-binding protein fusion protein produced by subcloning the same receptor domain in pMAL-c2 vector (NEB) and induction by IPTG.

Cell culture and transfection of COS-7 cells

COS-7 cells (ATCC# CRL 1650) were grown in 90% DMEM + 10% heat-inactivated fetal calf serum. Semi-confluent cells were transfected in 35 mm

dishes using the DEAE-dextran method (Luthman and Magnusson, 1983) using 1 μg of supercoiled plasmid coding for 5-HT_3-wt, 5-HT_3-FL or 5-HT_3-CM. Efficiency of transfection was controlled histochemically with reporter plasmid pCH110 (Pharmacia) coding for b-galactosidase. Cells were harvested for processing 48-72 hours after transfection.

RT-PCR on transfected COS-7 cells

Total RNA was isolated from transfected cells using the guanidine isothiocyanate lysis method. Plasmid DNA was removed by DNase I treatment and XhoI digestion before random-primed reverse transcription with MMLV-RT (Pharmacia), starting from 1 μg of total RNA template. PCR amplification (30 cycles) was performed on 25 ng of RT-cDNA using primers specific for 5-HT_3 subunit message (sense SP1, reverse RP2: AGAATAATGCCAAATGGA) or primers specific for C-terminally tagged 5-HT_3 subunit message (sense SP1, anti-sense RP3: GAGACACAGTCACTGTAG). Negative controls included either no-RT template (data not shown) or RT-cDNA from cells transfected with unrelated plasmid [FIG. 2.3].

Western blot of heterogously expressed 5-HT_3 receptors

Transfected COS-7 cells were washed in PBS and their membranes solubilized by incubation for 1 hour under vigorous agitation in ice-cold hypotonic Tris buffer containing 1% Triton X-100 (Sigma) and generic protease inhibitors PMSF (Sigma, 1 mM) and aprotinin (Sigma, 30 μg/ml). Reducing SDS-containing loading buffer was added to cleared lysate for 45 min at 65°C before loading on a 9% reducing PAGE followed by transfer to nitrocellulose. Detection using peroxidase-based ECL method (Amersham) was performed with GST-adsorbed primary serum 1232 (dil. 1/2000) or mAb M2 (IBI)

(1 μg/ml) and HRP-labelled secondary antibodies goat-anti-rabbit (dil. 1/4000) or goat-anti-mouse (dil. 1/2000), respectively.

Cellular localization of 5-HT_3 receptors in immunofluorescence

Transfected COS-7 cells were washed in PBS, then fixed at room temperature for 20 min in 0.1 M phosphate buffer pH 7.2 containing 4% paraformaldehyde. Cells were subsequently permeabilized or not with 0.05% Triton X-100 (Sigma) for 15 min at room temperature. Expressed 5-HT_3 receptors were visualized by indirect immunofluorescence using primary antibodies from adsorbed serum 1232 directed against FP5 fusion protein (dil. 1/500), anti-Flag epitope mAb M2 (1 μg/ml) or anti-c-myc epitope mAB 9E10 (2 μg/ml, Oncogene Sciences). FITC-labelled secondary goat anti-rabbit (dil. 1/700, Jackson) or Texas Red-labelled goat anti-mouse (dil. 1/200, Jackson) were used for secondary incubation.

2.4 RESULTS

Functional expression of mutant 5-HT_3 receptors

Oocytes injected with wild-type or mutant C-terminally tagged 5-HT_3 cDNA responded to 1-50 μM 5-HT with great sensitivity. Oocytes injected with buffer only or with cDNA coding for acetylcholine-gated channels did not respond to the application of 5-HT (data not shown). After 2-5 days of expression, the fast kinetics and the characteristic desensitization profile of the 5-HT-gated currents (Yakel et al., 1993) were recorded in mutant 5-HT_3-FL and -CM receptors cDNA-injected oocytes. Recordings were not qualitatively different between mutants (FL and CM) [FIG. 2.2] or between mutants and wild-type receptors (data not shown). The holding potential was kept very close to resting potential to avoid saturation of the clamp, an indication of the high efficiency of transcription, synthesis and translocation to the plasma membrane of these homomeric channels in oocytes. All mutant channels were reversibly sensitive to co-application of the antagonist d-tubocurarine with 5-HT, a hallmark of 5-HT_3 receptor pharmacology (Yakel and Jackson, 1988).

Expression of mutant 5-HT_3 receptors in COS-7 cells

To check the correct transcription of full-length 5-HT_3-FL message, RT-PCR was performed on lysed transfected cells after removal of contaminant plasmid DNA by DNase treatment and restriction enzyme digestion. The presence of both wild-type and hybrid receptor sequences was confirmed by amplification with oligonucleotide primers upstream of the natural stop codon and with primers spanning the junction site between the subunit and the artificial epitope, respectively.

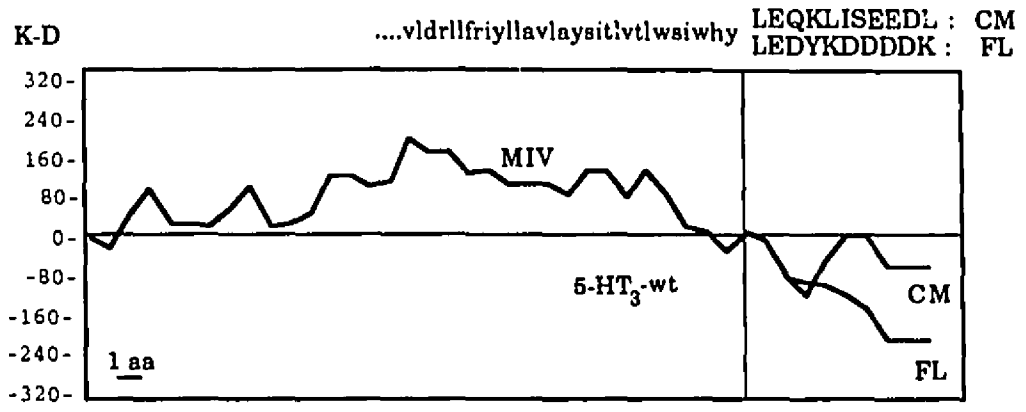

Figure 2.1: Kyte-Doolittle hydropathy profile of the C-terminal domain (including MIV) of wild-type 5-HT3 receptors compared to tagged 5-HT3-CM and 5-HT3-FL receptors.

Artificial epitope sequences inserted at the natural termination codon of the 5-HT3A receptor subunit are indicated in capital letters.

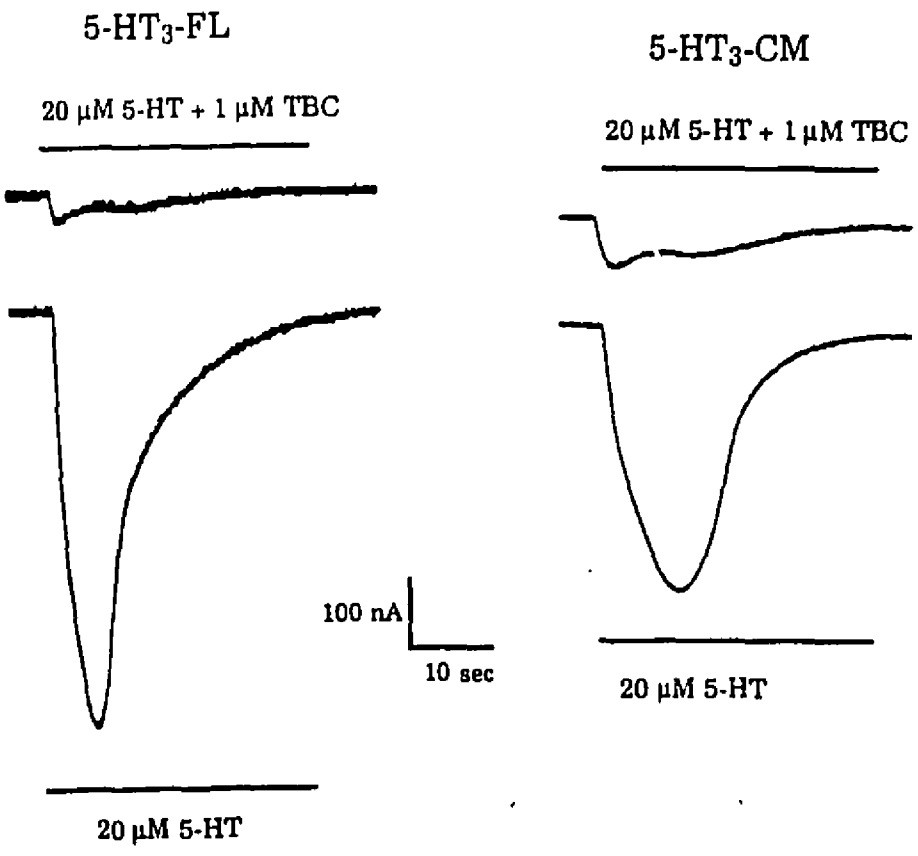

Figure 2.2: Functional expression of mutant C-terminally tagged 5-HT$_3$ channels in *Xenopus* oocytes. The characteristic 5-HT$_3$ antagonist d-tubocurarine (TBC) blocks activation of mutant 5-HT$_3$-FL and 5-HT$_3$-CM receptors when co-applied with agonist 5-HT.

Reverse-transcribed total RNA from cells transfected with α7 nicotinic receptor subunit as negative control did not produce any signal [FIG. 2.3], and no-RT samples gave only a light signal due to low amounts of residual plasmid DNA (data not shown). To visualize the expression of the translated protein, membrane proteins solubilized in Triton X-100 from transfected COS-7 cells were subjected to reducing SDS-PAGE, then blotted and visualized using appropriate receptor-specific immunoreagents. Antibodies against wild-type 5-HT_3A subunit and against Flag epitope detected the same major protein of Mr 61.2 ± 0.8 kDa [FIG. 2.4] corresponding to recombinant 5-HT_3-FL receptors (calculated MW of the mature mutant 5-HT_3-FL receptor subunit= 54.8 kDa). No specific labeling was observed in negative control samples from sham-transfected COS-7 cells or in samples from 5-HT_3-transfected cells processed with pre-immune serum 1232 or irrelevant mouse mAb (data not shown). The noticeable difference (12%) between the calculated and the observed molecular weight is likely due to glycosylation of this membrane protein that contains 3 consensus sites of Asn-linked glycosylation in its extracellular N-terminal domain.

Cellular localization of 5-HT_3 receptors

High levels of expression of wild-type and mutant 5-HT_3 receptors in 10-15% of COS-7 cells were observed between 48 and 72 hours of post-transfection time [FIG. 2.5]. The immunofluorescence signal in permeabilized cells was localized mainly in Golgi, endoplasmic reticulum, nuclear and plasma membranes. Most of the signal in permeabilized cells comes from intracellular compartments, suggesting that a low percentage of synthesized receptors are actually translocated to the plasma membrane. Using antibodies against

natural 5-HT_3 epitopes located in an intracellular domain of the subunit, immunofluorescence was visible only after treatment of the cells with detergent. With antibodies against artificial C-terminal epitopes and in the absence of permeabilization agent, the immunofluorescence signal was visible only in the plasma membrane, delineating a characteristic ring of labeling around positive cells [FIG. 2.5]. With all antibodies tested, patches of labeling corresponding to clusters of receptors on the plasma membrane were observed in transfected cells.

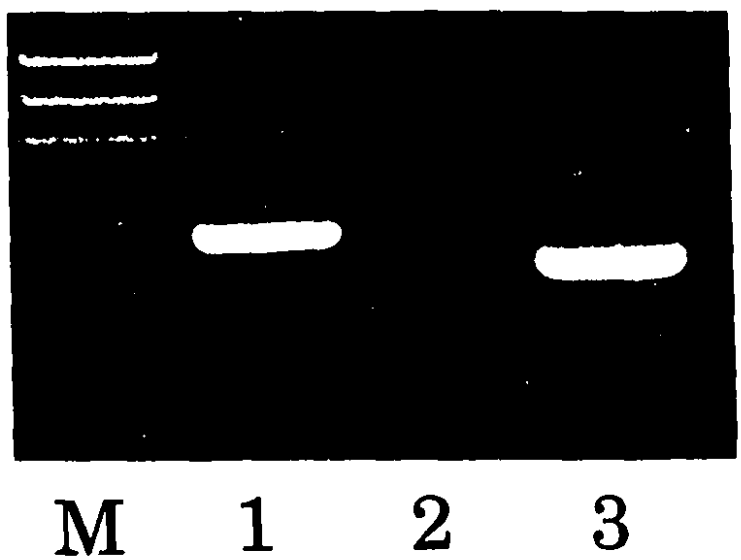

Figure 2.3: Transcription of mutant 5-HT_3 receptors mRNA in transfected COS-7 cells.

Agarose gel electrophoresis of amplified RT-PCR products from cells transfected with 5-HT_3-FL (1, 3) or rat a7 nicotinic receptor subunit (2), using primers specific for epitope-tagged 5-HT_3 subunit (481 bp product, lane 1) and primers specific for 5-HT_3 subunit (386 bp product, lanes 2 and 3) (see Materials and methods). M: PhiX-HaeIII DNA size markers.

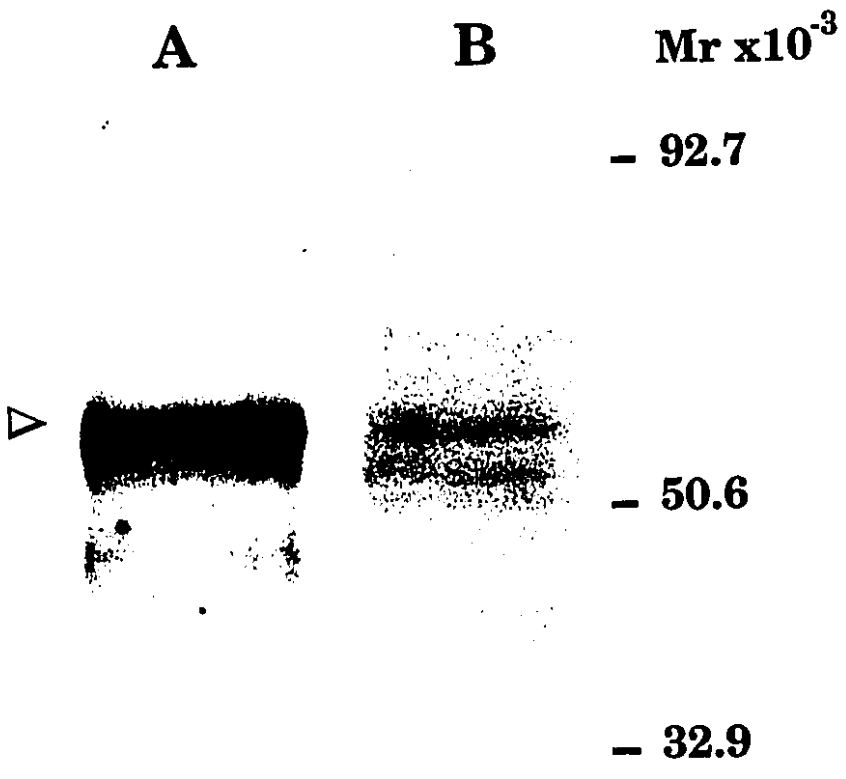

Figure 2.4: Western blot of mutant 5-HT$_3$ receptors in transfected cells.

5-HT$_3$-FL subunits of Mr 61.2 ± 0.8 K (arrowhead) expressed in transfected COS-7 cells are visualized in chemiluminescence with anti-5-HT$_3$ receptor polyclonal antibodies from serum 1232 (A) as well as with anti-Flag epitope mAb M2 (B).

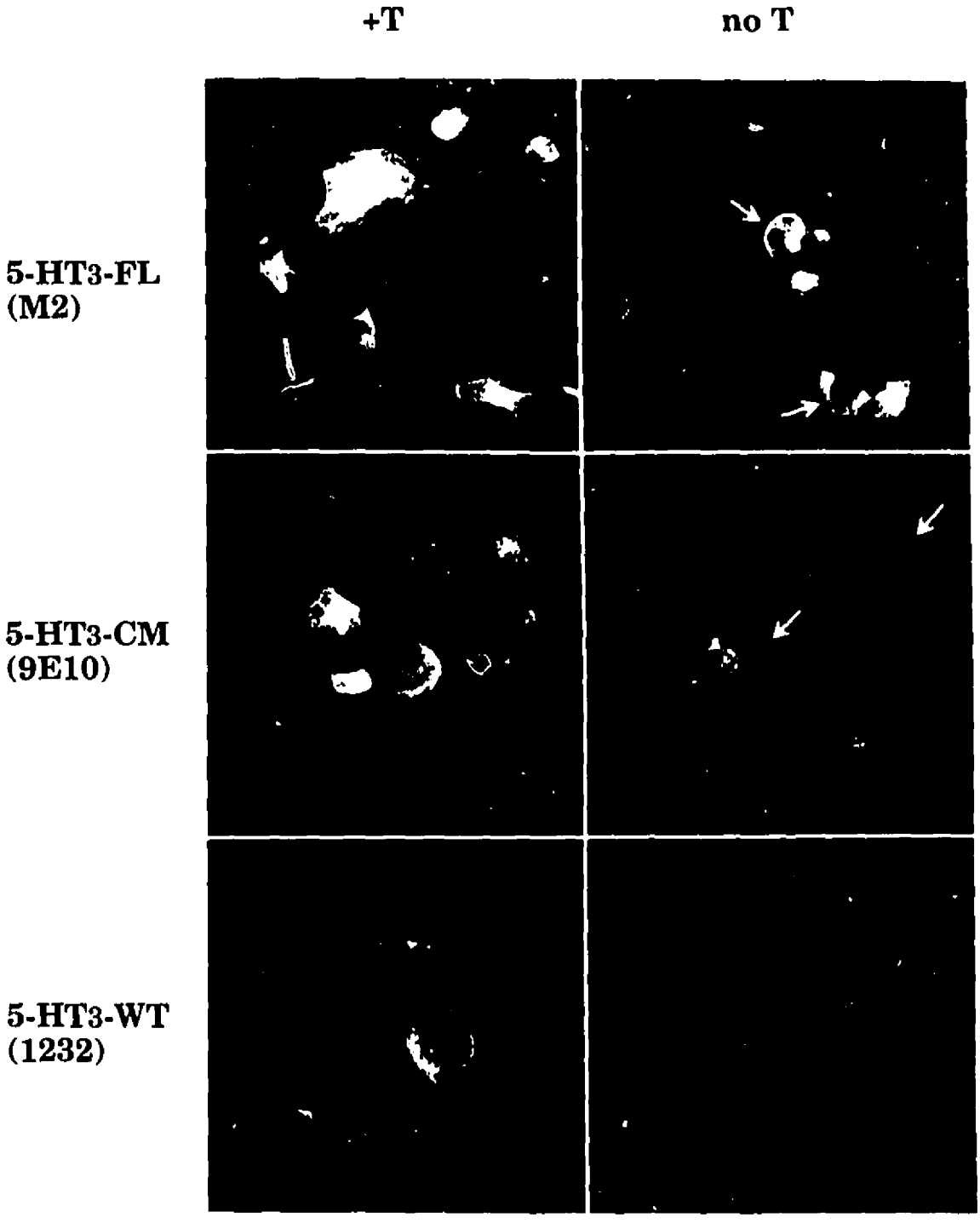

Figure 2.5: Immunolocalization of 5-HT$_3$ receptors in transfected cells. Detection by indirect immunofluorescence of mutant and wild-type 5-HT$_3$ receptors in Triton X-100-permeabilized (+T) vs non-permeabilized (no T) transfected COS-7 cells. Note the characteristic plasma membrane localization (arrows) of 5-HT$_3$ receptors in non permeabilized preparations.

2.5 DISCUSSION

The unambiguous localization of artificial epitopes on the outer side of the plasma membrane of COS-7 cells transfected either with 5-HT_3-FL or 5-HT_3-CM demonstrates that the distal C-terminal domain of the channel subunit is extracellular [FIG. 2.6].

The difference of sequence between the two artificial epitopes Flag and c-myc warrants that this extracellular location is not conferred by the structure of these hydrophilic additions, but is intrinsic to the primary structure of the channel itself [FIG. 2.1].

The fact that these modified channels are functional, i.e. gated by 5-HT and blocked by 5-HT_3 antagonists, demonstrates that the C-terminal domain does not participate critically in agonist-binding or gating, as suggested by the lack of conservation of this distal domain between members of the acetylcholine-gated channels [FIG. 2.7].

The absence of a specific immunofluorescence signal in non-permeabilized cells transfected with 5-HT_3-wt, -FL or -CM using anti-FP5 antibodies as immunoreagents demonstrates that this hydrophilic domain between putative TMDIII and TMDIV is intracellular. From the short distance (38 amino-acids) between the C-terminal portion of the FP5 domain and the artificial epitopes, we conclude that the putative TMDIV is the only possible stretch of hydrophobic amino-acids long enough to span the membrane [FIG. 2.7]. The presence of protein kinase A and tyrosine kinase phosphorylation sites (Maricq et al., 1991) in the hydrophilic domain of 5-HT_3 channels between

TMDIII and TMDIV seems in agreement with the intracellular location of residues 362-451 of the 5-HT_3A receptor subunit.

The alignment of TMDIV and preceding sequences in representative members of the nicotinic receptor gene superfamily suggests strongly that their membrane insertion during synthesis, and thus their membrane topology, is identical [FIG. 2.6]. The 5-HT_3 receptors and homologous acetylcholine-, GABA- and glycine-gated channels thus happen to have a tertiary structure with four TMDs and a global transmembrane organization drastically different from other classes of ligand-gated channels. Glutamate-gated channels of the AMPA and kainate subtypes have three transmembrane domains, with a distinctive hemi-loop between the first two, as demonstrated by the analysis of glycosylation mutants and epitope mapping (Hollmann et al., 1994; Bennett and Dingledine, 1995). The presence of protein kinase sites (Raymond et al., 1993) in what is assumed now to be an extracellular domain of the pharmacophore of glutamate-gated channels (Stern-Bach et al., 1994), confirms that biochemical arguments of topology have to be interpreted cautiously. On the other hand, ATP-gated channels of the P_{2X} subtypes, with physiological properties similar to nicotinic receptors, have a predicted structure with only two transmembrane segments and thus seem phylogenetically more related to K-selective inward rectifier channels than to other neurotransmitter receptors (Surprenant et al., 1995).

We show here that the use of artificial epitopes inserted in serotonin-gated channels allows their visualization in heterologous expression systems even in the absence of subtype-specific antibodies directed against the wild-type receptors. This methodology thus confers a unique specificity for 5-HT_3

channel subunits without altering the pharmacological or electrophysiological properties of the receptor complex. The C-terminal domains of most integral proteins not being functionally critical, this method of tagging with the hydrophilic peptides Flag and c-myc should be applicable to a wide range of neurotransmitter- and voltage-gated channels (Anand et al., 1993). C-terminal functional tagging should also be particularly helpful to characterize the stoichiometry and the native subunit composition of heteromeric receptors.

From our data obtained in transfected COS-7 cells, we observed that the subcellular distribution of these channels is confined to membrane compartments. However, the bulk of fully translated receptors, visualized with antibodies recognizing the most C-terminal part of the subunit, remains in intracellular compartments and is not translocated to the plasma membrane. This situation seems the rule in this family of proteins, as suggested by the accumulation of nicotinic receptors in intracellular stores of sympathetic (Jacob et al., 1986) as well as central neurons (Hill et al., 1993). The role of these non-translocated proteins and their status of functionality remain to be established.

Our observation of patches or clusters of immunoreactivity in the plasma membrane of transfected cells suggests that these receptors are subject to clustering in heterologous expression systems. Further investigations will have to determine if this aggregation is due to the presence of an internal protein motif of clustering or anchoring in the 5-HT_3 channel sequence and/or to a clustering factor found in non-neuronal as well as neuronal cells.

We report here for the first time the immunolocalization of 5-HT_3A channel subunits in mammalian cells using characterized anti-fusion protein polyclonal antibodies. *In situ* cellular and subcellular immunocytochemical localization of 5-HT_3 receptors in neuronal preparations allowed by these antibodies will facilitate the identification of neuronal populations expressing ionotropic 5-HT receptors for a better understanding of the role of 5-HT-mediated fast neurotransmission in central and peripheral nervous systems.

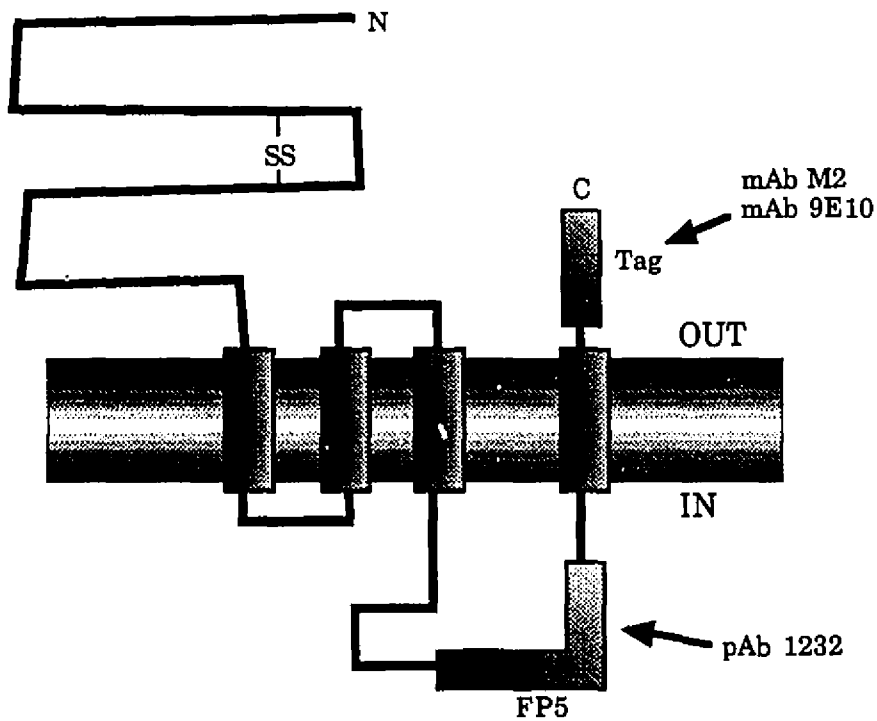

Figure 2.6: Topological model of 5-HT_3 receptor.

Proposed transmembrane organization of 5-HT_3 receptor subunit containing artificial C-terminal epitopes (Tag). Intracellular domain FP5 used for the development of anti-receptor polyclonal antibodies is located between TMDIII and TMDIV and corresponds to residues 362-451 of the 5-HT_3-A receptor subunit. The cysteine loop formed by Cys161 and Cys175 in the extracellular N-terminal domain of 5-HT_3 is symbolized by -SS-.

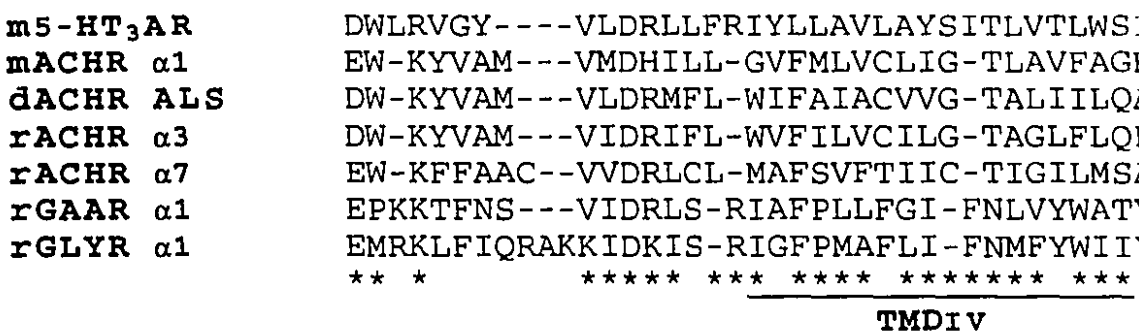

Figure 2.7: Alignment of TMDIV of 5-HT$_3$-A subunit with other neurotransmitter-gated channel subunits.
Significant homology of TMDIVs and upstream sequences is found between various members of the nicotinic receptor gene family. Amino acids are considered similar for alignment if in the same physico-chemical group: hydrophobic (A, I, L, M, F, P, W, V), polar uncharged (N, C, Q, G, S, T, Y), positively charged (R, K, H) or negatively charged (D, E). ACHR: acetylcholine receptor, GAAR: GABA-A receptor, GLYR: glycine receptor, ALS: alpha-like subunit. m: mouse; d: drosophila; r: rat.

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Immunolocalization and Transmembrane Topology of

Ligand-Gated Ion Channels By

Functional Epitope Tagging

CHAPTER THREE: Preliminary data on P_{2X} ATP-gated channel topology.

3.1 INTRODUCTION

As mentioned in sections 1.5 & 1.6, a putative transmembrane topology has been described for the P_{2X} ATP-gated ion channel group. Examination of the hydropathy plot [FIG. 1.2] of the deduced amino acid sequence shows two hydrophobic stretches of amino acids suitable in length to span the phospholipid bilayer (Surprenant et al., 1995). The lack of a leader peptide signal sequence leads to the assumption of an intracellular N-terminal domain. According to the plot, the first transmembrane domain would be followed by a large extracellular loop containing potential N-linked glycosylation sites, followed by the second transmembrane domain and an intracellular C-terminal domain (Brake et al., 1994; Valera et al., 1994).

In order to experimentally investigate the transmembrane topology of this channel subunit, several techniques may be used, some of which may be more time-consuming than others.

In the present study, using the method of reporter epitope tagging described in section 1.7, characterized antibodies to artificial epitopes [FIG. 1.6] are used to probe the transmembrane topology of P_{2X} receptors. This elegant method is much less laborious than raising monoclonal or polyclonal antibodies which may or may not recognize the receptor. A DNA cassette encoding the FLAG and c-myc epitopes is inserted into the coding region of the P_{2X} gene, maintaining the open reading frame. Subcloning of this mutant receptor into expression vectors allows for successful transfection into mammalian cells and for functional expression in *Xenopus* oocytes.

3.2 MATERIALS AND METHODS

Construction of C-terminally epitope tagged P_{2X} receptors

Full-length P_{2X} subunit cDNA was mutated using PCR amplification with Pfu DNA polymerase (Stratagene), a vector-specific primer and a mutagenic primer replacing the Stop codon with a unique XhoI site, then subcloned in pcDNAI (Invitrogen) previously digested with HindIII-XhoI. Two XhoI-XbaI cassettes containing either the in-frame human c-myc epitope GEQKLISEEDL (Evan et al., 1985) or the Flag epitope DYKDDDDK (IBI) followed by an artificial stop codon and a part of the natural 3' untranslated region of rat proton pump subunit SV-PP116 (Perin et al., 1991) were spliced alternatively to generate c-myc-tagged P_{2X} (P_{2X}-CM) or Flag-tagged P_{2X} (P_{2X}-FL). Subcloning of mutant receptors was checked by restriction pattern analysis and dideoxy sequencing with Sequenase (UBI).

Eletrophysiological recording of P_{2X} currents in Xenopus oocytes

Functional integrity of wild-type and mutant C-terminally tagged P_{2X} channels was verified using electrophysiological recording in two-electrode voltage-clamp configuration after expression in *Xenopus* oocytes. Collagenase-treated oocytes were incubated 3-5 days at 19°C in Barth's solution after nuclear injection of 8-10 ng of wild-type P_{2X} (P_{2X}-wt), and P_{2X}-FL supercoiled plasmids in injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES pH 7.4). During recording, cells were bathed in Ca^{++} (1 mM $CaCl_2$) Ringer's solution containing 97 mM NaCl, 3 mM NaOH, 2 mM KCl, 1 mM $CaCl_2$, 10 mg/ml Phenol Red, 10 mM HEPES pH 7.4. Channels were challenged with application by perfusion of 1 μM of ATP (Sigma) alone or in combination with 100 μM

Reactive Blue 2 (RBI), a characteristic P_{2X} antagonist. Oocytes were selected for a resting potential between -30 to -50 mV. Experiments were done with a two-electrode voltage clamp amplifier (OC-725B, Warner Instrument Corp.). Amplifier and acquisition were under the control of Axodata (Axon Instruments) on a Macintosh platform. The signals were sampled at 500 Hz and analyzed with Axograph (Axon Instruments) and Igor (WaveMetrics).

Cell culture and transfection of HEK-293 cells

HEK 293 cells were grown in DMEM + 10% heat-inactivated fetal bovine serum. 30-50% confluent cells were transfected in 100 mm dishes using the calcium phosphate method (Profection, Promega) using 10 µg of supercoiled plasmid coding for $P_{2X}3$-wt or $P_{2X}3$-FL. Cells were trypsinized and plated in eight chamber slides (SuperCell Culture Slides, Fisher) at 80 x 10^3 cells per chamber and grown 24 hours before fixation..

Cellular localization of P_{2X} receptors in immunofluorescence

Transfected HEK-293 cells were washed in PBS, then fixed at room temperature for 20 min in 0.1 M phosphate buffer pH 7.2 containing 4% paraformaldehyde. Cells were subsequently permeabilized or not with 0.05% Triton X-100 (Sigma) for 15 min at room temperature. Expressed P_{2X} receptors were visualized by indirect immunofluorescence using anti-Flag epitope mAb M2 (1 µg/ml, IBI). or anti-c-myc epitope mAb 9E10 (2 µg/ml, Oncogene Sciences). Texas Red-labelled goat anti-mouse (dil. 1/200, Jackson) was used for secondary incubation.

3.3 CELLULAR LOCALIZATION OF P_{2X} RECEPTORS

It has been convincingly demonstrated using monoclonal antibodies M2 which is directed against the FLAG epitope and 9E10 which is directed against the c-myc epitope, that the C-terminal domain of the P_{2X} receptor in transfected mammalian HEK-293 cells has an intracellular location due to the lack of an immunofluorescence signal in non-permeabilized cells and the presence of this signal in permeabilized cells [FIG. 3.1]. This result confirms the proposed C-terminal domain localization deduced from hydropathy plots [FIG. 3.2].

Considering this result, it seems that the P_{2X} ATP-gated ion channel has a similar transmembrane topology to the amiloride sensitive sodium channels which consists of only two membrane-spanning domains (Snyder et al., 1994; Renard et al., 1994), differing greatly from known topological profiles of other ligand-gated ion channels.

Both the wild-type and epitope-tagged mutant receptors have similar physiological profiles [FIG. 3.3] indicating that the insertion of the reporter epitopes do not perturb or significantly alter channel function. The physiological properties of wild-type versus mutant tagged P_{2X} receptors also ensures that the addition of the small hydrophilic sequence (10-11 amino acids) does not affect or modify the receptor's structure in terms of transmembrane topology.

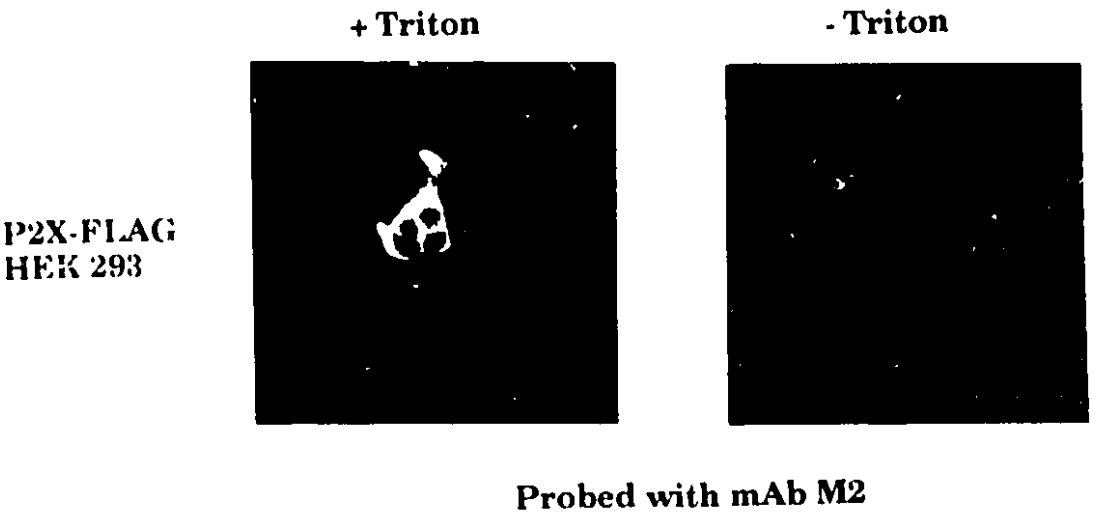

Figure 3.1: Immunolocalization of $P_{2X}3$ receptors in transfected cells.

Detection by indirect immunofluorescence of mutant $P_{2X}3$ receptors in Triton X-100-permeabilized (+ Triton) vs non-permeabilized (- Triton) transfected HEK 293 cells.

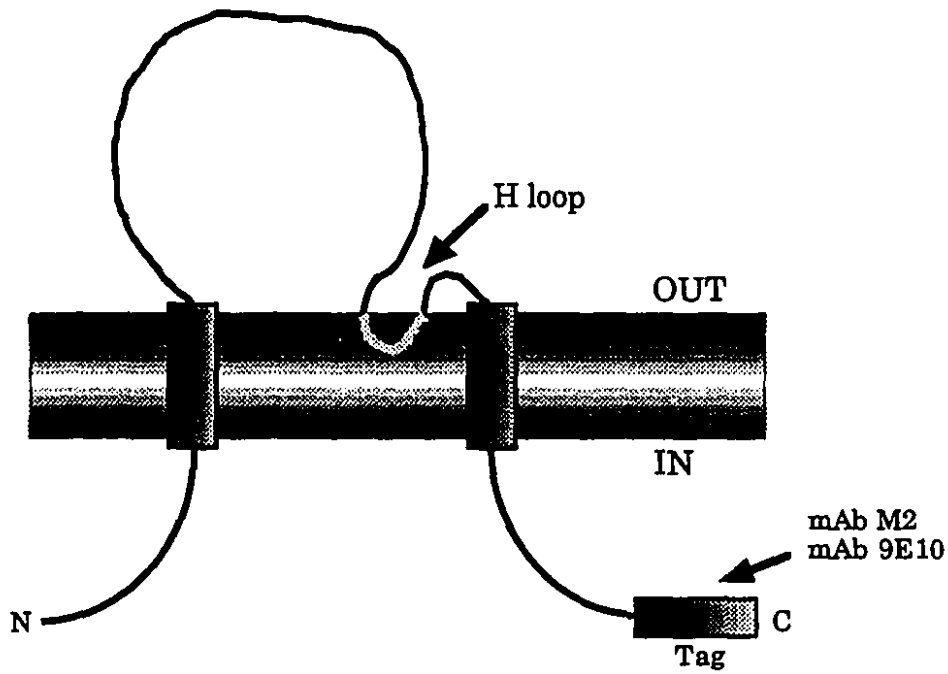

Figure 3.2: Topological model of the P_{2X} receptor.

Proposed transmembrane organization of P_{2X} receptor subunit containing artificial C-terminal epitopes (Tag).

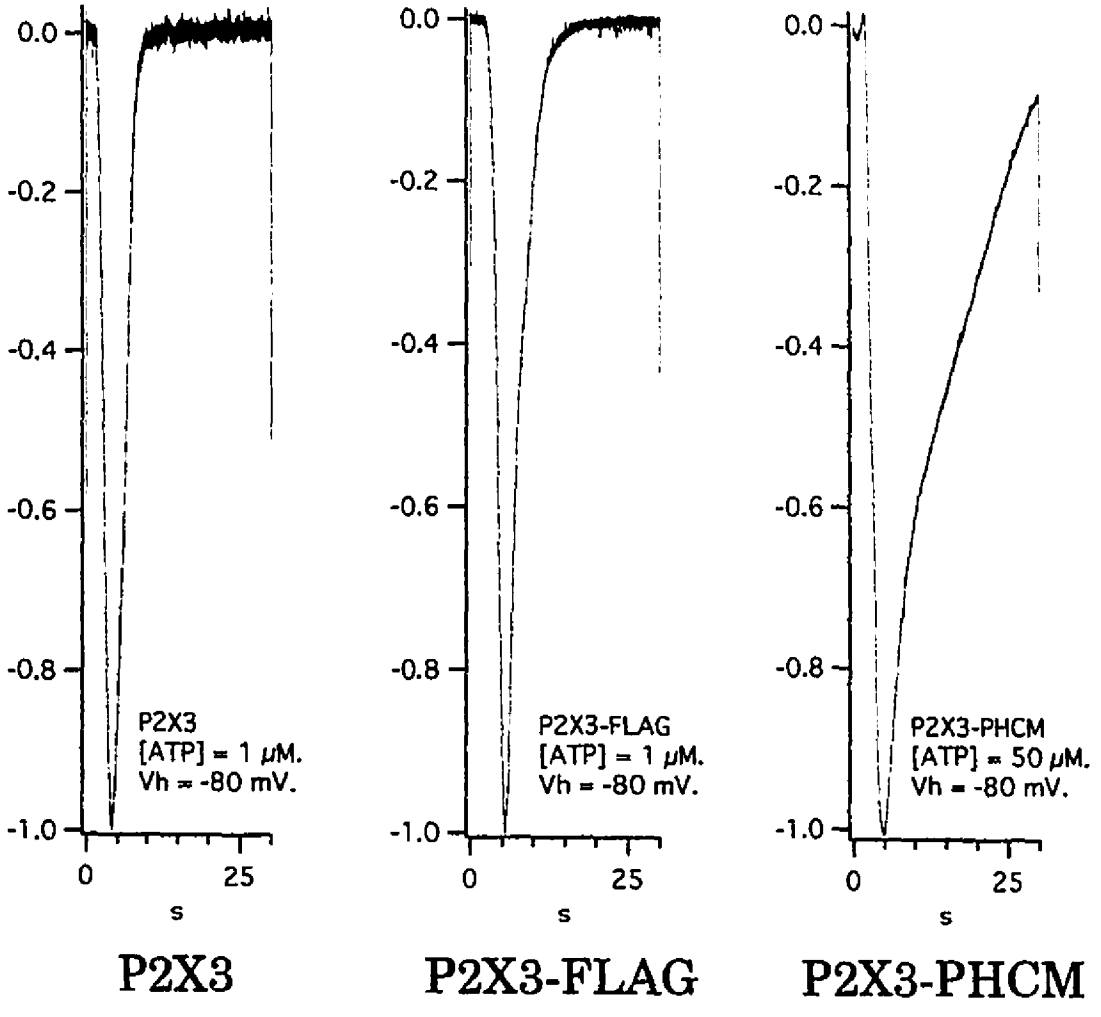

Figure 3.3: Functional expression of mutant C-terminally tagged and wild-type $P_{2X}3$ channels in *Xenopus* oocytes

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Ligand-Gated Ion Channels By

Functional Epitope Tagging

CHAPTER FOUR: Conclusion.

CONCLUSION

Neurotransmitter-gated ion channels mediate fast chemical synaptic transmission. This superfamily of channels includes the nAChR, ionotropic GluR, GlyR, 5-HT_3R, GABAR, and the P_{2X}R.

Plots analyzing the hydrophobicity of the deduced amino acid structure of receptor subunits can be used to construct a putative transmembrane topology profile. A span of approximately twenty hydrophobic amino acids arranged in a perpendicular a helix would be sufficient to span the phospholipid bilayer. However, without the support of experimental evidence, topological profiles deduced from this type of analysis can only be considered theoretical.

In this thesis, original data has been presented demonstrating the localization of the poorly conserved C-terminal domain of the 5-HT_3 serotonin-gated ion channel as well as the C-terminal domain of the P_{2X} ATP-gated channel, a channel which is a member of a new family of ligand-gated ion channels.

In order to probe the transmembrane topology of these channel subunits, the method of epitope tagging has been implemented. Receptor subunits were tagged at the C-terminal domain of the subunit with specific epitopes for which characterized antibodies are available. These reporter antibodies were used as tools to unambiguously demonstrate the localization of the reporter epitope with respect to the plasma membrane. In addition to reporter antibodies, polyclonal antibodies directed against the putative intracellular loop between MIII and MIV of the 5-HT_3 receptor were also raised.

Using the M2 reporter antibody directed against the FLAG epitope, and the 9E10 reporter antibody directed against the c-myc epitope, it was shown for the 5-HT_3 receptor that the C-terminal is located extracellularly in transfected mammalian cells [FIG. 2.6]. This finding confirms the expected topological location of the C-terminal domain with respect to the hydropathy plot [FIG. 1.1]. Also, using antibodies raised against the putative intracellular loop of the wild-type receptor, this region was found to be located, as expected, intracellularly. The short distance between the reporter epitope and the wild-type epitope allows us to conclude that only one membrane-spanning domain is present between this distance.

The P_{2X} receptor's primary structure defines a new motif for ligand-gated ion channels. Possessing only two putative membrane-spanning segments, this channel is phylogenetically closer to inward rectifier K channels than to nicotinic receptors. In order to convincingly demonstrate the cellular location of the C-terminal domain, the two reporter epitopes and their respective antibodies were used in transfected HEK 293 cells. Our data indicate that the C-terminal domain is located intracellularly, confirming the presence of a second membrane spanning domain [FIG. 3.2].

The fact that the channels are functional despite the epitope insertion allows us to conclude that the C-terminal domain is not involved in agonist binding or channel gating, and is not critical for subunit association..

This method of epitope tagging has the big advantage of being applicable to probe the topology of any cloned membrane protein, aside from

neurotransmitter-gated ion channels, as well as for their immunolocalization *in situ* or in western blot.

By studying the transmembrane topology and structure-activity relationships of neurotransmitter-gated ion channels, we gain more information about the operation of the channel, which is essential in the design of novel compounds for the treatment of most neurological diseases.

C-terminal functional tagging should also provide experimental tools for the purification and visualization of receptor channel subunits in order to characterize the stoichiometry and subunit composition of heteromeric receptors.