EXPRESSION OF WT AND MUTANT α3 NEURONAL NICOTINIC SUBUNIT CONSTRUCTS IN α3 KNOCK-OUT MOUSE SYMPATHETIC NEURONS

by

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate excitatory synaptic signaling in vertebrate autonomic neurons. Sympathetic neurons express five of the 12 known neuronal nAChRs genes: α3, α5, α7, β2 and β4 and therefore several potential receptor subtypes could exist. The purpose of my study is to investigate the function of α3. Specifically, I test the hypothesis that α3 is essential for the receptor ACh sensitivity. For that purpose, I use sympathetic neurons from α3 knock-out mice together with gene transfer technique with an adenovirus to express α3 genes in cultured sympathetic neurons from α3 null animals. Whole-cell electrophysiological recordings of these neurons do not show any sensitivity to ACh. Interestingly, infection of these neurons with an adenoviral vector containing the wild-type α3 construct restores ACh sensitivity to levels similar to that in wild-type neurons; this results suggest that all the nAChR components but α3 are present in the α3 null neuron. Moreover, this preparation is as a good model to investigate the role of α3 gene. In order to investigate the behavior of some mutated α3 constructs previously tested in *Xenopus* oocytes, I have built three new adenoviruses. In the first two the α3 ACh binding site has been modified to increase the affinity of the muscle blocker α-bungarotoxin for the receptor. Unexpectedly, α3 null neurons infected with these adenoviruses did not produce ACh sensitive receptors. In the third adenovirus, α3 bears a single amino acid mutation that modifies the calcium permeability of the channel. This latter adenovirus confirmed the data from the expression study in *Xenopus* oocyte. To

conclude, this system provides us with a new tool to investigate the major role played by the α3 nAChR subunit in mouse sympathetic neurons.

Résumé

Les récepteurs neuronaux à l'acétylcholine (nAChRs) sont des canaux ioniques liés au ligand qui transmettent les signaux synaptiques excitateurs dans les neurones autonomes des vertébrés. Les neurones sympathiques expriment cinq des 12 gènes codant pour les nAChRs neuronaux: α3, α5, α7, β2 et β4 et de ce fait plusieurs sous-types de récepteurs peuvent potentiellement exister. L'objectif de mon étude est d'examiner la fonction d'α3. Précisément, je teste l'hypothèse qu'α3 est essentielle pour la sensibilité du récepteur à l'acétylcholine. Pour ce faire, j'utilise des neurones sympathiques de souris "knock-out" pour la sous-unité α3 ainsi qu'une technique de transfert de gène basée sur un adenovirus de manière à exprimer les gènes d'α3 dans des neurones sympathiques provenant de souris manquant α3 et préalablement mis en culture. Les enregistrements électrophysiologiques en configuration cellule entière révèlent que ces neurones sont dépourvus de sensibilité à l'acétylcholine. De façon intéressante, l'infection de ces neurones par un vecteur adenoviral contenant la construction α3 sauvage rétablit la sensibilité à l'acétylcholine à des niveaux similaires à ceux trouvés dans les neurones sauvages; ce résultat suggère que tous les composants des nAChRs, hormis α3, sont présents dans les neurones manquant α3. De plus, cette préparation est un bon modèle pour examiner le rôle que joue le gène α3. De manière à examiner le comportement de certaines constructions mutantes de α3 préalablement testées dans les ovocytes de Xénope, j'ai bâti trois nouveaux adenovirus. Dans les deux premiers, le site de liaison de l' acétylcholine à été modifié dans le but d'augmenter l'affinité du bloqueur musculaire α-bungarotoxine pour le récepteur. De manière inattendue, les neurones manquant α3

infectés avec ces adenovirus ne produisent pas de récepteurs sensibles à l'acétylcholine. Dans le troisième adenovirus, α3 porte une mutation d'un seul de ces acide-aminés qui modifie la perméabilité du canal au calcium. Ce dernier adenovirus a confirmé les données provenant de l'étude d'expression dans les ovocytes de Xénope. Pour conclure, ce système constitue un nouvel outil pour examiner le rôle majeur joué par la sous unité α3 nAChR dans les neurones sympathiques de souris.

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En route pour la route 66.....

à Manue, Tinet, Philou et Mamée...

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Chapter 1: Introduction

1.1 The muscle nAChR

Cholinergic transmission occurs through two classes of receptors, the muscarinic and the nicotinic receptors. Muscarinic receptors are metabotropic receptors, whereas nicotinic receptors (nAChRs) are ligand-gated ion channels. These two kinds of receptors belong to different supergene families. A number of recent reviews have been dedicated to the nAChRs (Changeux, 1993; Sargent, 1993; Karlin & Akabas, 1995; McGehee & Role, 1995; Role & Berg, 1996; Colquhoun & Patrick, 1997; Keller & Taylor, 1999; McGehee, 1999; Corringer *et al.*, 2000). Below, I synthesize information, mainly from these reviews, in order to give an overview of the current knowledge of the structure and function of the neuronal nAChRs.

Muscle nAChR is the prototypical channel of the ligand-gated ion channels, a gene family which also includes receptors for α-aminobutyric acid ($GABA_A$), glycine (Gly), and 5-hydroxytryptamine ($5\text{-}HT_3$). The nAChR and the $5\text{-}HT_3$ receptors are selective for cations, whereas $GABA_A$ receptors and Gly receptors are selective for anions (Karlin & Akabas, 1995). Our current understanding of the structural, molecular, and biophysical properties of these multisubunit proteins has largely arisen from studies of the nAChR channels in vertebrate muscle cells and electric organs of the electric ray *Torpedo californica*. The break through in the nicotinic research field arose from the discovery by Lee that a snake venom α-toxin, α-bungarotoxin (α-BTX), binds with a very high affinity to the nAChR. Thus, the successful engineering of insoluble beads to which α-BTX was

attached, allowed the purification by affinity chromatography of the muscle-type electric fish nAChR (Changeux *et al.*, 1970).

From a structural point of view, the overall shape of the muscle nACh receptor is a complex composed of four homologous, but distinct, subunits, α ,β ,γ ,and δ that assemble into nAChR $\alpha_2\beta\gamma\delta$ pentamers (see **figure 1.A**). Its subunits are arranged around a central channel that is about 25Å wide in the extracellular domain but narrows to about 7Å close to the cytoplasmic end of the membrane spanning domain (Miyazawa *et al.*, 1999). Each subunit has a large extracellular domain, four transmembrane regions (M1-M4), a large intracellular domain between M3 and M4, there is a short stretch of amino acids on the subunit's extracellular side, just after M4. The main acetylcholine binding site is located in the N-terminal part of the α subunit and is formed by the vicinal cysteine residues Cys 192 and Cys 193 which are linked with a disulfide bridge. The binding of the ligand provides energy for a conformational change in the gate of the cation selective ion channel (Karlin & Akabas, 1995).

Combined protein purification, molecular biological and electrophysiological experiments have provided important insights into how the channel functions. Moreover, recent studies demonstrate that muscle nAChR subunit genes are part of a larger class of nAChRs family, most expressed by neurons and therefore called neuronal nAChRs (Sargent, 1993).

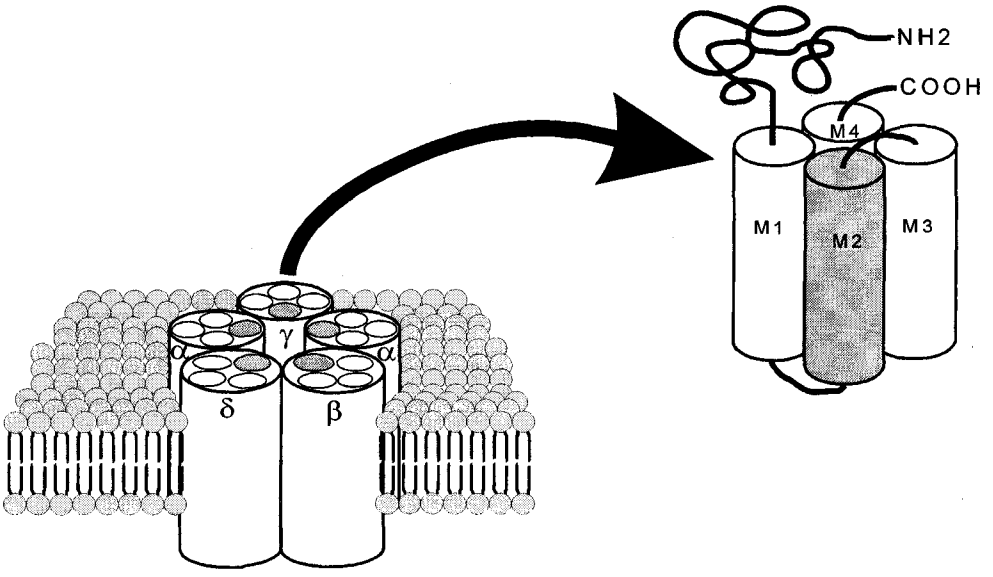
Figure 1 Anatomy of the nicotinic acetylcholine receptor.

A. Overall Structure of the nAChRs. Each nAChR subunit has four transmembrane domains (M1 to M4), a large amino-terminus and a short carboxy-terminus. Coassembly of five subunits forms the receptor. The receptor presented is the muscle nAChR with its $\alpha 2\beta\delta\gamma$ pentameric structure. The neuronal nAChRs have the same overall structure.

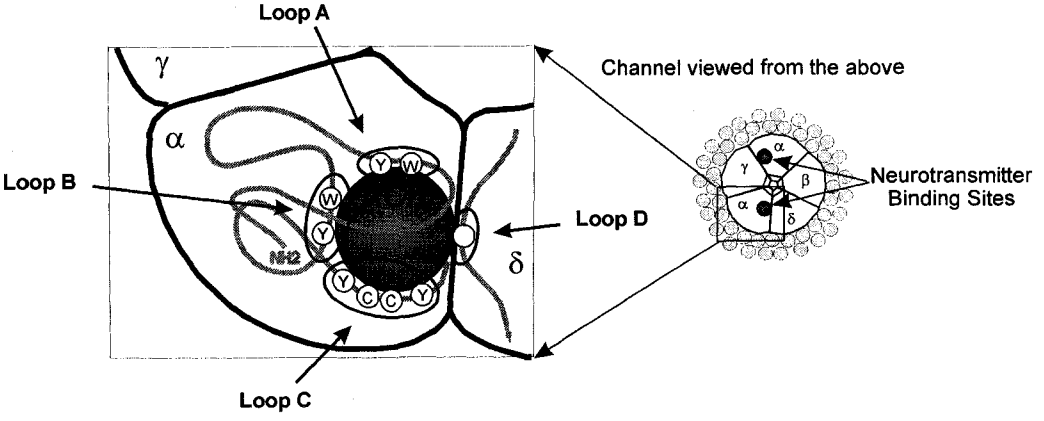
B Neurotransmitter binding site. The neurotransmitter binding sites consist primarily of amino acids (spheres) residing in the large hydrophilic region of the alpha subunits. Specific neighbouring amino acids form loops (A, B, C, and D *inset on the left*) that have been shown to be part of the ligand binding site.

C The ion channel. The M2 regions of the different subunits assemble together to form the funnel-shaped ion channel. There are three rings of charged amino acids neighbouring the M2 segment: extracellular ring, intermediate ring and the cytoplasmic ring. At these positions, the five charged residues corresponding to two α and three non-α subunits are shown. The circles connecting the charged residues are only to emphasize the ring-like formation of these residues.

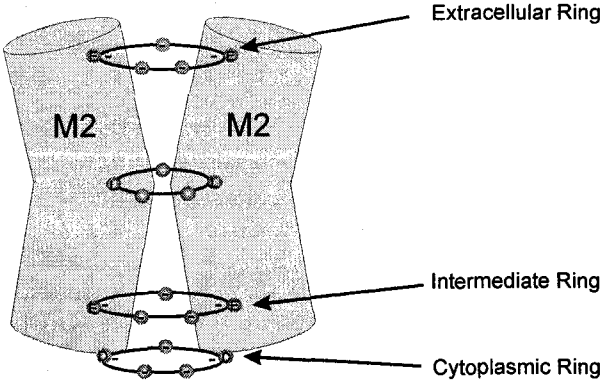
A


NH2
COOH
M4
M1
M2
M3
γ
α
α
δ
β

B


Loop A
γ
α
Loop B
M2
Loop D
δ
Loop C
Channel viewed from the above
Neurotransmitter
Binding Sites

C


Extracellular Ring
M2
M2
Intermediate Ring
Cytoplasmic Ring

1.2 The neuronal nAChR gene family

The neuronal nicotinic receptor subunit gene family has a total of 12 members that are divided in two categories: 9 α subunits (α2, α3, α4, α5, α6, α7, α8, α9, and recently α10) or ligand-binding subunits, and three β subunits (named β2, β3, β4) (see review by McGehee, 1999, see also Elgoyhen *et al.*, 2001).

The α subunits are highly homologous to the muscle nAChR α subunit (α1), including the cysteine residues that are known to be important for ACh binding (Cys 192, Cys 193) and the proposed transmembrane-spanning domains (McGehee, 1999). The neuronal β nAChR subunits are distinguished by the absence of the ACh-binding cysteine residues. However, this does not imply that neuronal β subunit do not contribute to ligand binding profiles.

Heterologous expression studies have been widely used to find out the different subunit combinations that give rise to functional nAChRs. Neuronal nAChRs can be divided in two groups, depending on which subunit combinations compose them. In the first group, nAChRs are formed by combinations of α/β pairs, and these receptors are not sensitive to nanomolar concentration of the muscle nAChR blocker α-BTX. In *Xenopus* oocytes, ACh-evoked currents have been recorded by two-electrode voltage-clamp after injecting the cDNA of α2, α3, or α4 with either β2 or β4. (Sargent, 1993). The second group is composed of homomeric α channels and these receptors are blocked by nanomolar concentrations of α-BTX. In *Xenopus* oocytes, α7 and α9 have been shown to form functional channels when expressed alone whereas α8 needs to be coexpressed with α7; and α10 with α9 (McGehee, 1999; Elgoyhen *et al.*, 2001).

Although the search for additional α- and β-like subunit genes has been extensive, some studies suggest that the nAChR family tree may not be complete. The pattern of subunit expression revealed by *in situ* hybridizations performed with probes for the different mRNA subunits still leaves some neuroanatomical regions apparently lacking an appropriate α or β partner (McGehee, 1999). For example, Deneris and colleagues (Deneris *et al.*, 1988) reported that β2 mRNA was observed in regions of the adult rat CNS, such as the supraoptic nucleus, that were not labeled by ^{3}H-labeled agonists and the monoclonal antibody MAb 270, specific for nicotine-binding sites, locations where neither the α2, the α3, nor the α4 mRNA were detected.

In addition, the different functional characteristics of native nAChRs, studied by patch-clamp recordings performed *in vivo*, are still poorly matched to the reconstituted nAChR channel characteristics obtained in heterologous expression studies to date. While the complexity of the known nAChR combinations is considerable, additional neuronal nAChR subunits may still be awaiting discovery (Mc Gehee, 1999).

The structure-function of the muscle and neuronal nAChRs have been extensively studied. I present below a brief summery of the current knowledge on the ligand binding region and the structure of the ion channel.

1.3 Structure function of the nAChRs

1.3.1 The ACh binding region

a) Muscle nAChRs

In the electric organ and muscle nAChRs, a number of studies demonstrate that the location of the binding site for nicotinic agonists and competitive antagonists is at the $\alpha 1/\gamma$ and $\alpha 1/\delta$ subunit interfaces (Corringer *et al.*, 2000).

First, affinity labeling experiments, which consist of using a traceable version of a molecule that binds irreversibly to the receptor and therefore highlights the amino acids that constitute the binding site, contributed to a better understanding of the structure of the ACh binding site. The use of a series of competitive antagonists of different chemical structures such as the aryl-cation p-(dimethyl amino) benzenediazonium fluoroborate (DDF) (Langenbuch-Cachat *et al.*, 1988; Dennis *et al.*, 1988), the alcaloid d-turbocurarine (dTC) (Pedersen & Cohen, 1990), the polypeptide α-BTX (Oswald & Changeux, 1982) and the agonist nicotine (Middlelton & Cohen, 1991), showed that these probes label primarily the $\alpha 1$ subunit, and to a lesser extent the γ and δ subunits.

Second, the mouse muscle $\alpha 1$ subunit expressed with either γ or δ subunits in fibroblasts yields an ACh binding pocket with pharmacology similar to that of the native muscle nAChRs, whereas all other pairwise coexpressions of subunit failed to give ACh binding sites (Blount & Merlie, 1989).

Third, the $\alpha\gamma$ and $\alpha\delta$ dimers display marked pharmacological differences, particularly for α-conotoxin M1 with a 10,000-fold higher affinity for the $\alpha 1/\delta$ compared

with the α1/γ binding sites of mouse muscle-type receptor (Sine, 1993; Sine *et al.*, 1995). The much stronger labeling of α1 compared with that of γ and δ subunits supports an asymmetric location of the binding site with respect to the interface. Therefore, the α1 subunit carries the "principal component" of the nicotinic binding site and the δ and γ subunits contribute to the "complementary component" (Corringer *et al.*, 1995)

The various residues that compose the principal component of the α1 subunit of the *Torpedo* nAChR were identified by affinity labeling, proteolysis and Edman degradation experiments (Corringer *et al.*, 2000). The affinity alkylation of the *Torpedo* nAChR with the 4-(N-maleimido) benzyltri[^{3}H]methyl ammonium by Kao and colleagues (Kao *et al.*, 1984) was the first demonstration that this compound specifically and uniquely labels the α subunit C192 and C193. Their results indicate that these two paired cysteines form a disulfide bridge and are located near the ACh binding site. Using DDF, Dennis and colleagues labeled amino acids in three distinct regions of the amino-terminal region: a tyrosine at position 93, two tryptophans at position 86 and 149, two tyrosines at position 190 and 198, and the two paired cysteines at position 192 and 193 (Dennis *et al.*, 1988; Galzi *et al.*, 1990). Their data suggest that these three regions form peptidic loops (referred to as loop A, B and C) that collectively form a negatively charged cup to which the positively charged quaternary ammonium group of ACh binds (shown on **figure 1.B**).

For the complementary component, the homologous tryptophans γW55 and δW57 (in loop D) were found labeled by nicotine and dTC, whereas homologous tyrosine γY111 and arginine δR113 (in loop E) were specifically labeled by dTC (Chiara *et al.*, 1998; Chiara *et al.*, 1999). Mutation of the aspartic acid δD180 (in loop F) to asparagine,

and of the homologous aspartic acid δD174, was found to decrease the affinity to ACh (Martin *et al.*, 1996).

b) Neuronal nAChRs

Sequence comparison of neuronal with muscle nAChR subunits indicates a high conservation of the loop A, B, C, and D motifs (Corringer *et al.*, 2000). The labeled residues from loop A, B, C are indeed present in the α2, α3, α4, α6, as well as α7 and α8 subunits; and the labeled residue from loop D are present in the β2, β4 as well as α7 and α8. In the homooligomeric α7 receptor, mutation of the corresponding tyrosine and Thryptophan residues (W54, Y92, W149, and Y188) in the α7 binding site alters the apparent affinities of binding and activation of ACh, establishing their contribution to the ACh binding site (Corringer *et al.*, 1995; Galzi *et al.*, 1990). Thus, in this case, the same subunit carries both the principal and the complementary components of binding. In the case of heterooligomeric nAChRs, sequence comparison data between muscle and neuronal nAChR subunits sequences suggest the presence of the principal component of the ACh binding site on the α-type subunit whereas the complementary component would be carried by the β-type subunit (Corringer *et al.*, 2000).

The pharmacological properties of nAChR vary markedly with subunit composition and species. For instance, the binding affinity of ACh for chick nAChR range from 5 nM (α4β2) to 1 μM (α7). α-toxins from snake venoms typically bind with subnanomolar affinities to the muscle-type receptor, whereas only a subgroup of them, long-chain toxins, which prototype is α-BTX, binds with high affinity to α7 receptor

(Servent *et al.*,1997). α-Conotoxin MII binds specifically to the α3β2 receptor (Harvey *et al.*,1997).

Numerous studies contributed to the mapping of the pharmacological diversity of nAChR binding sites using mutational analysis and expression heterologous system (Corringer *et al.*, 2000).

An example of this kind of study is provided by the work of Levandoski and colleagues where they investigated the molecular determinants responsible for α-BTX binding to nAChRs (Levandoski *et al.*, 1999). For that purpose, they used two homologous α subunits, one highly sensitive to α-BTX block, the *Torpedo* α1, and the other α-BTX insensitive, the neuronal α3. Using site directed mutagenesis, they tried to confer α-BTX sensitivity to the α-BTX insensitive α3 subunit. Rat α3 residues 184-191 have been replaced with the corresponding region from the *Torpedo* α1 subunit, therefore introducing a cluster of five α1 residues into the α3 subunit. Functional activity and α-BTX sensitivity have been assessed following co-expression in *Xenopus* oocytes of the chimeric α3 subunit (α3/α1) with either β2 or β4 subunits. Agonist-evoked responses of α3/α1-containing nAChRs were successfully blocked by nanomolar concentrations of α-BTX whereas wild-type α3-containing nAChRs were insensitive to the toxin. Another α3 chimeric subunit, α3/α7, similar to α3/α1, but incorporating the corresponding amino-acids from the α-BTX-sensitive α7 subunit, also conferred potent α-BTX sensitivity to chimeric receptors when co-expressed with the β4 subunit. Thus, Levandoski and coworkers concluded that the amino acid residues between position 184 and 191 of the α-BTX sensitive subunits α1 and α7 play a critical functional role in the interaction of the α-BTX with the nAChRs sensitive to this toxin (Levandoski *et al.*, 1999).

Taken together these studies support the notion that variable residues located at the vicinity of the affinity labeled amino acids are the major elements contributing to the pharmacological diversity of the nAChRs. The emerging picture is that the binding site consists of conserved core of specific amino acid residues, and that different amino acids neighbouring these positions, as well as several amino acids from the non conserved loop E and F, confer on each receptor subtype its individual pharmacological properties.

1.3.2 The nAChR ion channel

A number of experimental evidences indicate that the second transmembrane domain, M2, of each subunit lines the conductive pathway of either the muscle and the neuronal nAChR.

a) Muscle nAChRs

The ion channel was initially identified chemically with open channel blockers and the study of their interaction with the muscle-type nACh receptor (Corringer *et al.*, 2000). The main advantage of these blockers is that they interact with the channel pore without affecting the binding of the ligand to the receptor.

Among the variety of channel blockers that exist, the sedative antipsychotic drug chlorpromazine (CPZ) has the ability to covalently bind to the open nAChR only upon ultraviolet light irradiation (photolabeling) (Koblin & Lester, 1979). Therefore, radioactively labeled CPZ, ^{3}H-CPZ, can be used as a tool to label the receptor (Oswald & Changeux, 1981; Heidmann & Changeux, 1984). Thanks to this photolabeling method, Changeux and colleagues, identified the conducting pathway of the nAChR. Their first demonstration was that all four subunits of the *Torpedo* nAChR can be photolabeled by

^{3}H-CPZ in the presence of agonist, suggesting a site of interaction along the axis of the channel (Oswald & Changeux, 1981). Then, Changeux and coworkers submitted the purified CPZ photolabeled nAChRs subunits to trypsin cleavage, which was followed by HPLC fractionation and amino acids sequencing. They identified several residues on the α, β, δ, and γ subunits that interacted with CPZ (Giraudat *et al.*, 1986; 1987; Revah *et al.*, 1990). Interestingly, all these residues belong to the M2 segment of the subunits and correspond to a non polar ring of leucine residues and two polar rings of serine and threonine residues (Revah *et al.*, 1990). Revah and colleagues concluded that the simplest model accounting for this set of results was that the M2 segments, organized in α-helices, were arranged around the nAChR axis and composed the walls of the ion channel. Accordingly, in such a configuration, on each subunit the residues labeled by ^{3}H-CPZ and the negatively charged amino acids framing M2 would be aligned along one face of the α-helix and thus would point toward the channel lumen (Revah *et al.*, 1990).

Imoto and colleagues developed another approach to show the involvement of the M2 transmembrane domain in determining the conductance of the nAChR. They built chimeric δ subunit constructs containing different regions corresponding to the *Torpedo* or bovine δ subunits, expressed them in *Xenopus* oocytes and measured by two-electrode voltage-clamp their responses to ACh (Imoto *et al.*, 1986). Imoto's rationale was based on the finding that the coexpression of the bovine δ subunit with *Torpedo* α, β, and γ subunits gave rise to a receptor with channel conductance similar to that of the bovine nAChR. By expressing chimeric bovine δ subunit constructs that contain homologous regions of the *Torpedo* δ subunit, they were able to determine the domain of the subunit responsible for the change in the channel conductance and identified it as been M2. In

subsequent experiments, the same group identified the amino acid residues that interact with permeating ions (Imoto *et al.*, 1988). Using site directed mutagenesis, they introduced various point mutations into the *Torpedo* nAChR subunit cDNAs to alter the net charge of the charged residues around the M2 segments. The conductance properties of these nAChR mutants expressed in *Xenopus* oocytes indicated that three clusters of negatively charged residues neighbouring segment M2 of the α, β, γ, and δ, forming three anionic rings (an extracellular ring, an intermediate ring and a cytoplasmic ring), are major determinants of the rate of ion transport (Imoto *et al.*, 1988)(see **figure 1.C**).

A third approach, the substituted-cysteine accessibility method (SCAM), has confirmed the location of the M2 region in the conductive pathway of the nAChR. This method is based on the covalent interaction of small, charged, highly water soluble sulfhydryl-specific molecules (such as the positively charged methanethiosulfonate ethylamonium, MTSEA) with exposed sulfhydryl groups of cysteine residues (Akabas *et al.*, 1992). If candidate residues along the conducting pathway of the receptor are substituted with cysteine residues, MTSEA can potentially bind to those residues while passing through the pore and block the ACh-evoked current through the receptor. Akabas and colleagues substituted, one at the time, 22 amino acids spanning the M2 and the flanking region of the mouse α subunit (Akabas *et al.*, 1994). When expressed in combination with β, γ, and δ subunits in *Xenopus* oocytes, all but one construct gave rise to functional nAChRs. Ten mutant receptors containing a cysteine residue at one of 10 positions from the extracellular ring to the intermediate ring showed a significant reduction in their response to ACh following MTSEA treatment when compared to control ACh responses. These results suggest that MTSEA can interact with the closed

receptor at 10 different positions along the M2 segment, and assuming that substituted cysteines have similar accessibility to that of the corresponding wild-type residues, the authors concluded that these ten amino acids normally face the lumen of the nAChR pore (Akabas *et al.*, 1994).

b) Neuronal nAChRs

Several groups using site-directed mutagenesis in combination with electrophysiological techniques on heterologously expressed recombinant neuronal nAChRs have shown the major role played by the M2 segment in the conductivity pathway of these receptors.

In one of the initial studies, Cooper and colleagues demonstrated that removal or addition of negative charges at the extracellular ring of α4β2 neuronal nAChR subtype had a significant effect on the channel conductance of the receptor expressed in *Xenopus* oocytes (Cooper *et al.*, 1991). Substitution of the glutamic acid in the extracellular ring of the α subunit to a lysine residue ($\alpha4_{E266K}$) caused a 50% reduction in the channel conductance of the mutated receptor, whereas substitution of a lysine residue at the corresponding position in the β subunit ($\beta2_{K260E}$) doubled the channel conductance. Since each of this mutated α4 or β2 introduced a new channel conductance level, Cooper and colleagues reasoned that the number of channel conductance states resulting from coexpression of a mutant and a wild type α4 or β2 can be used as an indication of the number of α4 or β2 subunits incorporated to the receptor complex. Based on the possible arrangements of subunits in one complex, their results indicated that the neuronal nAChR is most likely a pentamer made up of two α4 and three β2 subunits (Cooper *et al.*, 1991).

This result has been confirmed by the study of Anand and colleagues who used the quantitation of the amount of radioactivity in individual subunits of ^{35}S-labeled nAChRs expressed in *Xenopus* oocytes to determine the subunit stoichiometry of the nAChRs (Anand *et al.*, 1991).

In subsequent studies, Galzi and colleagues (Galzi *et al.*, 1992) explored the regions of the neuronal nAChR that determine the cation-selectivity of the homomeric α7 receptor. As we have already seen, cation conductive nAChRs have a high degree of homology in their pore region with other members of the ligand-gated channel family to which the anion conducting $GABA_A$ and glycine receptors belong too. However, these two types of receptors differ by several residues in this common region. Thus, Galzi and coworkers reasoned that substituting these residues in the M2 of α7 with the corresponding residues of $GABA_A$ and glycine receptors may be sufficient to reverse the charge selectivity of α7 from cationic to anionic. The chloride permeabilities of the mutant and wild-type receptors were examined electrophysiologically upon expression in *Xenopus* oocytes. Their results indicated that only three amino acid replacements are sufficient to change the α7 nAChR to an anion selective channel: introduction of an additional proline adjacent to the intermediate ring ($\alpha7_{P236'}$), substitution of the negatively charged glutamic acid residue of the intermediate ring to neutral alanine ($\alpha7_{E237A}$), and substitution of a non polar valine in the M2 region to a polar threonine residue ($\alpha7_{V251T}$) (Galzi *et al.*, 1992). These results have been further studied by Corringer and coworkers (Corringer *et al.*, 1999). The main finding of their experiments is that the insertion of the proline residue can only be done between the positions 234 to 238 of α7 M2 segment in

order to convert the homomeric α7 nAChR to a functional anion selective receptor (Corringer *et al.*, 1999).

The inward rectification is the property of the neuronal nAChR channel to conduct inward currents at negative potentials, but little outward current at positive potentials. This phenomenon is not observed in the muscle-type nAChRs. Inward rectification provides an important mechanism to ensure that the receptors do not short circuit the action potential in the nerve terminal and reduce transmitter release. In two recent studies, Haghighi and Cooper have demonstrated that inward rectification of neuronal nAChRs can result from a voltage-dependant block by the intracellular polyamine spermine and that the intermediate ring contains negatively charged residues that are essential for inward rectification (Haghighi & Cooper, 1998; 2000). First, they examined recombinant α3β4 and α4β2 neuronal nAChRs subtypes expressed in *Xenopus* oocytes and native nAChRs expressed on Superior Cervical Ganglion (SCG) neurons (Haghighi & Cooper, 1998). Whole-cell ACh-evoked currents recorded from these receptors exhibited strong inward rectification. In contrast, the single channel currents from these neuronal nAChRs measured outside-out patches outwardly rectify. When spermine was added to the patch electrode in outside-out recordings, it caused a concentration- and voltage-dependant block of ACh evoked single-channel currents (Haghighi & Cooper, 1998).

They then showed that the negatively charged residues at the intermediate ring are essential for the interaction of polyamines with α4β2 and α3β4 neuronal nAChRs, using site-directed mutagenesis and electrophysiology on α4β2 and α3β4 receptors expressed in *Xenopus* oocytes (Haghighi & Cooper, 2000). For example, partial removal of negative charges of the intermediate ring in one of the subunit such as the substitution of the

glutamic acid at position 240 for a neutral alanine in the α3 subunit ($\alpha3_{E240A}$), diminished the high-affinity, voltage-dependant interaction between intracellular polyamines and the $\alpha3_{E240A}\beta4$ mutant nAChRs, abolishing inward rectification (Haghighi & Cooper, 2000). Furthermore, their results indicate that these negatively charged residues influence the calcium selectivity of the pore, indicating that a common structural element governs both inward rectification and calcium permeability of neuronal nAChRs (Haghighi & Cooper, 2000).

Taken together, these results indicate the importance of the M2 region in determining the conductance and selectivity of neuronal nAChRs, as it is the case for the muscle nAChRs.

Among the various neuronal subunits cloned to date, some have been shown to be more important than others in terms of expression in the nervous system and physiological implication. *In vivo*, the native nAChRs are widely expressed in the central nervous system (CNS) as well as the peripheral nervous system (PNS). *In situ* hybridization studies have shown that nAChRs subunits are differentially expressed among the nervous system. For instance, the α4 and the α7 are predominantly expressed in the CNS whereas the α3 in more expressed in the PNS. Since for my Masters research I have been focusing on one neuronal α subunit, I summarize below informations obtained in heterologous expression systems and *in vivo* regarding the main neuronal α nAChRs subunits: α3, α4, α5, and α7.

1.4 Nicotinic ACh receptors expressed in the CNS

1.4.1 α4-containing nAChRs

a) Localization of the α4 nAChRs

In the brain, the most abundant nAChR species has been shown to be composed of the α4 and β2 gene products, as demonstrated by the immunoaffinity-purification study performed by Schoepfer and colleagues in the chick (Schoepfer *et al.*, 1988). *In vivo*, α4 containing nAChRs purified from the brain have been shown to be affinity labeled with bromo[^{3}H]acetylcholine, and autoradiographic ligand-binding experiments using ^{3}H-nicotine have demonstrated that α4-containing nAChRs bind nicotine with high affinity (Whiting & Lindstrom, 1987).

b) α4 nAChRs expressed in heterologous systems general properties

Expression studies in *Xenopus* oocytes have shown that the α4 subunit forms functional nAChRs when coexpressed with either β2, β4, or β2 and α5 (McGehee and Role, 1995). Similarly to α3, α4 containing nAChRs will show different pharmacological properties depending on which partner subunit α4 is co-assembled with. As an example, the rat α4β2 and the rat α4β4 show different agonist potency profiles: for α4β2, ACh=Nicotine>Dimethylphenylpiperazinium(DMPP)>Cytisine, whereas for α4β4, Cytisine>Nicotine>ACh>DMPP (Mc Gehee & Role, 1995).

The α4 and β2 genes expressed in combination in *Xenopus* oocytes have been also used as a model to demonstrate the pentameric structure and the subunit stoichiometry of

the neuronal nAChRs. The gene products assemble in a two-to-three ratio to produce receptors, preserving the structural motif on muscle nAChRs with the two α and three non-α subunits (Cooper *et al.*, 1991; Anand *et al.*, 1991).

c) α4 and brain disorders

Mutations in α4 has been demonstrated to be involved in autosomal dominant frontal lobe epilepsy (ADFLE). ADNFLE patients suffer from a partial epilepsy causing frequent, violent, brief seizures at night usually beginning in childhood. Steinlein and colleagues have identified two distinct mutations in the α4 nAChR subunit in patients with ADNFLE (Steinlein *et al.*, 1995, Steinlein *et al.*, 1997). The first mutation is a missense mutation that replaces serine with phenylalanine at codon 248 ($\alpha4_{S248F}$), a strongly conserved amino acid in the M2 region (Steinlein *et al.*, 1995). In the second mutation three nucleotides (GCT), which encode a leucine residue, were inserted at nucleotide position 776 ($\alpha4_{776ins3}$) into the coding region for the C terminal end of the M2 domain (Steinlein *et al.*, 1997).

Studies performed in *Xenopus* oocytes have reported that the receptors formed by co-expressing $\alpha4_{S248F}$ or $\alpha4_{776ins3}$ subunits with β2 subunits differ from α4β2 nAChRs in several ways but no common effects of the two mutations on the response to ACh have been reported (Weiland *et al.*, 1996; Steinlein *et al.*, 1997; Kuryatov *et al.*, 1997; Bertrand *et al.*, 1998). It is only recently that Figl and colleagues demonstrated the common effect of the two mutations on the ACh response (Figl *et al.*, 1998). They constructed two rat homologues $\alpha4_{S252F}$ and $\alpha4_{+L264}$ of the human ADNFLE mutations $\alpha4_{S248F}$ and $\alpha4_{777ins3}$, respectively. They coexpressed them with rat β2 subunits in *Xenopus* oocytes, and studied

the properties of the expressed receptors. First, these mutations caused use-dependent potentiation of the response during a train of brief 100 nM ACh pulses. Second, these mutations delayed the rise times of the 5-15 nM (+L264) and 30 nM (S252F) ACh responses. Third, they reduced extracellular Ca^{2+}-induced increases in the 30μM response. From their results, Figl and coworkers proposed that a slow unblocking of the mutant receptors in its closed state would be the cause of the use-dependent potentiation and the delay in the rising phase of the mutant ACh response. The authors suggest that the use-dependent increases in the amplitude of the mutant synaptic currents could trigger ADNFLE seizures by suddenly increasing nicotinic transmitter release in the brain (Figl *et al.*, 1998).

1.4.2 α5-containing nAChRs

Heterologous expression of any one of the neuronal nicotinic acetylcholine receptor α-type subunits, either alone or with any β-type subunit, typically yields functional nAChR channels. A striking exception to this rule is the nAChR α5 subunit.

Transcripts from this subunit have been detected at high levels in the rat brain by *in situ* hybridization in a small number of regions (Wada *et al.*, 1990), whereas low levels of the α5 subunit mRNA have been found in the rat sympathetic ganglia (Mandelzys *et al.*, 1994). But heterologous expression of α5, either alone or with any β-type subunit has failed to yield functional channels (Boulter *et al.*, 1987; Couturier *et al.*, 1990). However, when α5 is coexpressed with other α and β subunits, the properties of the resultant channels are substantially altered.

a) α5α4β2 nAChRs

Ramirez-Latorre and colleagues were the first to report a triplex combination involving α5 that forms a functional neuronal nAChR in heterologous systems and in primary neurons (Ramirez-Latorre *et al.*, 1996). First, they showed that coexpression of α5, α4, and β2 subunits cDNA in *Xenopus* oocytes gave rise to an α5α4β2 nAChR that is less sensitive to ACh than α4β2 nAChR, and potently activated and desensitized by nanomolar concentrations of nicotine. Single channel analysis revealed that this α5α4β2 nAChR had an additional large conductance state when compared to α4β2 nAChR. Second, α5 subunit specific antisense oligonucleotides applied to chick sympathetic neurons and assay by single channel recording revealed a selective deletion of the largest conductance state without affecting the two other conductance states also present in these neurons. Finally, Ramirez-Latorre and coworkers showed the involvement of the M2 segment of α5 in the channel pore using the SCAM method on a series of mutant forms of the α5 subunit coexpressed with α4 and β2 in *Xenopus* oocytes (Ramirez-Latorre *et al.*, 1996).

b) α3α5β2 and α3α5β4 nAChRs

Fucile and colleagues reported a different triplex combination involving the α5 subunit (Fucile *et al.*, 1997). They transfected human cells with the subunits α3, α5 and β4 and this resulted in the expression of functional α3α5β4 nAChRs. This α5-containing receptor had a 9 fold decrease in the apparent affinity for ACh when compared to α3β4 nAChRs (Fucile *et al.*, 1997).

More recently, Nelson and Linstrom, in a study combining expression in *Xenopus* oocytes and single channel analysis, reported that coexpression of α5 with α3β2 or with α3β4 gave rise to functional nAChRs with different biophysical properties that the nAChRs formed by the dimers α3β2 or α3β4 (Nelson & Lindstrom, 1999). Indeed, they showed that α3α5β2 nAChR had one longer open time different from those of α3β2. The nAChRs composed of α3α5β4 had an additional conductance, and open time and burst time were longer from those for α3β4 nAChRs. However, when Nelson and Lindstrom performed the same analysis on native nAChRs from human neuroblastoma cells, that endogenously express these four subunits, and compared their results with those obtained in heterologous expression systems, they concluded that the predominant functional nAChRs *in vivo* were probably only composed of α3β4 subunits (Nelson & Lindstrom, 1999).

Taken together, these studies show that the α5 subunit can be part of functional neuronal nAChRs. The coexpression of this peculiar subunit with any α/β subunit strongly modifies the biophysical properties of the original heterodimeric α/β nAChRs.

1.4.3 α7-containing nAChRs

a) Localization of the α7 nAChRs

The α7 nAChR subtype is present in both the CNS and the PNS and accounts for high affinity α-BTX binding. Indeed, *in situ* hybridization experiments results have shown that the distribution of α7-transcipts in the adult rat brain does not correlate with the distribution of high-affinity ^{3}H-nicotine-binding sites but overlapped the pattern of

^{125}I-α-BTX-binding sites observed by ligand binding autoradiography (Séguéla *et al.*, 1993). In the CNS, α7-containing receptors have been shown to have a presynaptic localization (McGehee *et al.*, 1995). McGehee and colleagues have shown that nanomolar concentrations of nicotine enhance both glutamatergic and cholinergic synaptic transmission by activation of presynaptic nAChRs. Moreover, this activation was blocked by α-BTX and abolished by α7 antisense oligonucleotides treatment (McGehee *et al.*, 1995). In the chick sympathetic ganglia, α7 nAChRs have been localized extrasynaptically, as shown by electron microscopy studies using labelled α-BTX and α7 specific monoclonal antibody (Jacob & Berg, 1983; Jacob *et al.*, 1986).

b) α7 nAChRs expressed in heterologous systems general properties

Expression studies in *Xenopus* oocytes have shown that injection of the α7 subunit cDNA alone resulted in a functional cation channels gated by nicotinic agonists (Couturier *et al.*, 1990). One exception to this observation is that chick α7 and α8, which have a very high degree of sequence identity, will form heteromeric complexes with pharmacology very similar to α7 homomers (Gerzanich *et al.*, 1994). The α7 receptor is moderately sensitive to ACh with an EC_{50} for ACh of 200 μM and displays the following order of agonist potency: nicotine > cytisine > DMPP > ACh (Séguéla *et al.*, 1993).

c) α7 nAChRs are sensitive to α-BTX

Another unique pharmacological feature for a neuronal nAChR is that α7 receptors expressed in *Xenopus* oocytes were found to be highly sensitive to α-BTX and were completely blocked by nanomolar concentrations of this muscle nAChR blocker whereas expression of other neuronal α/β subunits combinations are blocked by thousandfold higher concentrations of this toxin (Couturier *et al.*, 1990).

d) α7 nAChRs desensitize rapidly

In response to agonist exposure nicotinic AChR undergo two types of transitions: the first is a rapid (in milliseconds) opening of the channel, resulting in membrane depolarization; the second is a slower (in seconds) progressive decline of the current response, corresponding to the stabilization of nAChRs in refractory closed forms called the desensitized state (Katz & Thesleff, 1957). Desensitization includes fast and slow components. Desensitization has also been observed *in vitro* when nAChR subunits have been expressed in *Xenopus* oocytes and interestingly, the different subunit combinations have clear differences in the rate and voltage dependence of nAChR desensitization (Mc Gehee, 1999). Indeed the α3β4 currents desensitize relatively slowly, in seconds or tens of seconds, whereas α3β2, α7α8 heteromeric, and α7 homomeric nAChRs desensitize much more rapidly, in hundreds of milliseconds (Mc Gehee, 1999).

A number of studies have been performed to identify the molecular bases of these differences in desensitization depending the nAChR subunits composition. Revah and coworkers investigated the structural elements of the homomeric, α-BTX sensitive α7 nAChR that could be involved in the desensitization of the receptor (Revah *et al.*, 1991).

Using site-directed mutagenesis and expression in *Xenopus* oocytes they showed that substitution in the chick α7 subunit of a highly conserved uncharged leucine at position 247 to either a phenylalanine ($\alpha7_{L247F}$), a valine ($\alpha7_{L247V}$), a threonine ($\alpha7_{L247T}$), or a serine ($\alpha7_{L247S}$) suppressed the inhibition by the open channel-blocker QX-222. This indicated that the residue at position 247 in the M2 domain faces the lumen of the ion channel, since QX-222 does not obstruct the channel pore of the mutated α7 nAChRs. Unexpectedly, the authors reported that these mutations of Leu 247 modify the physiological response of the receptor to ACh: these mutations decreased the rate of desensitization of the mutant nAChR response to ACh, increased the apparent receptor affinity for ACh and abolished inward rectification. Moreover, the amplitude of these changes always increased in the order phenylalanine, valine, threonine, serine, with the polar mutations giving rise to the most pronounced modifications.

In a subsequent study, the same group investigated the pharmacological properties of the $\alpha7_{L247T}$ mutant receptor (Bertrand *et al.*, 1992). They reported that this mutant possess a paradoxical pharmacology because antagonists of the wild type receptor such as dihydro-β-erythroidin, hexamethonium, or (+)-turbocurarine elicit ionic currents when applied to the mutant $\alpha7_{L247T}$ and these responses are blocked by α-BTX. The authors also reported that prolonged application of ACh, for 10 seconds, causes desensitization in the wild-type but leads to a potentiation of the responses to ACh or dihydro-β-erythroidin in the mutant. The authors concluded that these data are consistent with a scheme in which mutation of Leu-247, and the substitution of this bulky leucine residue with the shorter threonine renders the channel permeable when the receptor is in its desensitized conformation (Bertrand *et al.*, 1992).

Interestingly, Labarca and coworkers in a similar study performed on the α4 subunit, reported that the substitution of the leucine 9' to a serine ($\alpha4_{Leu9'Ser}$), a position homologous to $\alpha7_{L247}$, gave rise to a hypersensitive mutant receptor that had a slower desensitization rate and an increased sensitivity to agonist when expressed with the β2 subunit in *Xenopus* oocytes (Labarca *et al.*, 1995).

e) α7 nAChRs are very Ca^{2+} permeable

Séguéla and coworkers have shown that the homooligomeric receptor formed with the α7 subunit expressed in *Xenopus* oocytes has an unusually high permeability to calcium ions with an estimated permeability ratio (P_{Ca}/P_{Na}) close to 20, greater than that of the other nicotinic receptors and of the NMDA subtype of glutamate receptors, which values are around 1.5 and 5, respectively (Séguéla *et al.*, 1993). Bertrand and colleagues have identified the structural determinants responsible for this high Ca^{2+} permeability by expressing in *Xenopus* oocytes various forms of the α7 subunit that were bearing different amino acid substitutions (Bertrand *et al.*, 1993). They report that substitution of the negatively charged glutamic acid at position 237, at the cytoplasmic end of M2, for a neutral alanine $\alpha7_{E237A}$ abolishes the high Ca^{2+} permeability in the mutant $\alpha7_{E237A}$ nAChR without affecting the other properties of the pharmalogical and physiological response to ACh (Bertrand *et al.*, 1993).

1.5 Nicotinic ACh receptors expressed in the PNS

1.5.1 Localization of the α3 nAChRs

In vivo, the α3 subunit is widely expressed in autonomic ganglia: its transcripts have been detected in sympathetic ganglia (Mandelzys *et al.*, 1994), parasympathetic ganglia (Poth *et al.*, 1997), trigeminal ganglia (Flores *et al.*, 1996), and adrenal gland (Campos-Caro *et al.*, 1997); whereas it has a lower abundance in the CNS (Mulle *et al.*, 1991; Connolly *et al.*, 1995). An α3 antisense oligonucleotide treatment decreased the number and altered the properties of the normally expressed nAChRs in the chick sympathetic neurons (Listerud *et al.*, 1995). The major expression of the α3 subunit in the PNS has been confirmed by Yeh and colleagues who recently developed a novel monoclonal antibody raised against the C-terminus of the rat α3 nAChR subunit (Yeh *et al.*, 2001). Yeh and coworkers immunobloted samples from different tissues with the α3 antibody. By comparing immunoblots intensities with immunoblots from serial dilutions of known amounts of α3β4 extracted from transfected HEK cells, Yeh and colleagues report that the level of α3 protein is the highest in the superior cervical ganglia (SCG), the interpedoncular nucleus and the pineal gland, moderate in habenula and superior colliculi and lower in cerebellum, cerebral cortex, and hippocampus (Yeh *et al.*, 2001).

Taken together, these results show the consistent expression of the α3 nAChR subunits in autonomic neurons.

1.5.2 α3 nAChRs expressed in heterologous systems general properties

Expression studies in *Xenopus* oocytes have shown that expression of the α3 subunit in combination with either β4 or β2, and sometimes α5 give rise to functional nAChRs with different physiological properties (Boulter *et al.*, 1987, Luetje & Patrick, 1991; Nelson & Lindstrom, 1999). The nAChRs composed of α3β4 desensitizes more slowly than the α3β2 nAChRs (Bohler *et al.*, 2001), and they show different agonist potency profiles: for the rat α3β2 nAChR, DMPP>ACh>Nicotine>Cytisine whereas for α3β4, Cytisine>Nicotine=ACh=DMPP (Luetje *et al.*, 1990; Papke *et al.*, 1993).

1.5.3 The role of M3-M4 loop in the specific targeting of the α3 subunit at the synapse of the chick autonomic neurons

A recent study by the group of Jacob determined the structural determinant for specific targeting of the α3 subunit to the synapse of autonomic neurons and showed that this phenomenon involved the α3 M3-M4 cytoplasmic loop (Williams *et al.*, 1998). In chick ciliary ganglia (CG), nAChRs which contain α3 and α5 subunbits are concentrated at the specialized postsynaptic membrane; whereas α7-containing nAChRs are excluded from the synapse, being localized perisynaptically. Molecular interactions that mediate the accumulation of nAChRs at neuronal synapses are still undefined. Jacob and coworkers wanted to test the hypothesis that particular domains of neuronal nAChR subunits specify their *in vivo* localization. The large loop region between M3 and M4 shows the greatest divergence in sequence and length between the individual subunits. Jacob's group therefore tested the ability of the long cytoplasmic loop to influence the localization of nAChRs relative to synapses on CG neurons. They generated chimeric

subunits by replacing the long cytoplasmic loop of α7 with the α3 or α5 cytoplasmic loop between M3 and M4 (α7/α3 and α7/α5 subunits, respectively). Thus, a difference in the distribution of chimeric α7 as compared to wild-type α7 on the expressing CG neuron surface would be due to the added α3 or α5 cytoplasmic loop. To distinguish the exogenous subunits, a sequence encoding the myc proto-oncogene tag (10 amino acids) was added to the end of the C-terminus, being readily accessible for external surface labeling.

Chimeric subunits were overexpressed in CG neurons developing *in vivo* by using avian–specific replication competent retroviral vector variants of the B envelope subgroup (RCASB-P). RCASBP-B was injected into the neuronal tube of Stage 9-10 chick embryos *in ovo* to infect neural crest precursors that give rise to CG neurons. The viral vector stably integrates into the genome of dividing cells and leads to high levels of exogenous gene expression in the progeny throughout development. Using this injection protocol, almost all CG neurons and very few brain regions were infected. For all receptor subunit constructs, age-matched chick embryos were injected with equivalent viral titers.

A key aspect of Jacob's study is the demonstration that α3- and α5-containing nAChRs and α7-containing nAChRs have restricted and largely exclusive surface localizations on normal developing CG neurons (Jacob & Berg, 1983; Jacob *et al.*, 1986). Again α3- and α5-containing nAChRs are concentrated in the postsynaptic membrane. In contrast, α7-containing nAChRs are excluded from the synapse, and are present perisynaptically on the surface membrane of dendrites that emerge from the postsynaptic cell in the region of innervation.

The α7/α3 chimeric subunit is preferentially localized to the postsynaptic membrane, demonstrating that the long cytoplasmic loop of α3 is sufficient to target extrasynaptic α7 to the synapse. The ultrastructural distribution of the chimeric subunits was established in the CG at stages when all the neurons are functionally innervated by using an anti-myc monoclonal antibody, followed by a biotinylated antimouse antibody and a streptavidin-biotinylated horseradish peroxidase complex to achieve maximum sensitivity. In contrast, overexpression of α7/α5 chimeric subunit or myc-tagged wild-type α7 (as a negative control) does not result in myc labeling of the synapse.

In conclusion, this study shows that the long cytoplasmic loop of α3, but not α5, can target nAChRs to the synapse during normal *in vivo* synapse formation. This is the first demonstration that a particular domain of one subunit plays an essential role in targeting receptor subtypes to the synapse in intact animals.

Taken together, these results suggest that the α3 subunit may be an important component of the nAChRs mediating fast synaptic transmission in the autonomic nervous system (Mc Gehee, 1999).

1.6 Knock-out and knock-in mouse models for the main α nAChRs subunits

1.6.1 α4 knock-out mice

Two groups have independently generated α4 knock-out mice (Marubio *et al.*, 1999; Ross *et al.,* 2000). In both cases, gene targeting and homologous recombination resulted in the deletion of the acetylcholine binding site as well as three of the four

transmembrane-spanning domains (M1, M2 and M3) of the α4 subunit. In each case, homozygous mutants were born in the expected Mendelian proportion. The main finding of these two studies is that homozygous mutant mice were capable of reproduction and had no obvious physical abnormalities. This result demonstrates that the physiological role of the α4 subunit in the brain is not needed for normal growth and behavior. However, in both studies, ligand-binding autoradiography using ^{3}H-nicotine showed the loss of high affinity nicotine binding sites throughout the brain of α4-null mouse.

Since nicotine and other neuronal nicotinic agonists have been reported to reduce anxiety (Costall *et al.*, 1989), Marubio and colleagues examined the behavioral responses of α4 -/- mice to administration of nicotine and epibatidine, a nicotinic agonist. The results showed that α4 -/- mice displayed a reduced antinociceptive effect of nicotine on the hot-plate test and diminished sensitivity to nicotine in the tail-flick test. The raphe magnus and the thalamus are two brain areas that have been shown to be implicated in the supraspinal nicotine-elicited antinociception (Tripathi *et al.*, 1982). Patch-clamp recordings on these neurons revealed that the neurons from α4 -/- mice do not respond to nicotine whereas neurons from wild-type animal do. This indicated a contribution of the α4 subunit to functional nAChRs in these specific brain areas. The authors concluded that the α4 nAChR subunit, possibly associated with the β2 nAChR subunit, is therefore crucial for nicotine-elicited antinociception (Marubio *et al.*, 1999).

Ross and colleagues confirmed the results of Marubio and co-workers (Ross *et al.*, 2000). The authors undertook a detailed analysis of spontaneous and nicotine stimulated behavior of the α4 -/- mice. Their results suggest that, in a stressful setting, α4-null mice have a heightened basal level of anxiety-like behavior that can be reduced by nicotine

administration. In conclusion, their study suggest that the α4-containing nAChRs may be intimately involved in mediating anxiolytic-like effects (Ross *et al.*, 2000).

Taken together, the results from these two separate studies confirm that the high affinity binding sites for nicotine in the brain are the α4-containing nAChR. They also suggest a role for the α4 nAChR in mediating anxiolytic-like effects. But the main result remains that in both cases the homozygous α4 -/- mice are normal which demonstrates that the role of the α4 subunit in the brain is not needed for normal growth and behavior.

Interestingly, Labarca and colleagues developed another approach to get more insights for the role of α4 in the brain, and they generated a knock-in mice for the α4 gene which resulted in a gain of function of the α4 nAChRs (Labarca *et al.*, 2001).

1.6.2 α4 **knock-in mice**

Labarca and coworkers generated a line of mice expressing α4 subunit where the leucine 9' has been substituted to a serine ($\alpha4_{Leu9'Ser}$) (Labarca *et al.*, 2001). The same group already studied this mutation in *Xenopus* oocytes expression system. Their data showed that $\alpha4_{Leu9'Ser}$ gave rise to a hypersensitive mutant receptor that had a slower desensitization rate and an increased sensitivity to agonist when expressed with β2 (Labarca *et al.*, 1995). Labarca and colleagues generated the α4 knock-in mice by gene targeting and homologous recombination. They report that homozygous mutant mice displayed a dominant neonatal mortality (Labarca *et al.*, 2001). Immunocytochemistry showed that the most anatomical change in the brain of mice homozygous for the mutant $\alpha4_{Leu9'Ser}$ was a severe deficit of dopaminergic neurons in the substantia nigra. Since these dopaminergic neurons normally express α4-containing nAChRs, the authors speculated

that constant activation of the hypersensitive mutant nAChRs leading to increased calcium influx in the cell was the cause of the deficit of dopaminergic neurons (Labarca *et al.*, 2001). They also reported that a strain that retained the neo selection cassette in an intron had reduced expression of the hypersensitive receptor and was viable and fertile. The viable mice display increased anxiety, poor motor learning, excessive ambulation that is eliminated by very low levels nicotine. These last results support a role for α4-containing receptors in the control of baseline anxiety (Labarca *et al.*, 2001).

1.6.3 α7 **knock-out mice**

The identification of the α7 containing nAChR as a major target for α-BTX binding in the brain combined with the high calcium permeability of the receptor led researchers to assess the physiological importance of this subunit by generating mice lacking the α7 gene.

Mice deficient in the α7 neuronal nAChR subunit have been generated by Orr-Urtreger and colleagues by introducing a 7 kb deletion into the murine α7 subunit gene (Orr-Urtreger *et al.*, 1997). This mutation deleted the last three exons of the α7 locus which completely eliminates its potential for participation in an ion channel. Although this mutation might allow for the synthesis of a truncated protein with ligand-binding capacity, Northern blotting and immunoblotting studies confirmed the absence of detectable mRNA or protein and ensured that this mutation produced a null allele. The α7-null mice are present in the expected proportion of matings of heterozygote mice and as it has been the case for the α4-null mice, the main finding of this study is that homozygous α7 -/- mice are viable, grow to normal size, are fertile and show no obvious

physical or neurological deficit. The authors reported that autoradiography with ^{3}H-nicotine revealed no detectable abnormalities of high affinity binding sites in the brain of α7 -/- mice, but, as expected, that there was an absence of high-affinity ^{125}I-α-BTX sites. Electrophysiological study of cultured hippocampal neurons from α7 -/- mice showed that these neurons lack the rapidly desensitizing, nicotinic currents that are normally present in this structure (Orr-Urtreger *et al.*, 1997).

Taken together, the results from this study confirm the high affinity binding sites of α-BTX to α7-containing nAChR in the brain. Equally important, results demonstrate that the α7 subunit is not essential for normal development and behavior.

1.6.4 α7 **knock-in mice**

To learn more the role of α7 in the brain, Orr-Urtreger and coworkers generated a line of mice expressing a mutated form of the α7 subunit where the leucine at position 250 as been substituted to a threonine ($\alpha7_{L250T}$) (Orr-Urtreger *et al.*, 2000). This mutation results in $\alpha7_{L247F}$ nAChRs that are hypersensitive to ACh and have a slower desensitization rate and an increased sensitivity to agonist (Bertrand *et al.*, 1992). Orr-Urtreger and colleagues report that the homozygous L250T mice die within 2 to 24 hours after birth and Western blot analysis showed that their brain exhibit a marked reduction α7 nAChR protein levels. They also report that brains from homozygous mutant show extensive apoptotic cell death throughout the somatosensory cortex. Furthermore, electrophysiological recordings confirmed that $\alpha7_{L250T}$ nAChRs are functionally expressed on neurons within the brains of mutant neonatal mice and have physiological properties consistent with those observed for the $\alpha7_{L250T}$ nAChRs expressed in oocytes.

The authors concluded that these findings indicate that neurons in the developing brain expressing only $\alpha7_{L250T}$ nAChRs are susceptible to abnormal apoptosis and they speculated that this might be due to increased calcium influx conferred through the "gain of function" mutant nAChR (Orr-Urtreger *et al.*, 2000).

1.6.5 α3 **knock-out mice**

Genetically engineered mice lacking the α3 neuronal nicotinic acetylcholine receptor subunit have been generated by Baudet and colleagues (Xu *et al.*, 1999). Insertion of an HPRT cassette in the α3 gene deleted three of the four transmembrane domains, the amphipathic helix domain implicated in channel formation, and the four cysteines essential for ligand binding. The mutation was transmitted to the germline, and Northern blot analysis showed that homozygous -/- mice did not express any detectable mRNA of the α3 subunit. No phenotypic abnormalities were noted in heterozygous mice, and matings of +/- mice produced -/- mice in the expected ratio of 25% at birth. However, about 40% of -/- mice died from unknown causes in the first 3 days of life and almost all remaining animals died over an interval of 6-8 weeks after weaning. All surviving mice were substantially growth impaired , with a mean weight approximately 40% of wild type at the time of weaning.

The α3-/- mice have a very severe phenotype. First, they have megacystis which is an extreme bladder enlargement accompanied by dribbling urination, bladder infection, urinary stones. Immunohistochemistry study showed that ganglion cells and axons were present in the bladder of α3 -/- mice. Bladder strips from α3 -/- mice failed to contract in response to 0.1 mM nicotine, but did contract in response to electrical field stimulation or

the muscarinic agonist carbamylcholine. These results suggest that smooth muscle responses in the α3 -/- mice are normal and that bladder voiding is impaired because of the lack of expression of α3-containing nAChRs in neurons of pelvic ganglia. As suggested by Xu and colleagues, it is probable that the urinary tract dysfunction, bladder infection, and bladder stones contributed to the mortality of mice beyond weaning.

Second, -/- mice have severe mydriasis, very widely dilated ocular pupils with no contraction in response to light. This suggest that the autonomic input to the eye is compromised in the α3 -/- mice. Whereas in normal mice eyes open by two weeks after birth, in -/- mice, the eyelids remain closed or nearly closed throughout life.

Altered eye and bladder function observed *in vivo* in α3 -/- mice suggested defects in their autonomic ganglionic transmission. Therefore, Xu and colleagues recorded at the single channel ACh-evoked currents in SCG neurons from α3 -/- mice using patch-clamp technique. Comparison of -/- and +/+ mice indicated that α3 is required for ACh-activated channels normally detected in these neurons.

In conclusion, genetic analysis of the function of the α3 subunit indicates that it is essential for autonomic control of some peripheral organs, with the most obvious phenotypic effects on survival, growth, bladder function, and pupillary contraction. The delay in mutant animal death might be explained by the fact that α3 is essential for neuronal transmission in some autonomic ganglia that is not immediately lethal when blocked (Xu *et al.*, 1999).

1.7 Hypothesis

My goal is to test the hypothesis that the α3 subunit is crucial for the appearance of functional nAChRs in mice SCG neurons.

1.8 Overview of the approach

In order to test this hypothesis, I first have to confirm that SCG from α3 -/- mice developed by Xu and colleagues had no sensitivity to ACh. Then, can I rescue functional nAChRs by overexpressing the α3 subunit in sympathetic neurons from α3 -/- mice? If so, then I plan to express mutant forms of the α3 subunit to allow me to investigate further aspects of targeting α3-containing receptors to postsynaptic domains in these neurons.

Chapter 2: Materials and methods

2.1 α3 knock-out mice: genotyping protocol and colony maintenance

2.1.1 Tail lysates preparation

After identification of the newborn mice by finger removal, a piece of tail of about 0.5 cm was cut and kept in a 1.5 ml Eppendorf tube. Each tail sample was then rapidly immersed in 0.5 ml of lysis buffer containing 150 μg/ml of Proteinase K and put at 55°C in the shaking water bath until complete lysate. The lysates were then vortexed and boiled for 5 min to inactivate the Proteinase K (that would otherwise degrade the *Taq* polymerase). Lysates were then diluted to 1/30 in glass-distilled water before use.

2.1.2 PCR reaction

The three-way polymerase chain reaction (PCR) was carried out in a 25 μl reaction mixture containing 1 μl of the 1/30 dilution of the boiled tail lysate, 1.5 U of *Taq* polymerase, 1X PCR Buffer (MBI Fermentas), 2.5 mM $MgCl_2$ (MBI Fermentas), 0.2 mM dNTP, 1.25 μl DMSO (Sigma), 0.2 μM of each of the three primers. To ensure the reliability of the results, 4 controls were added for each PCR batch: one tube without DNA, one with known +/+ DNA, one with known +/- DNA, and one with known -/- DNA. The PCR was performed on a thermal controller (PTC-100, MJ Research Inc.) using the following parameters: an initial step of 3 min at 94°C, then 35 cycles of 30 sec at 94°C/30 sec at 65°C/30 sec at 72°C, and a final step of 10 min at 72°C. Once the PCR

completed, PCR samples were mixed with loading dye and subjected to migration on a 2% agarose-TBE-ethidium bromide gel for 30 min at 100 V.

2.1.3 Colony maintenance

Original heterozygous breeders, one male and two females, received from Baylor college of medicine, were mated, their progeny genotyped and the heterozygous pups kept for further breeding. The colony reached a size of 60 heterozygous mice (20 males and 40 females) that ensured a production rate of around 5 surviving mutant mice per week.

2.1.4 Genotyping solutions

Lysis Buffer: mix 5 ml Tris HCl 1 M (pH 7.5-8), 10 ml EDTA 0.5 M (pH 8), 2 ml NaCl 5 M, 0.5 ml DTT 1 M, 50 µl Spermidine 1 M, 20 ml 10% SDS and complete to 100 ml with glass-distilled water. Lysis buffer was stored at 4°C.

Proteinase K: Lyophilized Proteinase K (Fisher) was resuspended in glass-distilled water to a stock solution of 10 mg/ml. Aliquots were stored at -20°C.

Primer solutions: Three different primers were used:

wild-type forward: 5'-GTGGATCCCTCCGGCCATCTTTAAGAG

wild-type reverse: 5'-GACTGTGATGACAATGGACAAGGTGAC

mutant reverse: 5'-TGGCGCGAAGGGACCACCAAAGAACGG

Each primer (Gibco BRL) was resuspended in DEPC water to a stock concentration of 100 μM. Aliquots of 10 μM were stored at -20°C.

Taq polymerase: *Taq* polymerase (MBI Fermentas) 5,000 U/ml was diluted to 1/10 in the PCR mix solution (free of DNA samples). Aliquots were stored at -20°C.

dNTP mix: 100 mM solutions of each dNTP (MBI Fermentas) were mixed together to a final stock at 25 mM. Aliquots were stored at -20°C.

2.2 Sympathetic neurons in culture

Superior cervical ganglia (SCG) neuronal cultures were performed by adapting protocols from Mains and Patterson (1973), Hawrot and Patterson (1979) and Hawrot (1980).

2.2.1 Modified culture dishes

6 mm holes were bored in 35 mm tissue culture Petri dishes (Corning) and sulphuric acid-washed Aclar (Allied Signals and Plastics) coverslips were glued with silicone rubber (3140 RTV coating, Dow Corning) and left to air dry overnight. The well capacity of these modified dishes was approximately 50 μl. Before use, the dishes were sterilized via 20 min exposition to UV light and manipulated in sterile conditions from then on.

2.2.2 Dissection

Superior Cervical Ganglia were dissected under sterile conditions from 2 to 5 days old mice killed by cervical dislocation. SCG were collected in a 15 ml conical polystyrene tube (Fisher) containing plating medium at room temperature.

2.2.3 Dissociation and plating

Plating medium was replaced by 3 ml of trypsin solution and the ganglia were left incubating at 37°C in a water bath for 45 min, flicking every 5 min. Meanwhile, dishes were prepared for plating. The laminin solution was removed from the dishes that were rinsed twice with HBSS and filled with 1.5 ml fresh growth medium. Sterile glass rings were deposited around the well to restrict the cell suspension to the centre. After the enzymatic incubation, SCG were triturated with a fire-polished Pasteur pipette until complete dissociation. Cells were then rinsed twice with plating medium and resuspended in a small volume of growth medium in order to plate 2 drops/well. The cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5 % CO_2 and 95 % air.

2.2.4 Culture maintenance

The next day, glass rings were removed and cells were fed with growth medium supplemented with cytosine arabinofuranoside (ARA-C, 10 μM, Sigma) to kill the dividing cells and to let the neurons grow in the virtual absence of non neuronal cells. ARA-C treatment was maintained during three days and growth medium was replaced every three days.

2.2.5 Culture solutions and media

Fresh Vitamin Mix (FVM): it is composed of 1.25 mg/ml ascorbic acid, 65 μg/ml glutathione and 12 μg/ml 6,7-dimethyl-5,6,7,8 tetrahydropterine ($DPMH_4$; Sigma), supplemented with 100 mg/ml glucose (Fisher), L-glutamine (50 mM, Gibco), penicillin (12,000 units/ml, Gibco) and Streptomycin (12,000 μg/ml, Gibco).

Growth Medium: $L_{15}CO_2$ medium with 5% rat serum, 4% FVM, and NGF (1/20,000 dilution) (from Hawrot and Patterson, 1979).

10X Hank's Balanced Salt Solution (without Ca^{2+}, Mg^{2+} nor $NaHCO_3^-$)(10X HBSS): mix 2 g KCl, 40 g NaCl, 237.5 mg Na_2HPO_4, 300 mg KH_2PO_4, 5 g glucose, 5 ml Hepes 1 M and 0.5 mg phenol red in 500 ml water.

L15Air: Sterile L15 medium (Leibovitz 15, Sigma).

$L_{15}CO_2$: For 5 l, dissolve 68.7 g L15 powder (Gibco) in 3951 ml glass-distilled water. Add 25 ml SVM and 300 g Imidazole (Sigma) and stir the mixture for 1 hr and adjust pH to 7.4 using 2 N HCl. Complement the medium with a sodium bicarbonate solution and filter through 0.2 μm membrane (Nucleopore). Stored at 4°C.

NGF solution: reconstitute 2.5 S Nerve Growth Factor (Cedarlane) in 0.02% acetic acid to a stock concentration of 1 mg/ml.

Plating medium: L15 Air with 10 % Horse Serum (Gibco) and 4% FVM.

Rat serum: anaesthetised retired male breeders rats (Sprague Dawley, C.D strain, Charles River Canada Inc.) have been bled and the blood allowed to clot on ice for 30 min. After a 30 min centrifugation at 30,000 g at 4°C, the supernatant (serum) was kept a 4°C over night. After a second centrifugation, the serum was filtered through a 0.22 mm membrane and aliquoted and stored at -20°C.

Sodium bicarbonate solution: to 1024 ml glass-distilled water, 12.6 g $NaHCO_3$ (0.15 M) and 2.05 ml phenol red (0.5 %) were added and pH was adjusted by bubbling the solution with CO_2 for 20-30 sec.

Stable Vitamin Mix (SVM): for 60 ml, 180 mg glutamic acid, 180 mg proline, 120 mg inositol, 180 mg aspartic acid, 180 mg cysteine, 60 mg β-alanine, 24 mg vitamin B_{12}, 120 mg choline chloride, 6 mg lipoic acid, 0.24 mg biotin, 60 mg β-aminobenzoic acid, 300 mg fumaric acid (all from Sigma) and 4.8 mg coenzyme-A (Pharmacia) have been diluted in glass-distilled water, aliquoted and stored at –20°C.

Trypsin solution: Trypsin-3X (Worthington Biochemical Corporation) was diluted in 1X HBSS at a concentration of 1 mg/ml, filtered and pH colour-adjusted with 1 M Hepes KOH (pH 7.4).

2.3 Building the adenovirus constructs

2.3.1 Basic principle of adenovirus production

To overexpress different α3 nAChR subunit constructs in SCG neurons, adenovirus (Ad) has been used as the gene transfer vector. Briefly, in viral transfection, the DNA of interest is inserted into the virus genome and infection with the recombinant virus induces the transfer of the foreign gene. The adenoviral genome consists of a linear double-stranded 36 kb DNA molecule, surrounded by an icosahedral capsid (**figure 2**). The genome is divided into "early genes" and "late" ones, depending on when their products are made in the adenovirus replication cycle. The early genes are arranged in four regions, termed E1-E4. E1 corresponds to the first viral proteins produced after infection and governs the expression of all other viral genes (Hitt *et al.*, 1995; Shenk, 1995). Thus a deletion in E1 makes the virus unable to replicate, except in complementing cells stably expressing the E1 genes such as HEK 293 cells (Hitt *et al.*, 1995). The replication deficiency and its compensation by the HEK 293 cells are the foundations of the methods used to make adenoviral vectors containing my different α3 constructs.

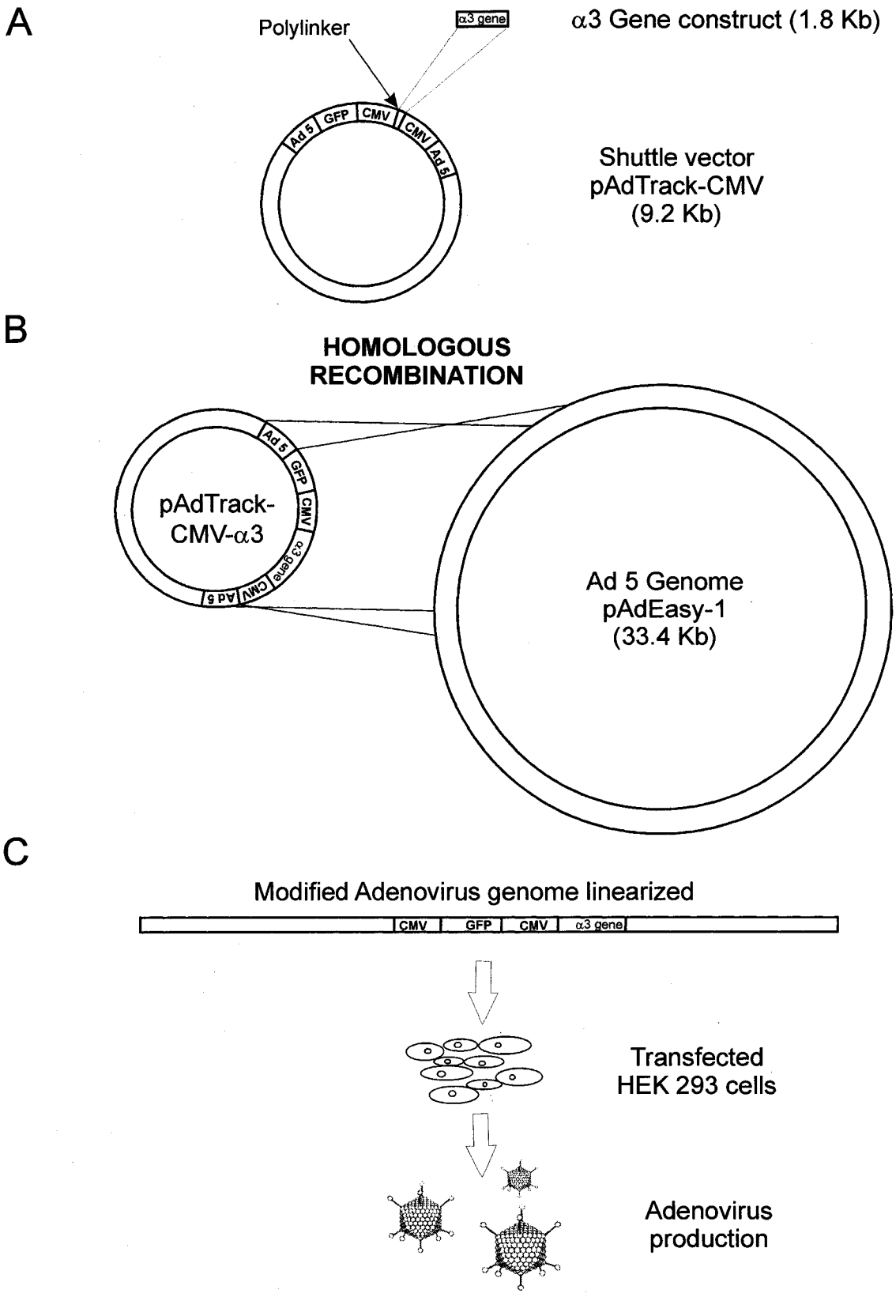
The different α3 constructs have been cloned into plasmids that include small parts of the adenovirus serotype 5 genome (Ad5; **figure 2 A**). Because the plasmids serve as carriers for my genes before virus formation, these vectors are called "shuttle plasmids". Electrocompetent bacteria were then cotransformed with a shuttle plasmid and another plasmid consisting of most of the Ad5 genome, except for the E1 region. Recombination between homologous sequences of the two plasmids sometimes occurred

Figure 2 Schematics of the construction of an Adenovirus containing an α3 gene construct.

A. Insertion of the α3 gene construct into the shuttle vector. The α3 gene has been inserted in the polylinker of the shuttle vector by ligation.

B Homologous recombination between the shuttle plasmid and the vector containing the Ad 5 genome except the E1 gene. The shuttle vector containing the α3 gene and the vector containing the Ad 5 genome are used to cotransform electrocompetent bacteria in order to get recombinants that have integrated the α3 gene construct into the Ad 5 genome lacking the E1 gene.

C Adenovirus production. The recombinant has been linearized with the rectriction enzyme Pac I and transfected into HEK 293 cells that express the E1 gene and therefore allow the replication of the adenovirus containing the α3 gene construct.


A
Polylinker
α3 gene
α3 Gene construct (1.8 Kb)
Ad 5
GFP
CMV
CMV
Ad 5
Shuttle vector
pAdTrack-CMV
(9.2 Kb)
B
HOMOLOGOUS
RECOMBINATION
pAdTrack-
CMV-α3
Ad 5
GFP
CMV
α3 gene
CMV
Ad 5
Ad 5 Genome
pAdEasy-1
(33.4 Kb)
C
Modified Adenovirus genome linearized
CMV
GFP
CMV
α3 gene
Transfected
HEK 293 cells
Adenovirus
production

in these bacteria and created an Ad5 genome with my gene instead of E1 (**figure 2 B**). Antibiotic resistance selection and restriction digests confirmed the existence of recombinant viral DNA with my insert. Liposome-mediated transfection into HEK 293 cells, which express E1 gene products, allowed the complete fabrication of a virus with the modified genome (McGrory *et al.*, 1988; **figure 2 C**). Along with the gene of interest, the construct also contains the Green Fluorescent Protein (GFP) gene, therefore viral plaques in the HEK 293 cell monolayers can be assayed by monitoring GFP expression with fluorescence microscopy. Larger amounts of virus were then grown in HEK 293 cells and were used to infect cultured SCG neurons. In specific conditions, the cells stayed in a relatively good health while expressing the transferred gene: they show clear nuclei and nucleoli when observed under phase contrast microscopy (Slack *et al.*, 1996). By overexpressing different α3 constructs in neurons from α3-null mice, that do not express any ACh sensitivity, I expected to see ACh sensitivity reappear with the specific properties of the α3 construct expressed.

2.3.2 Methods

Five different virus vectors have been generated in my experiments: one with the wild type α3 nAChR subunit coding sequence; a second with a chimeric α3 subunit where 5 amino acids (W184, W187, V188, Y189 and T191) come from the ligand binding site of the *Torpedo* α1 subunit (called α3/α1); a third with another chimeric α3 subunit where 6 amino acids (K184, R185, N186, K188, F189 and E191) come from the ligand binding site of the rat α7 subunit (called α3/α7); a fourth with a single amino acid mutation in the intermediate ring of the M2 domain of the α3 subunit (called $\alpha3_{E240A}$);

and finally a fifth one with the wild type α3 bearing a c-myc tag at its C-terminus (called α3 myc). To generate these recombinant adenoviruses, He's protocol (1998) has been followed.

Preparing the vectors used to generate adenoviruses

AdTrack-CMV and AdEasy-1 vectors were obtained from Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore). Both were used to transform DH5αF' bacteria by classical heat-shock procedures (Sambrook *et al.*, 1989). The bacteria were plated onto LB/agar plates with the appropriate antibiotics (i.e. 100 μg/ml ampicillin for AdEasy-1 and 50 μg/ml kanamycin for AdTrack-CMV). Picked colonies were grown in 5 ml of LB with the appropriate antibiotics. Plasmid DNA has then been extracted from ~2-3 ml of bacterial suspension with the Qiaprep Spin Miniprep Kit (Qiagen). Briefly, the procedure consists of lysing the bacteria under alkaline conditions and clearing the lysate of cell debris. The plasmid DNA is adsorbed onto a silica-gel membrane, washed and eluted into 2 x 50 μl water or Tris solution. Such preparations yield up to 20 μg DNA. Finally, larger DNA stock (200 to 400 μg) have been generated with a Qiagen Plasmid Maxi Kit, which uses columns containing ion exchange resin. The DNA has been resuspended in 200 μl of water. The final DNA concentration has been evaluated by spectrophotometry (an OD_{260} of 1 corresponding to 50 μg/ml DNA).

Meanwhile, the different α3 constructs have been amplified, extracted from their vectors and inserted by ligation in the polylinker of the shuttle vector. The same optimized procedure has been used to generate all the different α3 constructs. As an example, the full-length rat α3 gene (GenBank accession number X03440; courtesy of

Dr. Séguéla, McGill University, Montréal) has been amplified in the pcDNAI/Neo plasmid (Invitrogen). The α3 sequence has then been extracted from the plasmid with the double digestion of 10 μg of the plasmid DNA with the restriction enzymes Not I and Xba I (MBI Fermentas) overnight at 37°C. After checking that the restriction product had the appropriate size when ran on an 1.5% agarose gel, the band of interest has been excised and gel extracted with the Spin-X column (Fisher) in a benchtop cenrifuge at 8,000 rpm for 10 min. The DNA has then been purified through a classical Tris phenol chloroform amyl/ammonium acetate purification procedure. The purified DNA has been resuspended in 40 μl of glass distilled water. In parallel, the shuttle vector has been linearized with the same two enzymes and the DNA insert prepared the same way. The only difference is that consecutively to the enzymatic restriction, the linearized shuttle vector has been dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP, 0.5 U/μg of vector; MBI) to avoid its recircularization and therefore increase the ligation yield. The pure α3 Not I/Xba I fragment has then been ligated into pure Not I/Xba I AdTrack-CMV:~2 μg of the insert were mixed with ~0.5 μg AdTrack-CMV, and 5 Units of T4 DNA ligase (MBI). The ligation was left to proceed for 1 hour at room temperature and the ligase was heat-inactivated for 10 min at 65°C to increase the transformation yield. Later on, the mixture was used to transform DH5αF' bacteria by a classical heat-shock protocol (Sambrook *et al.*, 1989). A small preparation of shuttle plasmid DNA was made and the insert's presence and orientation confirmed by restriction analysis. In some cases, to ensure that the subcloned α3 insert coding sequence was still functional in the shuttle plasmid, expression in *Xenopus* oocytes have been performed by David Davachi,

or myself, according to the methods described by Bertrand and coworkers (Bertrand *et al.*, 1991). The same procedure has been used to make the four other α3 constructs.

The chimeric α3/α1 (gift from Dr. Hawrot, Brown University, Providence) as well as chimeric α3/α7 have been amplified in the pcDNA3.1/ zeo (+) plasmid (Invitrogen). The insert has been extracted in both case with the restriction enzymes Kpn I and Not I. In the case of the mutant $\alpha3_{E240A}$, previously generated in the laboratory by Dr. Haghighi, it has been amplified in the plasmid pcDNA1/Neo and the insert extracted for ligation using the Sal I and Xho I sites. Finally, the C-terminus myc tagged α3, generated by Brigitte Pié, has also been amplified in the plasmid pcDNA1/Neo and the insert extracted using the Xho I and Xba I sites.

Generating recombinants in bacterial cells

Prior to the cotransformation, 5 μg of the shuttle plasmids were linearized with Pme I (New England Biolabs). After running the digestion products on a 0.8% agarose gel, the DNAs were extracted and purified as described previously. Between 0.02 and 0.1 μg of the Pme I-digested shuttle plasmid were then cotransformed with ~1 μg of the adenoviral backbone vector Adeasy-1. The cotransformation has been performed by electroporating BJ5183 electrocompetent *E. Coli* bacteria (prepared as described below) in 1 mm cuvettes at 1,800 V, 200 Ω and 25 μF in a Bio-Rad Gene Pulser electroporator. The transformation mix was resuspended in 500 μl LB, incubated it at 37°C and centrifuged at 225 rpm for 15-20 minutes, then plated it on 4 or 5 LB/kanamycin plates (50 μg/ml) and put at 37°C overnight (~20 hrs). 12-24 of the smallest colonies were picked up and grew in 5 ml LB containing 50 μg/ml kanamycin for 10-15 hours.

Minipreps were then performed as described previously, using the Qiagen kit and the sizes of Pac I-digested (New England Biolabs) plasmids were checked by running one fifth of a cut miniprep on 1% agarose gel. Good candidate clones yielded a large fragment (near 30 kb), plus a smaller fragment of 3.0 kb or 4.5 kb, depending on the site of homologous recombination. DH10β bacteria were then transformed with the correct recombinant plasmids and plasmid DNA purified.

Viral production in HEK 293 cells

HEK 293 cells were grown in 35 mm culture dishes (Corning). The cell confluency was about 50% to 70% at the time of transfection. For each virus, on the day of transfection, the recombinant adenoviral plasmid was submitted to restriction with Pac I, purified and resuspended it in sterile water. Generally, 3 to 4 μg DNA were used to transfect one 35 mm dish. For the LipofectAmine (Gibco BRL) transfection: for each dish, on the one hand, the Pac I-digested plasmid was mixed to 100 μl Opti-MEM I medium (Gibco BRL) and, on the other hand, 3 μl of LipofectAmine reagent to 100 μl Opti-MEM I. Both solutions were left for incubation at room temperature for 45 minutes. They were then combined gently and let stand for 15 minutes. Meanwhile, HEK 293 cells have been washed once with Opti-MEM I. Opti-MEM I was then added to the LipofectAmine-DNA mix up to 1 ml, transferred to the dish, and the cells returned to the 5% CO_2 incubator at 37°C. Transfection was stopped after 8 hours by replacing the medium with fresh complete α-MEM.

Since AdTrack-based vectors contain the subcloned GFP gene Transfections efficiency and viral productions were monitored by GFP expression. Green plaques were

often seen under fluorescence as soon as 24 hours post-transfection and CPE (cytopathic effect) started around 5 days after transfection. Cells were scraped off the dish with a rubber policeman at ~8 days post-transfection, when most cells were rounding and coming off, and transferred to a 15 ml conical tube. The cells were then spun down in a benchtop centrifuge at 8,000 rpm for 1 minute and the pellet resuspended in 1.5 ml sterile PBS. Some supernatant was kept at -80°C. In order to break down the cell membranes and harvest the virus produced in the cell, the cells were subjected to four alternated freeze/thaw/vortex cycles. First, they were quickly frozen in a mix of dry ice/methanol, and immediately thawed in a 37°C water-bath and vortexed vigorously. These freeze/thaw/vortex cycles were repeated 3 more times without letting the virus supernatant warm up. The cell lysate was then briefly centrifuged (45 sec, 15,000 rpm) to get rid of the cell debris and the virus-containing supernatant stored at -80°C after a quick-freeze. Two thirds of the viral supernatant produced by one 35 mm Petri dish were used to infect two 35 mm dishes and cell lysis appeared at 2 to 3 days post-infection. The virus was then harvested the same way as it has been after the transfection and viral amplification was done by increasing step by step the size of the next infected Petri dish, to reach a final total number of four 150 mm infected dishes. Each round of amplification gave at least 10-fold more viruses than the previous one. Consecutively to the final round, the viral stock was aliquoted and stored at -80°C. To perform the titration of the viral stock, HEK 293 cells in 35 mm Petri dishes have been infected with serial dilutions of viral supernatant (three 35 mm dish/virus dilution) and the number of green cells (i.e. infected cells) was counted 18 hours later. The different α3 construct viruses that I produced have the following titers:

α3:	2.9×10^{11} pfu/ml (Plaque Forming Units per milliliter)
α3/α1:	2.3×10^{11} pfu/ml
α3/α7:	4.6×10^{11} pfu/ml
$\alpha 3_{E240A}$:	6.3×10^{11} pfu/ml
α3-myc:	9.7×10^{11} pfu/ml

Making electrocompetent bacterial cells

10 ml of LB medium have been inoculated with frozen stocks of DH10β cells (courtesy of Dr. D. Morris, McGill University) in a 50 ml tube and left to grow in a rotating incubator (250 rpm) overnight at 37°C. After diluting 1 ml of culture broth into 1 l of LB medium in eight 1 liter flasks (125 ml each), bacteria were left to grow more for 4 to 5 hours at 37°C, until OD_{550} reached ~0.8. They were then harvested in four 250 ml conical centrifuge tubes and left them on ice for 30 minutes to 1 hour. The cells were then centrifuged at 2,600 g and 4°C for 10 minutes and the pellet was washed by resuspending the cells in 1,000 ml of ice-cold WB. The cell suspension was then spun down (2,500 g for 30 minutes) and the wash step repeated. Most of the supernatant was discarded, and the rest of it, the last 20 ml, was used to resuspend the bacteria that were transferred to a 50 ml tube. The cells were then subjected to a last centrifugation at 2,600 g for 10 minutes and all but 5 ml of the WB was pipetted out. The cell pellet was resuspended in the supernatant, aliquoted in 20 µl and stored at -80°C.

To check the cells's competency, one aliquot of competent cells was transformed by electroporation, with 1 µl of purified plasmid DNA at a known concentration. The transformed bacteria were grown and plated on appropriate antibiotic LB/agar plates as

described previously. After an overnight incubation at 37°C, over 10^8 colonies/μg of DNA have been obtained.

HEK 293 cell cultures

Low passage HEK 293 cells (E1-transformed human embryonic kidney cells; generous gift of Dr. P. Branton, McGill University) were put in culture in complete α-MEM. When they reached 80-90% confluency, they were harvested using citric saline. They were then either replated at ~1/5 – 1/3 of their density or frozen in liquid nitrogen in medium containing 10% sterile DMSO (Sigma). The cells were maintained at 37°C in a 5% CO_2 humidified incubator and fed with fresh medium every three days. For gene transfection, rather low passage number cells that grow more slowly were used and, for viral amplification, higher passage number cells that divide faster were preferred.

Solutions and media

Citric saline (10X): 50 g of potassium chloride and 22 g of sodium citrate dissolved into 500 ml of distilled water and then filtered.

Complete α-MEM: α-MEM complemented with 10% FBS, 1% Penicillin-Streptomycin, 1% L-glutamine (200 mM), 1% MEM-Vitamin solution and 1% Fungizone (all from Gibco BRL).

PBS (10X): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 mixed to a total volume of 1 l of distilled water. Sterilization of the solution by autoclaving.

WB: Sterile WB was made of 10% ultra pure glycerol in distilled water.

2.4 Electrophysiology

I assisted David Davachi for all the electrophysiological recordings performed in this study.

Whole-cell recordings from SCG neurons

Whole-cell patch clamp recordings on SCG neurons were performed at room temperature (22-24°C) using a List EPC-7 amplifier (Hamill *et al.*, 1981). Throughout the recordings neurons were perfused with the external solution at a rate of 1 ml/min, and Acetylcholine was applied by pressure ejection from pipettes with tip diameters of 20-30mm (Mandelzys *et al.*, 1995). Currents were filtered at 1.5 kHz with an eight-pole Bessel filter (Frequency Devices Inc.), sampled at 2.5-5 kHz, displayed and stored on-line with a Pentium 100-PC computer. The program PATCHKIT was used for stimulation and data acquisition. The resistance of patch pipettes ranged from 2 to 6 MΩ, and 50-60% of the series resistance was compensated.

Electrodes and solutions

Glass patch pipettes: they were made from capillary tubes (Frederick Haer & Co., Brunswick) using a DMZ-Universal Puller (program 10). Recording electrodes were filled with intracellular solution.

Intracellular solution: it contained 70 mM KF, 65 mM K Acetate, 5mM NaCl, 1 mM $MgCl_2$, 10 mM EGTA and 10 mM HEPES; pH 7.4 was adjusted with KOH.

External perfusing solution: It contained 140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 2.8 mM $CaCl_2$, 10 mM HEPES, 5.6 mM glucose, 2 mM glutamine, 0.5-1 μM TTX (Sigma) and 1 μM atropine; pH was adjusted to 7.4. ACh (Acetylcholine iodine, Sigma) was dissolved in the same external perfusing solution (100 μM).

Chapter 3: Results

3.1 SCG neurons from α3-null mice develop normally in culture but lack ACh-evoked currents

Several lines of evidence suggest that α3 subunits are important for nAChRs in sympathetic neurons, but their precise role has not been determined. For example, rat superior cervical ganglion (SCG) neurons express five nAChR transcripts: α3, α5, α7, β2 and β4, and over the first 2 postnatal weeks, mRNA levels for α3 and α7 subunits increased by more than threefold which correlates with an increase in ACh current densities (Mandelzys *et al.*, 1994). In contrast, no significant change in mRNA levels occur in α5, β2 and β4 transcripts. Moreover, pharmacological experiments characterizing the ACh-evoked currents on SCG for their sensitivity to different nicotinic agonists, as well as to α-BTX and neuronal BTX (n-BTX) suggest that most functional nAChRs have coassembled with α3, β2 and β4 (Mandelzys *et al.*, 1995). In addition, work from Jacob and colleagues showed that the M3-M4 domain of the α3 subunit specifically targets the nAChRs to the synapse (Williams *et al.*, 1998). My interest is to determine whether the α3 subunits are critical for the appearance of functional nAChRs on sympathetic neurons. To do so, I investigated nAChRs on neurons from mice with a disrupted α3 gene (Xu *et al.*, 1999).

3.1.1 Set up of the breeding colony

First, I set up a breeding colony to generate mice that lack the α3 subunit. Since mice homozygous for the α3 mutation do not breed, I mated heterozygous animals. For

genotyping the offspring of these matings, I used the PCR method developed by Xu and coworkers. Briefly, to disrupt the α3 gene, Xu and colleagues replaced a portion of the exon 5 of α3 with a sequence from the hypoxanthine phosphoribosyltransferrase (HPRT) gene. I amplified a portion of genomic DNA using primers that spanned the deleted region in exon 5 of the α3 gene, as well as a primer for the inserted portion of the HPRT gene. In this way, the primer sets are capable of amplifying a portion of the intact α3 gene and a portion of the disrupted α3 gene. **Figure 3.1 A** shows an example of an ethidium bromide stained agarose gel of the different amplimers resulting from the PCR of DNA from wild-type, heterozygous and homozygous mutant mice. The 500 bp band corresponds to the amplimer of a portion of the intact α3 gene whereas the 200 bp band corresponds to the amplimer of a portion of the disrupted α3 gene. The heterozygous mice have one copy of each allele and their PCR products have two bands, one of 500 bp and one of 200 bp. The fastest migrating band present in all the lanes corresponds to primer dimers (**figure 3.1 A**).

3.1.2 α3 -/- SCG neurons in culture

α3 -/- mice have a severe phenotype, half of them die within the first three days after birth; those that survived this critical stage die gradually over the next eight weeks (Xu *et al.*, 1999). To investigate nAChRs in sympathetic neurons from α3 -/- mice, I turned to tissue culture.

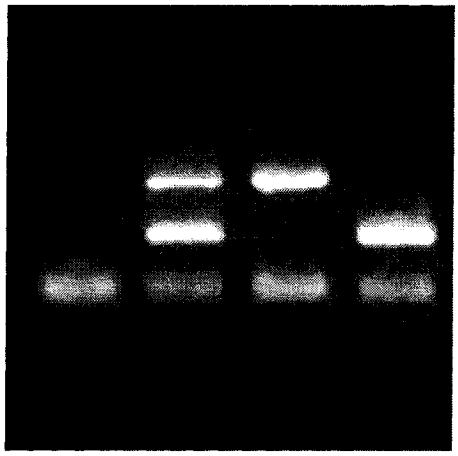
I adapted the protocol used in our laboratory for rat SCG to culture mice SCG neurons. Briefly, I dissociated the ganglia in a trypsin containing media for 45 min, rinsed and plated the neurons on laminin-coated coverslips. **Figure 3.1 B** shows a phase contrast

Figure 3.1 Primary culture from sympathetic neurons from α3 -/- mouse.

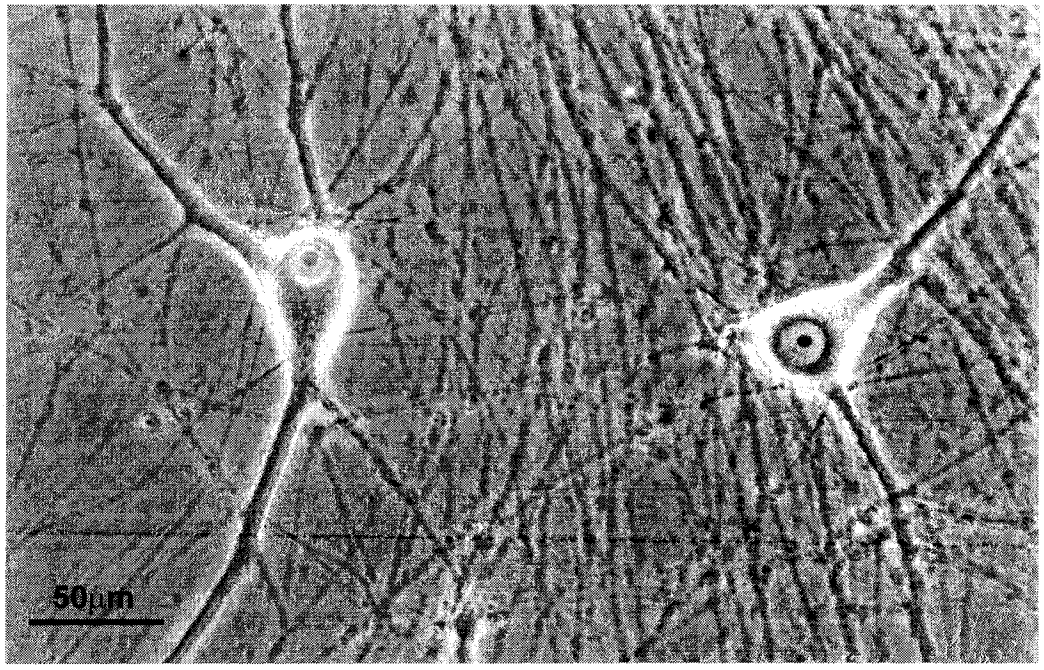
A. Picture of an ethidium-bromide stained agarose gel showing the different amplimers resulting from the PCR-based genotyping procedure. The different amplimers corresponding to the three genotypes are present: heterozygous (+/-), homozygous wild-type (+/+), and homozygous mutant (-/-). The first lane is a control PCR that did not contain any DNA sample.

B Phase contrast photomicrograph of SCG neurons from a P3 α3 -/- mouse grown in culture for two weeks. The two neurons look healthy: they have clear nuclei and nucleoli and they extend many processes: both axons and dendrites.

A



B



photomicrograph of SCG neurons from a three days old (P3) α3-null mouse grown in culture for two weeks. These neurons did not show any particularity in terms of growth when compared to wild-type neurons: their cell bodies extend numerous processes, both axons and dendrites and the neurons survive well in culture for at least one month. This indicates that the disruption of the α3 gene does not interfere with the neurons' ability to grow and extend processes.

3.1.3 α3-null SCG neurons in culture are insensitive to ACh

To test whether the α3 subunit is crucial for the appearance of functional nAChRs, we investigated the sensitivity to ACh of these neurons by whole-cell patch-clamp technique. The results are shown in **figure 3.2**. Neurons from α3 -/- mice were insensitive to 100 μM ACh. In contrast, application of 100 μM ACh produced a large depolarization in the SCG neurons from wild-type littermates (**figure 3.2 A**). **Figure 3.2 B** shows the mean ACh-evoked currents densities recorded in wild-type or α3-null SCG neurons. No ACh-evoked current were recorded from 16/16 neurons from α3-/- mice. On the other hand, all 18 neurons from wild-type animals had ACh-evoked currents; the mean ACh-evoked current density was 71.7 ± 11.2 pA/pF (mean ± S.E.M), when the membrane potential was held at –60 mV.

3.2 Rescue ACh sensitivity in neurons from α3-null mice by expressing the α3 gene

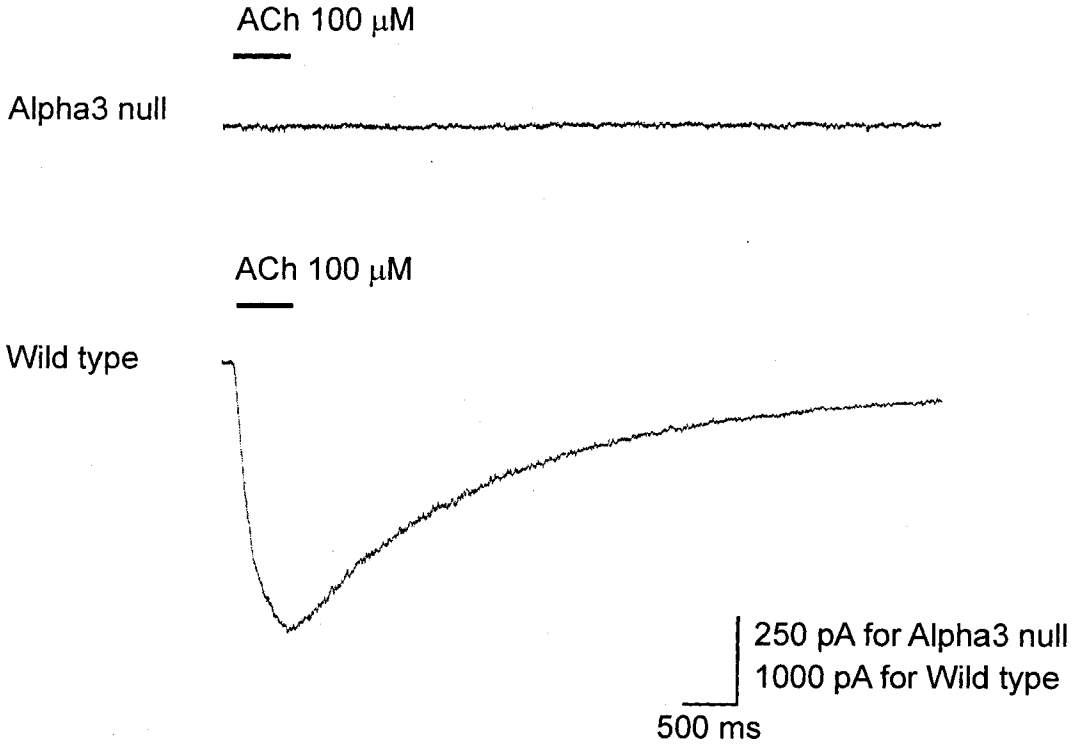
The fact that α3-null SCG neurons are insensitive to ACh indicates that the α3 subunit is critical for nAChR function. Therefore, I asked whether I could obtain functional currents in these neurons by overexpressing the α3 gene in neurons from α3-/-

Figure 3.2 Sympathetic neurons from α3 -/- mouse are not sensitive to ACh.

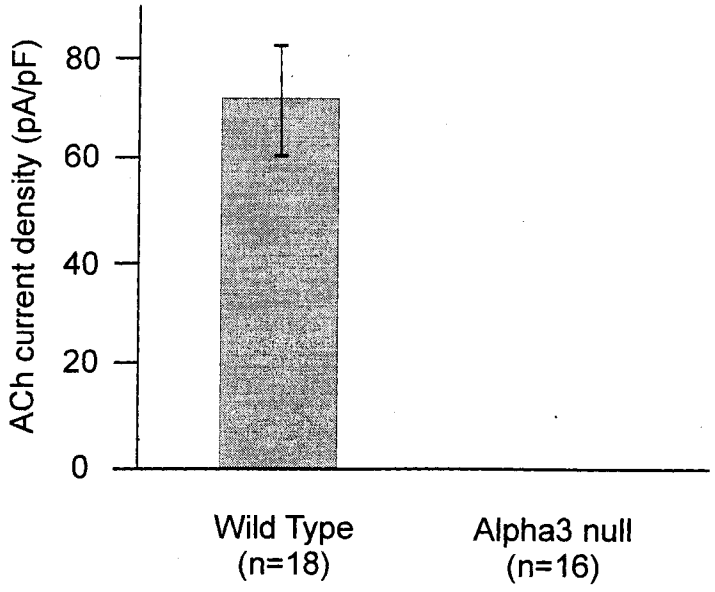
A. Currents traces from whole-cell patch-clamp after ACh stimulation. SCG neurons from α3 -/- mouse are not sensitive to 100 μM of ACh (top trace), whereas neurons from a wild-type littermate are sensitive to the same concentration of ACh, with in this case an ACh-evoked current of 3,000 pA (lower trace).

B. Bar graph showing the mean current densities in response to 100 μM ACh in wild-type and α3 -/- neurons. P1 to P3 mouse SCG neurons maintained in culture for two weeks. The wild-type neurons are sensitive to ACh with a mean ACh-evoked current density of 71.7 ± 11.2 pA/pF (mean ± S.EM), whereas the α3 null neurons are not sensitive to ACh. These data were obtained with a membrane potential held at –60 mV.

A


ACh 100 μM
Alpha3 null
ACh 100 μM
Wild type
250 pA for Alpha3 null
1000 pA for Wild type
500 ms

B


ACh current density (pA/pF)
80
60
40
20
0
Wild Type
(n=18)
Alpha3 null
(n=16)

mice. Previous work in our laboratory has shown that the use of adenoviruses was the most appropriate technique to transfer foreign genes into primary cultured neurons (Rosenberg, 1998). I generated an adenovirus containing an α3 construct (Ad α3) following the procedures developed by He and coworkers, as described in the Methods (He *et al.*, 1998). In He's method, the gene of interest is first ligated into a small plasmid, (9.2 Kb) called shuttle vector, instead of directly cloning the gene of interest in the large plasmid (33.4 Kb) containing the most of the adenovirus genome. The reason for this is that the shuttle vector is small which makes it easy to manipulate and it also contains the polylinker to ligate the gene of interest in. The homologous recombination step allow the integration of the gene of interest in the adenovirus backbone.

Briefly, I ligated the full length α3 sequence into the polylinker of the shuttle plasmid AdTrack-CMV which I refer to as AdTrack-CMV-α3. I then cotransformed *E.Coli* bacteria with AdTrack-CMV-α3 with AdEasy, the plasmid consisting of most of the Ad 5 genome except for the E1 region that contains genes required for virus replication. I then selected the recombinants plasmids for antibiotic resistance. Since these recombinants lack the E1 region, they can not replicate. Therefore to grow them, I transfected the recombinants in HEK 293 cells because these cells have been engineered to express the gene products from the E1 region. The recombinant adenovirus (Ad α3) possesses two CMV promoters, one governing the transcription of GFP, the other one governing the gene of interest which is the full length α3 gene.

To express the full length α3 cDNA in cultured SCG, I infected the neurons with $3x10^8$ pfu/ml of Ad α3 for 24 hours. No precise dose/response study has been performed, but at this concentration, Ad α3 infected 70 to 80% of the neurons, whereas with ten fold

less virus few green cells were detected. Next, I replaced the medium with fresh growth medium containing NGF and stimulated them with 40 mM KCl for 16 hours because the CMV promoter activity is highly regulated in neurons and depolarization up-regulates its activity by several folds (Wheeler & Cooper, 2001). After the "high potassium" treatment, the neurons were switched to regular 5 mM KCl growth medium, and examined 24-48 hours later. **Figure 3.3 A** shows an example of three neurons from α3 -/- mice infected with Ad α3; viewed in phase-contrast, and fluorescent optics. In this example, two neurons out of three expressed visible amount of GFP and therefore were clearly expressing genes from Ad α3.

Next, we examined these neurons electrophysiologically, using whole-cell voltage clamp recording techniques. **Figure 3.3 B** shows representative traces of the ACh-evoked current from both GFP positive and negative neurons. The GFP-positive neuron produced a 1.2 nA ACh-evoked current in response to 100 μM ACh. In contrast, no ACh sensitivity was detected from GFP-negative neuron. In all, we examined twelve GFP-positive neurons from α3 -/- mice infected with Ad α3: all 12 were sensitive to 100 μM ACh and the mean ACh-evoked current density was 67.1 ± 8.7 pA/pF (mean ± S.E.M), as shown in **figure 3.4**. These important results show that expression of the α3 gene can confer ACh sensitivity to the originally ACh insensitive α3-null SCG neurons.

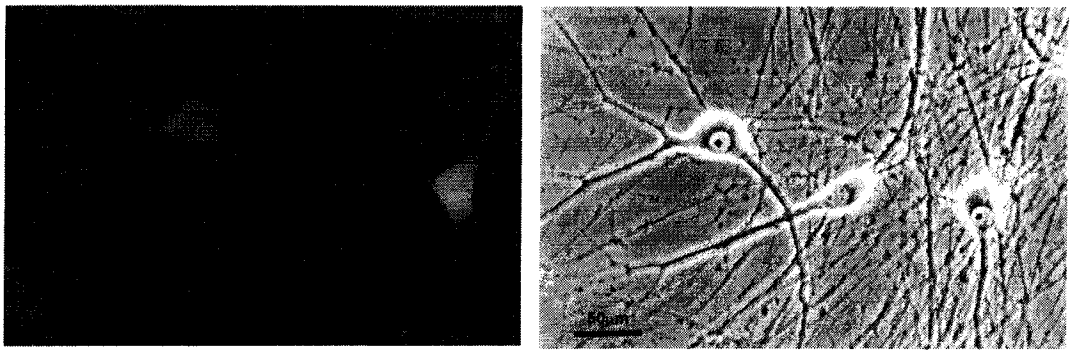
To ensure that the rescue of ACh sensitivity was due to the expression of the α3 gene, I infected sister cultures with similar concentration of Ad GFP, an adenovirus that contains only the GFP gene. We examine 6 infected neurons: none of the 6 GFP-positive neurons infected with Ad GFP had ACh-evoked current.

Figure 3.3 Sympathetic neurons from α3 -/- mouse infected with an adenovirus containing the α3 gene are sensitive to ACh.

A. Photomicrographs of SCG neurons from an α3 -/- mouse infected with Ad α3. Phase contrast photomicrograph of three P3 SCG neurons cultured for two weeks, infected for 24 hours, and stimulated with high potassium for 16 hours (picture on the right). After infection, the neurons still look healthy : one can easily distinguish their nuclei and nucleoli (even in the cell in the middle which is a bit out of focus). Fluorescence photomicrograph of the same field showing that two out of three neurons are GFP-positive, which confirms their infection by the virus.

B. GFP-positive neurons are sensitive to ACh. As depicted on the scheme on the left, the GFP-positive neuron from the same field is sensitive to ACh and produces a 1,200 pA current in response to 100 μM ACh. To the contrary, the GFP-negative neuron, as shown on the right, is not sensitive to ACh. For all the recordings, the membrane potential was held at –60 mV.

A



B

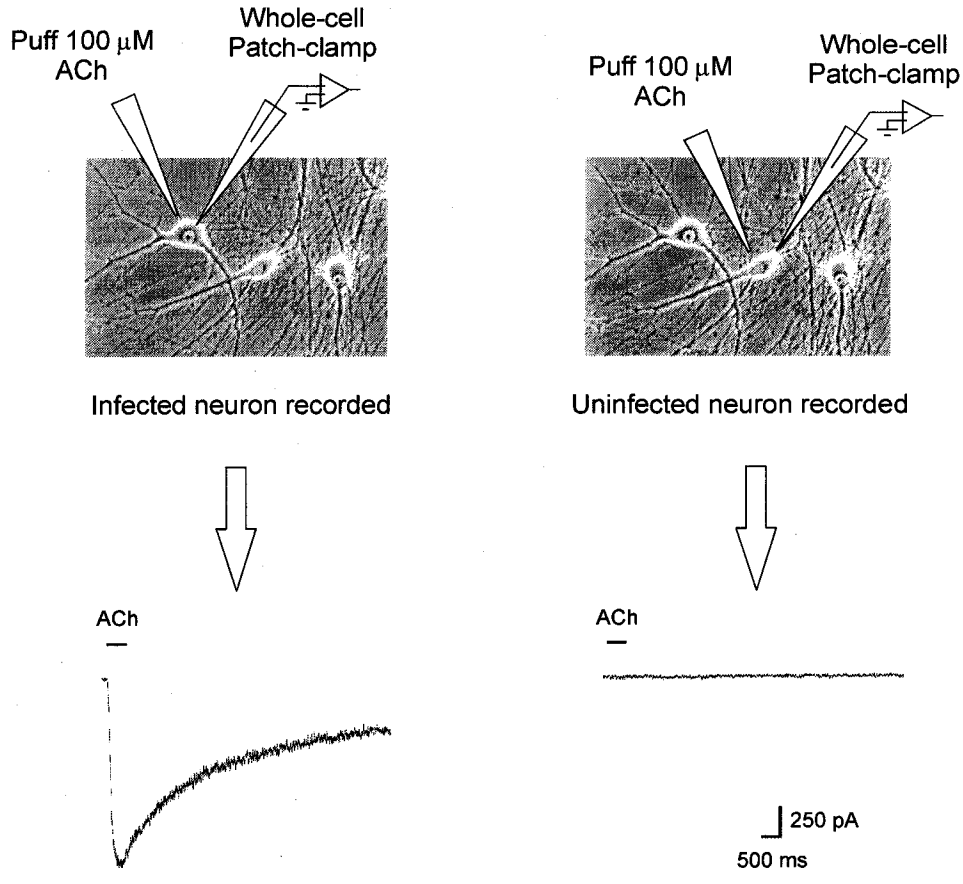
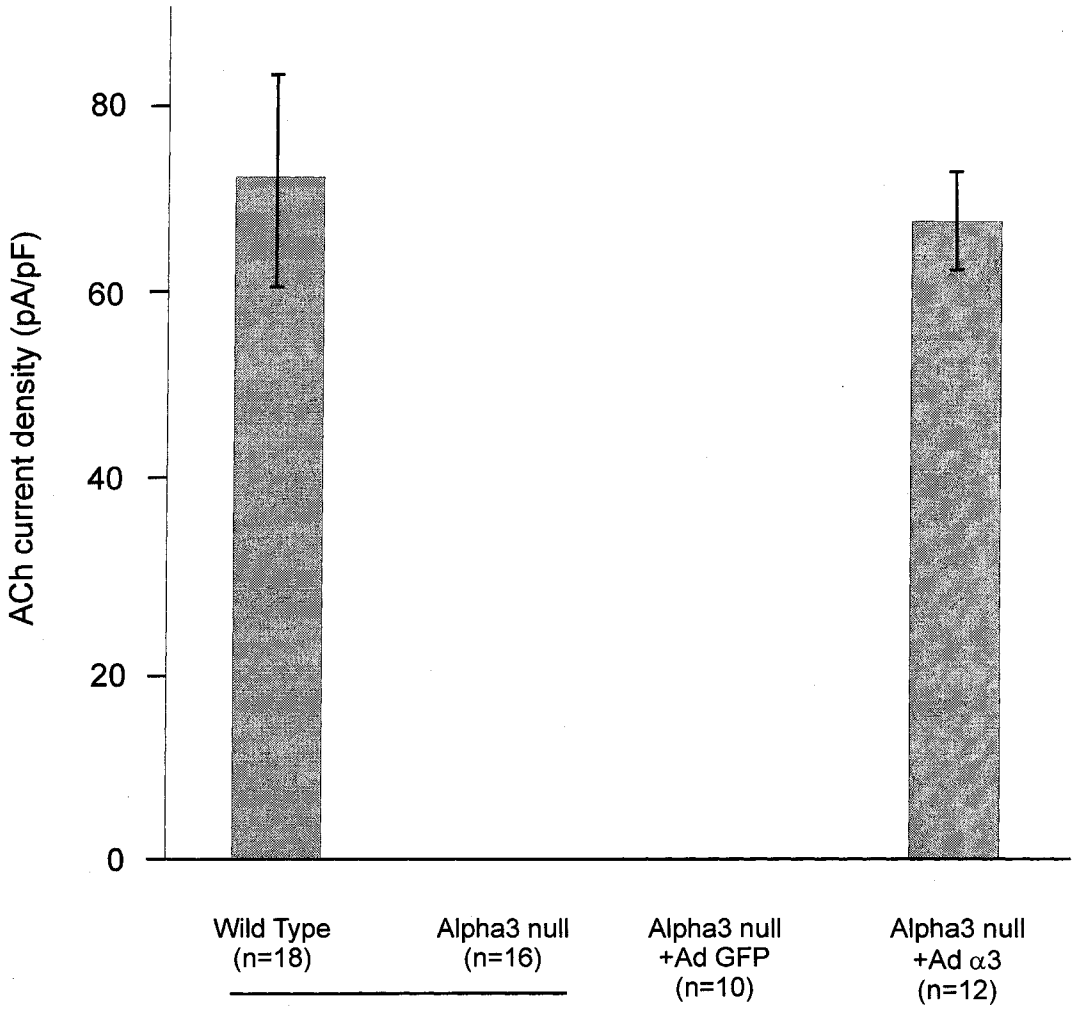

Puff 100 μM ACh
Whole-cell Patch-clamp
Infected neuron recorded
ACh
Puff 100 μM ACh
Whole-cell Patch-clamp
Uninfected neuron recorded
ACh
250 pA
500 ms

Figure 3.4 Sympathetic neurons from α3 -/- mouse infected with an adenovirus containing the α3 gene are sensitive to ACh, whereas α3 -/- mouse infected with an adenovirus containing only the GFP gene are not sensitive to ACh.

Bar graph showing the ACh-evoked current densities in response to 100 μM ACh in neurons from α3 -/- mouse infected with Ad α3 or Ad GFP. Only Ad α3 infected GFP-positive Ad α3 infected neurons were sensitive to ACh with a mean ACh-evoked current density of 67.7 ± 8.7 pA/pF (mean ± S.E.M). In contrast, GFP-positive neurons infected with Ad GFP are not sensitive to ACh. For all the recordings, the membrane potential was held at –60 mV.


ACh current density (pA/pF)
80
60
40
20
0
Wild Type
(n=18)
Alpha3 null
(n=16)
Alpha3 null
+Ad GFP
(n=10)
Alpha3 null
+Ad α3
(n=12)

From Fig 3.2 B

3.3 α3-null mice SCG neurons infected with an adenovirus containing the chimeric α3/α1constuct

Taken together my results suggest that for SCG neurons to have functional nAChRs, they must express α3 subunits. In addition, these results suggested that it may be possible to express altered forms of the α3 subunits that incorporate some type of tag or recognition sequence to learn more about how their receptors target to their appropriate domains.

One such tag could be the binding site for α-BTX. For example, recently, Levandoski and colleagues performed a study on the molecular determinants responsible for the α-BTX binding to nAChRs (Levandoski *et al.*, 1999). They reported the construction of a chimeric α3/α1 subunits in which residues 184-191 have been replaced by site-directed mutagenesis with the corresponding region of the *Torpedo* α1 subunit. This substitution led to a change of 5 amino acids in the α3 sequence (Y184W, E187W, I188V, K189Y and N191T). Receptors that incorporated this α3/α1 subunit were now blocked by α-BTX when expressed in *Xenopus* oocytes in combination with the β2 or the β4 subunit. Therefore, we hypothesized that expressing the α3/α1 construct in α3-null SCG neurons, might allow us to localize the α3/α1 nAChRs by using labeled α-BTX. Therefore, I built an α3/α1 containing adenovirus (Ad α3/α1) as described in the Methods.

3.3.1 α3-null mice SCG neurons infected with Ad α3/α1 are ACh insensitive

To express these Ad α3/α1-containing vectors, I infected with $3x10^8$ pfu/ml of Ad α3/α1 for 24 hours. At this concentration, Ad α3/α1 infected 70 to 80% of the neurons. The neurons were then stimulated as described previously and then examined electrophysiologically 24 to 48 hours later. Unexpectedly, no ACh-evoked currents were recorded in the Ad α3/α1 infected α3-null SCG. As shown in **figure 3.5**, 12/12 neurons from α3 -/- mice infected with Ad α3/α1 were not sensitive to 100 μM ACh.

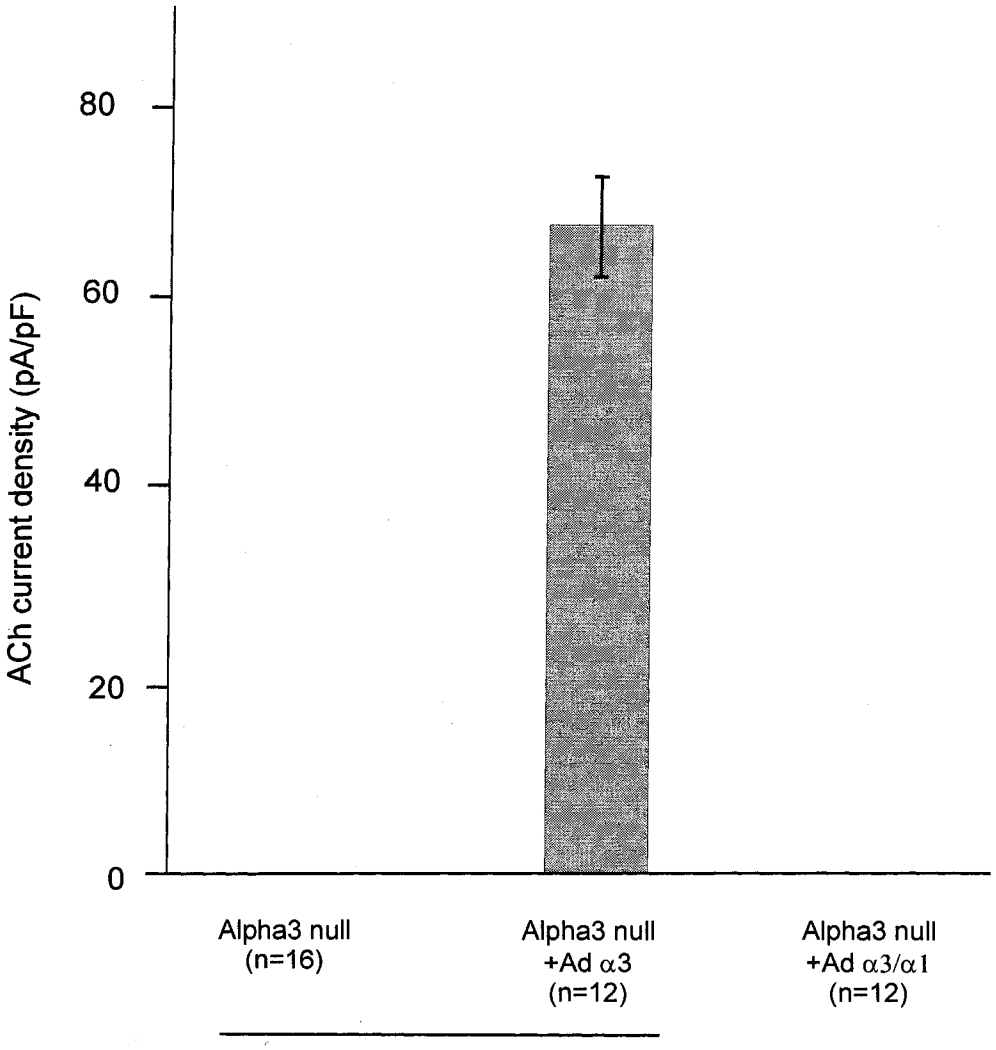
Some of these neurons were even tested with up to 1 mM ACh and still had no detectable ACh-evoked currents.

To ensure that these neurons were capable of expression ACh-evoked currents when expressing the full length wild-type α3, I infected sister cultures with $3x10^8$ pfu/ml of Ad α3. As expected, 6/6 positive controls α3-null SCG infected with Ad α3 were sensitive to 100 μM ACh.

Since α3/α1 was reported to give rise to functional receptors when expressed with β subunits in *Xenopus* oocytes but did not give rise to functional receptors when expressed in neurons, we worried whether there might be a problem with the particular α3/α1 construct that we were working with. To ensure that the construct that I inserted in the Ad genome was correct, we expressed the shuttle plasmid containing α3/α1 (AdTrack-CMV-α3/α1) in *Xenopus* oocytes together with β4. This experiment confirmed that the α3/α1 construct was forming functional nAChRs: the α3/α1 construct expressed in combination with β4 gave rise to channels that were sensitive to ACh and blocked by nanomolar concentrations of α-BTX.

Figure 3.5 Sympathetic neurons from α3 -/- mouse infected with an adenovirus containing the chimeric α3/α1 gene construct are not sensitive to ACh.

Bar graph showing the absence of response to 100 μM ACh in neurons from α3 -/- mouse infected with Ad α3/α1 compared to previous data from fig 3.4. 12/12 GFP-positive Ad α3/α1 infected neurons were insensitive to ACh, whereas 6/6 positive controls α3-null SCG infected with Ad α3 were sensitive to 100 μM ACh. For all the recordings, the membrane potential was held at –60 mV.


ACh current density (pA/pF)
80
60
40
20
0
Alpha3 null
(n=16)
Alpha3 null
+Ad α3
(n=12)
Alpha3 null
+Ad α3/α1
(n=12)

From Fig 3.4

Next, I worried that the α3/α1 gene might have been disrupted somehow during the recombination between the shuttle vector containing α3/α1 (AdTrack-CMV α3/α1) and the vector containing the adenovirus backbone (AdEasy). I therefore built three different Ad α3/α1 in parallel with Ad α3 as a positive control. None of the four Ad α3/α1 led to functional nAChRs when infecting SCG neurons from α3-/-mice, whereas each positive control Ad α3 conferred ACh sensitivity to these neurons. In addition, we send the Ad α3/α1 recombinant construct for sequencing at the Sheldon Biotechnology Center (McGill University). The results confirmed the presence of the intact insert with the correct sequence in the recombinant AdTrack-CMV α3/α1-AdEasy. Therefore, I concluded that Ad α3/α1 does not give rise to functional nAChRs in mammalian neurons, even though the products of this constructs are capable of co-assembling into functional nAChRs when expressed in *Xenopus* oocytes.

3.3.2 Incubation at room temperature does not restore α3/α1 nAChR function

Xenopus oocytes have been injected and incubated at room temperature whereas the SCG neurons have been kept at 37°C after infection, I wondered whether the chimeric α3/α1 protein was only capable of expressing at room temperature, and not at 37°C. Such cases have been reported for various mutant proteins such as the mutation ΔF508 in the cystic fibrosis conductance regulator (CFTR) (Denning *et al.*, 1992), and the mutant N470D of HERG that encodes the rapidly activating delayed rectifier potassium channel, responsible for the congenital long QT syndrome (LQT2) (Zhou *et al.*, 1999).

We therefore tested the expression of the chimeric α3/α1 subunits in neurons incubated at room temperature for 24 or 48 hours after high potassium treatment. To do

this, I cultured SCG neurons from P1 to P3 α3 -/- animals for two weeks, and then infected sister cultures with $3x10^8$ pfu/ml of Ad α3 as a positive control, while others were infected with a similar concentration of Ad α3/α1 for 24 hours. After the "high potassium" treatment, the medium was then switched to NGF supplemented growth medium where L15 CO_2 has been substituted to L15 air to allow the neurons to survive outside the incubator. After 24-48 hours of incubation at room temperature the neurons were examined electrophysiologically. Both groups of neurons, Ad α3 and Ad α3/α1 infected, looked healthy when observed under phase contrast and I did not observe any differences in GFP expression.

Using electrophysiological techniques, we found that all 6 Ad α3 infected α3-null neurons were sensitive to 100 μM ACh, whereas no ACh response was detected in any of the 10 Ad α3/α1 infected neurons; this experiment has been performed three different times. Therefore, incubation at room temperature does not rescue the ACh sensitivity in Ad α3/α1 α3-null infected neurons.

To conclude, we were unable to get expression from the α3/α1 construct even though it only has 5 amino acids different from α3, and these 5 residues from α1 constitute a conservative substitution.

3.4 α3-null mice SCG neurons infected with an adenovirus α3/α7 are insensitive to ACh

Since *Torpedo* is markedly different from mice, we speculated that the five amino acids from the *Torpedo* α1 subunit into the rat neuronal α3 subunit prevented α3 for expression in a neuronal context. To test this hypothesis, we decided to use another

construct, the chimeric α3/α7 subunit generated by Levandoski and coworkers. In this chimeric α3/α7 subunit, the α3 residues 184-191 have been replaced by site-directed mutagenesis with the corresponding region of the rat α7 subunit. This amino acid substitution resulted in a change of 6 amino acids in the α3 sequence (Y184K, K185R, H186N, I188K, K189F and N191E). Since α7 is expressed by SCG neurons, we reasoned that α3/α7 chimeric construct should be capable of expressing in neurons. This α3/α7 construct also conferred α-BTX sensitivity to the containing nAChRs when expressed in *Xenopus* oocytes in combination with either β2 or β4 (Levandoski *et al.*, 1999). I built an α3/α7 adenovirus (Ad α3/α7) as described in the Methods.

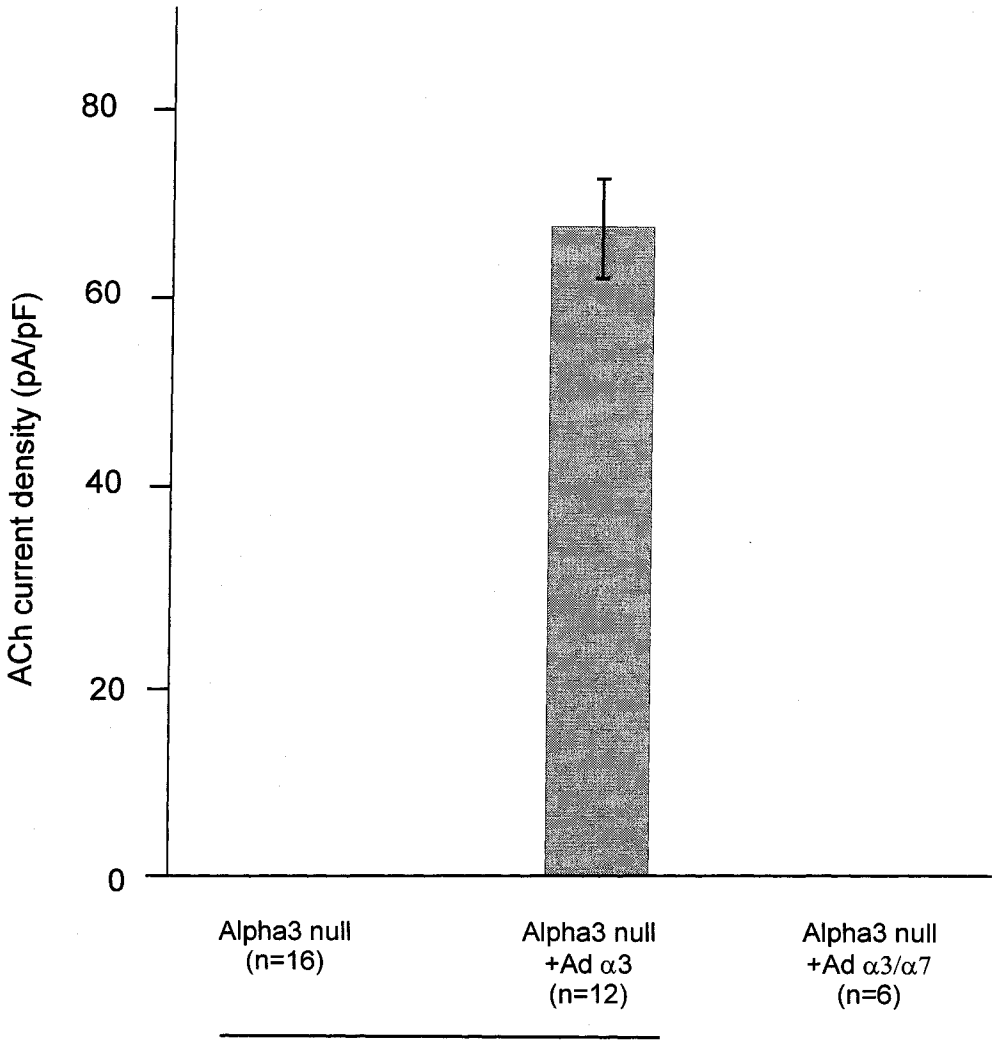
To express these adenovirus α3/α7-containing vectors, I cultured SCG neurons from P1 to P3 α3 -/- animals for two weeks, and then infected sister cultures with $23x10^8$ pfu/ml of Ad α3/α7, while others were infected with Ad α3 as a positive control for 24 hours. At this concentration, Ad α3/α7 infected 70 to 80% of the neurons. The neurons were examined electrophysiologically 24 to 48 hours after the high potassium stimulation.

Surprisingly, none of the 6/6 neurons from α3 -/- mice infected with Ad α3/α7 infected α3-null SCG neurons were sensitive to 100 μM ACh (**figure 3.6**). In contrast, 6/6 α3-null SCG infected with Ad α3 were sensitive to 100 μM ACh.

Taken together, my results on the expression of chimeric α-BTX sensitive α3 subunit show that neither α3/α1 nor α3/α7, lead to appearance of functional nAChRs in α3-null SCG neurons whereas their expression in *Xenopus* oocytes in combination with β2 or β4 subunits have been reported to form functional nAChRs that are blocked by nanomolar concentrations of α-BTX.

Figure 3.6 Sympathetic neurons from α3 -/- mouse infected with an adenovirus containing the chimeric α3/α7 gene construct are not sensitive to ACh.

Bar graph showing the absence of response to 100 μM ACh in neurons from α3 -/- mouse infected with Ad α3/α7 compared to previous data from fig 3.4. 6/6 GFP-positive Ad α3/α7 infected neurons were insensitive to ACh, whereas 6/6 positive controls α3-null SCG infected with Ad α3 were sensitive to 100 μM ACh. For all the recordings, the membrane potential was held at –60 mV.


ACh current density (pA/pF)
80
60
40
20
0
Alpha3 null
(n=16)
Alpha3 null
+Ad α3
(n=12)
Alpha3 null
+Ad α3/α7
(n=6)

From Fig 3.4

3.5 α3-null mice SCG neurons infected with an adenovirus $\alpha3_{E240A}$ are sensitive to ACh and do not inwardly rectify

To rule out the possibility that any mutation in the α3 subunit will give rise to a nonfunctional nAChR complex, I developed another α3 mutated adenovirus.

Previous studies from Haghighi and Cooper on the molecular bases of inward rectification of neuronal nAChRs have demonstrated that partial removal of negative charges of the intermediate ring in one of the subunit abolished inward rectification (Haghighi & Cooper, 2000). For example, the substitution of the glutamic acid at position 240 for an alanine in the α3 subunit ($\alpha3_{E240A}$) diminished the high-affinity, voltage-dependant interaction between intracellular polyamines and the $\alpha3_{E240A}\beta4$ receptor when expressed in *Xenopus* oocytes (Haghighi & Cooper, 2000). To test out if this mutant α3 would confer the same property to the $\alpha3_{E240A}$ nAChRs when expressed in neurons, I made an adenovirus with this construct.

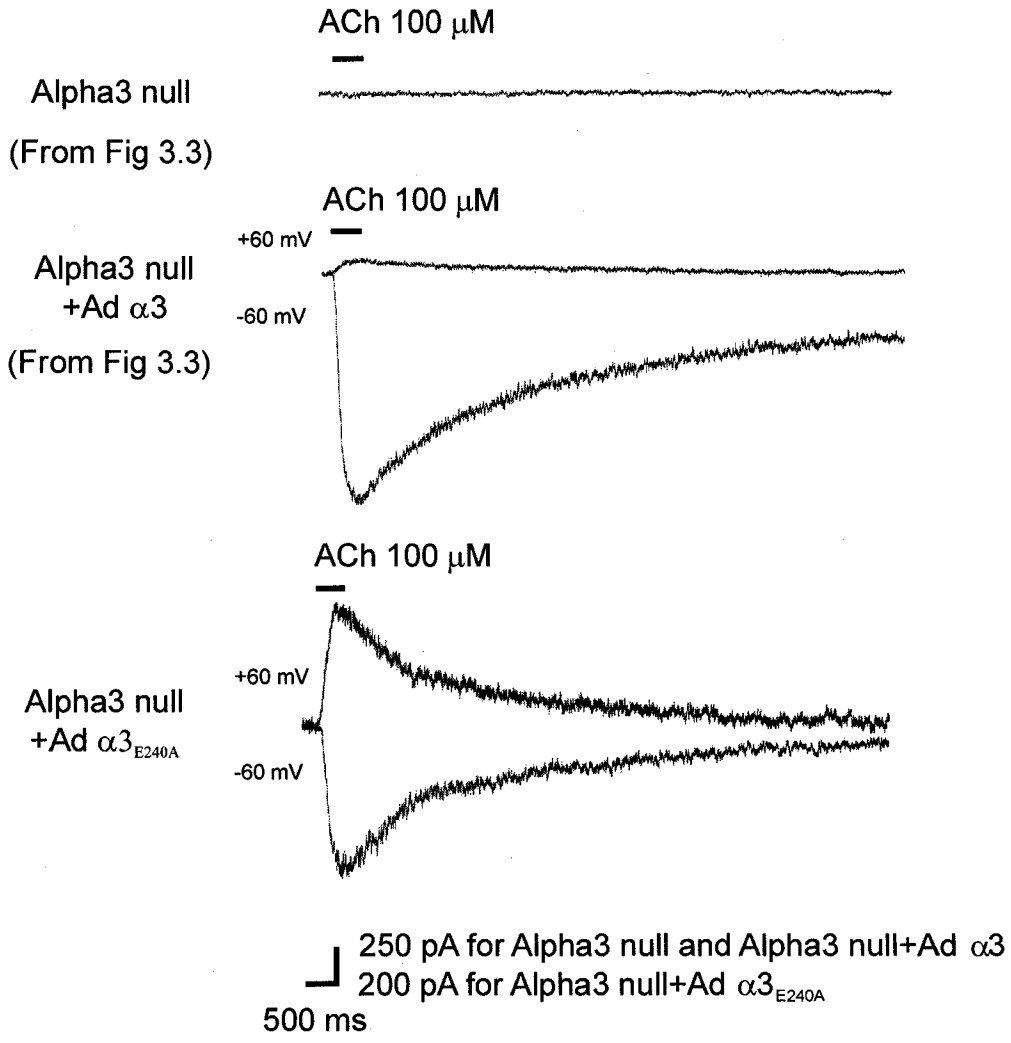
To express these adenovirus $\alpha3_{E240A}$-containing vectors, I infected the neurons with $3x10^8$ pfu/ml of Ad $\alpha3_{E240A}$ for 24 hours. At this concentration, Ad $\alpha3_{E240A}$ infected 70 to 80% of the neurons. The neurons were examined electrophysiologically 24 to 48 hours after the high potassium treatment. Neurons from α3 -/- mice infected with Ad $\alpha3_{E240A}$ were sensitive to ACh as the wild-type neurons; however unlike nAChRs in wild-type, the ACh-evoked current did not rectify (see **figure 3.7.A**). When the membrane potential hold at +60mV, a large outward ACh-evoked current was detected in the α3 -/- mice SCG infected with Ad $\alpha3_{E240E}$, whereas little or no response was recorded in wild-type SCG neurons. To give a better representation of the loss of inward rectification in

Figure 3.7 Sympathetic neurons from α3 -/- mouse infected with an adenovirus containing the chimeric $\alpha3_{E240A}$ gene construct are sensitive to ACh and do not rectify.

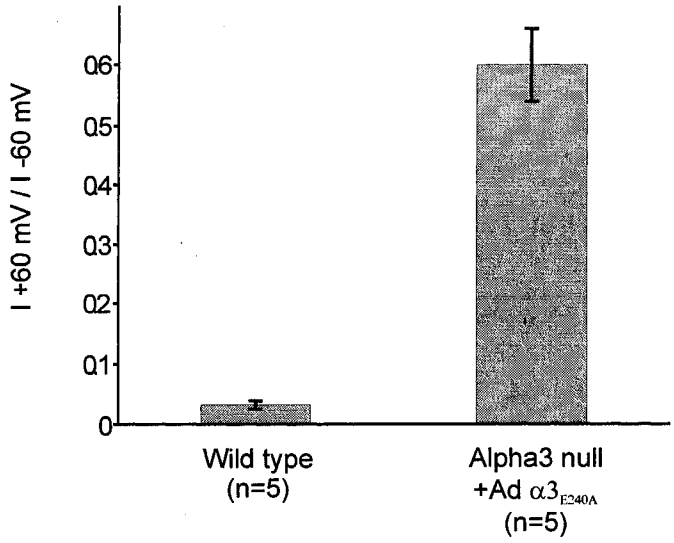
A. Representative traces of the ACh-evoked current recorded in an α3 -/- neuron infected with Ad $\alpha3_{E240A}$. The top two traces come from fig 3.3 as a reference. The third set of traces shows that the α3-null neuron infected with Ad $\alpha3_{E240A}$ produces a 800 pA current in response to 100 μM ACh when the membrane potential was held at –60 mM (bottom trace). When the membrane potential was switched to +60 mV, the same neuron gave rise to a 650 pA current in response to 100 μM ACh, and therefore do not rectify. The second set of traces from the top shows values obtained in an Ad α3 infected α3 -/- neuron. This neuron produces a 1,200 pA current in response to ACh when the membrane potential was held at –60 mV, but it produced a 100 pA current in response to ACh when the membrane potential was held at +60 mV.

B. Bar graph showing the ratio of the ACh-evoked current recorded at +60 mV versus the ACh-evoked current recorded at –60 mV. The 5/5 endogenous wild-type neurons let very little current pass through the channel at positive membrane potential, with an I +60/-60 mV of 0.03 ± 0.01 (mean ± S.E.M). In contrast, 5/5 Ad $\alpha3_{E240A}$ infected neurons from α3 -/- mice did not rectify at positive membrane potentials with an I +60/-60 mV of 0.6 ± 0.07 (mean ± S.E.M).

A


ACh 100 μM
Alpha3 null
(From Fig 3.3)
ACh 100 μM
+60 mV
Alpha3 null
+Ad α3
-60 mV
(From Fig 3.3)
ACh 100 μM
Alpha3 null
+Ad α3E240A
+60 mV
-60 mV
250 pA for Alpha3 null and Alpha3 null+Ad α3
200 pA for Alpha3 null+Ad α3E240A
500 ms

B


I +60 mV / I -60 mV
0
0.1
0.2
0.3
0.4
0.5
0.6
Wild type
(n=5)
Alpha3 null
+Ad α3E240A
(n=5)

$\alpha3_{E240A}$ nAChRs, I calculated the ratio of ACh-evoked current recorded at +60 mV versus the ACh-evoked current recorded at –60 mV (I +60 mV/I –60 mV). As shown in **figure 3.7.B**, the endogenous wild-type neurons let very little current pass through the channel at positive membrane potential, with a mean I +60 mV/I -60 mV of 0.03 ± 0.01 (mean ± S.E.M), whereas, the 5 Ad $\alpha3_{E240A}$ infected α3 -/- mice SCG neurons did not rectify at positive membrane potentials with an a mean I +60 mV/I –60 mV of 0.6 ± 0.07 (mean ± S.E.M).

This last result is important for two reasons. First, it confirmed the results from the study in *Xenopus* oocytes of Haghighi and Cooper on the molecular determinants responsible for inward rectification in neuronal nAChRs. It shows that one specific amino acid substitution in the M2 domain of the α3 subunit is enough to change completely the physiological properties of the nAChR channel. Second, the functional expression of $\alpha3_{E240A}$ in α3 -/- mice SCG neurons also rules out the possibility that any mutation is the α3 subunit modifying the properties of the nAChRs when expressed in *Xenopus* oocytes is not giving rise to functional nAChRs when expressed in a neuronal context.

3.6 α3-null mice SCG neurons infected with an adenovirus α3-myc are sensitive to ACh

The strategy to use α-BTX as a marker for nAChRs using α3/α1 or α3/α7 did not work. Therefore, we needed a different approach. We included an epitope tag on the extracellular portion of the α3 subunit to locate the receptor. I started this work by making an α3-myc adenovirus (Ad α3-myc) in which the full length α3 subunit gene has been modified with the insertion in its C-terminus of the coding sequence for the 10

amino acids of the myc proto-oncogene tag. We tested its expression in neurons and whole-cell patch-clamp recording showed that this construct gives rise to functional channels. Work is in progress to use this Ad α3-myc in immunocytochemical experiments.

Chapter 4: General discussion and conclusion

4.1 The appearance of functional nAChRs in mouse SCG neurons depends on the expression of the α3 nAChR subunit

My first objective was to test whether the α3 subunit was crucial for the appearance of functional nAChRs in sympathetic neurons. From the whole-cell patch-clamp analysis data, I found that α3 -/- SCG neurons in culture do not show ACh-sensitivity. This indicates that the appearance of functional nAChRs on SCG neurons depends on the expression of the α3 gene. Our results from the whole-cell patch clamp differ from the single-channel currents recorded by Xu and colleagues on α3 -/- neurons. It is unlikely that currents detected at the single-channel level would not be detected at the whole-cell level. Therefore, it is conceivable that the single-channels reported in the study of Xu *et al.* are not ACh-elicited currents but have another origin.

Several lines of evidence suggest that α3 subunits are important for nAChRs sympathetic neurons. However, studies in heterologous expression systems have shown that the α5 subunit can form triplex combination with α3 and β2 or β4 (Fucile *et al.*, 1997; Nelson & Lindstrom, 1999). Moreover, antisense oligonucleotides experiments have shown that depletion of the α3 gene product causes the appearance of a new nicotinic-induced current that can be blocked by α-BTX suggesting the presence of α7 nAChRs, in chick sympathetic neurons (Listerud *et al.*, 1991). Recently, Cuevas and colleagues have reported functional α7-containing nAChRs on rat SCG neurons (Cuevas *et al.*, 2000). The results of this last study are matter of debate because they are in

contradiction with previous work done in our laboratory where no α7- or α-BTX sensitive-currents have been detected (Mandelzys *et al.*, 1995; De Koninck & Cooper 1995).

Since SCG neurons from α3 -/- mice should still be expressing the α5 and α7 subunits, do they make functional receptors? Did the deletion of the α3 subunit up regulate the α4 or another α nAChR subunit that would give functional nAChRs?

The results from our whole-cell patch-clamp analysis showed that neurons from α3 -/- mice do not express functional nAChRs. This important results show that no other α nAChR subunit compensate the absence of the deleted α3 subunit.

Moreover, functional nAChRs expression was rescued in neurons from α3 -/- mice infected with an adenovirus containing the full length α3 subunit gene, and the receptors function properly.

Taken together our results demonstrate the crucial role played by the α3 subunit for the appearance of functional nAChRs in mouse SCG neurons.

4.2 SCG neurons from α3 -/- mouse combined with use of adenoviruses containing α3 subunit constructs as a model system to study domains of α3 required for nAChRs functionality

4.2.1 Neurons from α3 -/- mouse

SCG neurons from α3 -/- mouse should only be lacking the α3 gene since the mutation has been specifically targeted to the α3 gene. Therefore, the α5, α7, β2 and β4 subunits, normally expressed in wild-type SCG, should also be present in the neurons

from α3 -/- mice. Our results show that neurons from α3 -/- mice grow well in culture for at least one month and that we are able to get functional nAChRs upon adenoviral expression of the α3 subunit. This latter result suggest that the other nAChRs subunits required for the nAChR complex formation are present in the α3 -/- neurons. However, Xu and coworkers did not report the precise subunits nAChR expression pattern in the α3-null neurons (Xu *et al.*, 1999).

To compensate this lack of important information, I collected mRNA from P1 to P7 wild-type and α3 -/- animals. I isolated 100 μg of total mRNA from SCG neurons of each category of mice, which required more than 180 α3 -/- and 90 wild-type animals.

This samples will allow further comparison of the transcripts levels for the different nAChRs subunits expressed in the α3-null, and wild-type SCG. Precise mRNA measurements will be obtained by Ribonuclease Protection Assay.

Part of this precious material (10 μg) has been recently sent to Incyte Genomics for DNA micro array study. We do not know yet the results of this study, but they should give us more insights concerning the effect of the deletion of the α3 subunit on the genes expression pattern in the mouse α3 -/- SCG.

4.2.2 Choice of adenovirus as a gene transfer technique

To determine whether I could rescue functional currents in these neurons by overexpressing the α3 gene in neurons from α3 -/- mice, I infected them with an adenovirus containing the full length α3 coding sequence.

Several methods exist for gene transfer but only a few are efficient in post-mitotic cells such as primary neurons, glia, cardiac and liver cells, which are very resistant to

gene transfer (Castro *et al.*, 1996). To transfer gene into neurons, three techniques are mostly used: generation of transgenic animals, use of viral vectors, biolistic transfection (also called "Gene gun"). The Gene gun consists in bombarding the neurons with microprojectiles coated with the foreign DNA (Wellmann *et al.*, 1999). The generation of transgenic animals allows the stable integration of the mutated form of the gene of interest in the genome of the animals. However, I did not consider using them because this approach is long, expensive and only a small number of genes can be manipulated. To the contrary of transgenic animals, the Gene gun and adenovirus vectors only allow the transient expression of the gene of interest. I decided to use the adenoviral vectors rather than the Gene gun because even though both methods are better than classical gene transfer techniques (reviewed by Rosenberg, 1998), the Gene gun has a low transfection efficiency of about 10% of the cultured neurons, whereas adenoviral vectors infect over 80% of them (Slack *et al.*, 1996). Moreover, the use of adenoviruses as a gene transfer technique has been successfully employed in our laboratory to transfer the α3 nAChR subunit in cultured rat SCG neurons (Rosenberg, 1998). In addition work done by Damian Wheeler in our laboratory on the regulation of the CMV promoter which is present in the adenovirus system we use, allowed us to optimize the expression of their proteins (Wheeler & Cooper, 2001).

4.2.3 Use of our model system

The results from the electrophysiology analysis showed that all the Ad α3 infected neurons were sensitive to ACh. This important results show that expression of the α3 gene can confer ACh sensitivity to the originally ACh insensitive α3-null SCG neurons.

a) Expression of chimeric α-BTX-sensitive α3 subunits

My goal was then use this powerful system to learn more about nAChRs targeting and domains important for receptor function. For this purpose, I infected α3-null neurons with an adenovirus containing the chimeric α3/α1 construct that would allow us to mark the α3/α1 nAChRs with labelled α-BTX. Unexpectedly, none of the neurons from α3 -/- mice infected with Ad α3/α1 were sensitive to 100 μM ACh, whereas sister cultures were capable of expressing functional nAChRs when infected with Ad α3. Despite a rigorous troubleshooting strategy and the construction of four different Ad α3/α1, no functional receptors were obtained when α3-null neurons were infected with this viruses. I concluded that Ad α3/α1 does not give rise to functional nAChRs in mammalian neurons, whereas this construct produces functional nAChRs when expressed together with β4 in *Xenopus* oocytes. Incubation of the Ad α3/α1 infected neurons at room temperature for 24 to 48 hours did not rescue ACh sensitivity either.

I then infected α3-null neurons with another adenovirus that contain the chimeric α3/α7 construct. Since α7 is expressed in wild-type neurons, the chimeric α3/α7 subunit should be expressed correctly in a neuronal context. However, none of the neurons from α3-/- mice infected with Ad α3/α7 were sensitive to 100 μM ACh, whereas sister cultures infected with Ad α3 expressed functional nAChRs.

b) Expression of the $\alpha 3_{E240A}$ subunit

To rule out the possibility that any mutation in the α3 subunit is giving rise to a non-functional nAChR complex, I infected neurons from α3 -/- mice with an adenovirus

that contains the single amino acid mutated α3 subunit $\alpha3_{E240A}$. This mutation has been shown to give rise to nAChRs that do not inwardly rectify when expressed together with β4 in *Xenopus* oocytes. The data from the electrophysiological study show that α3 -/- neurons infected with Ad $\alpha3_{E240A}$ are expressing functional nAChRs sensitive to 100 μM ACh that do not inwardly rectify when the membrane potential was held at positive potentials. This last results confirmed the results from the study on the molecular determinant responsible for inward rectification performed in *Xenopus* oocytes. This last results also validate our model of expressing with adenoviruses mutated forms of the α3 subunit in α3 -/- neurons as an appropriate model to learn more on domains in the α3 subunit important for nAChRs function.

4.2.4 Future experiments on nAChRs targeting

The successful rescue of the α3 subunit allows the use the α3 -/- neurons in culture in combination with adenovirus vectors as a model to study domains in the α3 subunit that are involved in nAChRs functionality. The validation of this model has been obtained by the successful expression of non rectifying nAChRs in neurons from α3 -/- mice infected with the $\alpha3_{E240A}$ mutated form of the α3 subunit.

However, we do not know if the receptor is targeted to the appropriate place, but our model system could allow us to study this point. To do so, we simply need a way to mark the α3 subunit construct at its extracellular C-terminus with an epitope tag that we can detect by immunocytochemistry. Therefore, we would be able to have a picture of the pattern of receptor targeting on the neuronal membrane, but we could also study the effect of physiological changes on the receptor targeting.

For instance, a very interesting point to address is the effects of synaptic activity on the receptor targeting at the active synapse. The data from the whole-cell patch-clamp study give us an estimate of the number of functional receptors present at the cell surface, but it does not tell us if the receptor has been targeted to its appropriate location: under a presynaptic nerve terminal. SCG neurons in culture form a neuronal network that is not synaptically active. This is due to the mismatch between the receptors expressed on their cell body which are cholinergic (nAChRs) and the neurotransmitter released by their nerve endings which are adrenergic (Norepinephrine). However, one can make this SCG neuronal culture synaptically active by treating them with the Ciliary Neurotrophic Factor (CNTF) which has been shown to induce changes in the neurotransmitter phenotype converting it from adrenergic to cholinergic (Saadat *et al.*, 1989). By infecting CNTF-treated neurons from α3 -/- mice with an adenovirus containing an epitope-tagged α3 subunit, we could evaluate the effect of synaptic activity on the targeting of the receptor to the synapse. If there is an effect, we could then study the possible involvement of the M3-M4 domain of the α3 subunit in this targeting, as suggested by the study of the group of Jacob (Williams *et al.*, 1998), by expressing different adenoviruses containing various α3 subunit mutated in the M3-M4 loop.

4.3 The substitution of the amino acids 184-191 of the α3 subunit is critical for the expression of functional nAChRs in mice SCG neurons

Our strategy to express α-BTX-sensitive chimeric forms of α3 in SCG neurons from α3 -/- did not give rise to ACh sensitive nAChRs. This very intriguing result raises many questions concerning the role played by this amino acid sequence.

4.3.1 Did the mutation in the α3 subunit modify the ACh binding site?

Since our assay for the functionality of the α3/α1 and α3/α7 nAChRs is based on the electrophysiological measurements of the response to ACh, one could argue that the mutation introduced in the α3 subunit could have modified the conformation of the binding site of ACh. Although the sequence substituted contained 7 amino acids from 184-191, this substitution led to a change of 5 residues in the α3/α1 construct and 6 residues in the α3/α7 construct. The only amino acid in this sequence that has been shown to be part of the ACh binding site, the tyrosine at position 190 (in loop C, see introduction) is highly conserved among the α subunits and has not been mutated. The other residues involved in this substitution have not been reported to be important for the binding of the ligand (see introduction). Therefore, the substitution of this 7 amino acids sequence should not affect the binding of ACh. Moreover, the results from the study of Levandoski and colleagues where α3/α1 or α3/α7 were coexpressed with β2 or β4 subunit show that these chimeric α3 subunit give rise to functional nAChRs in *Xenopus* oocytes and prove that the binding pocket for ACh is intact (Levandoski *et al.*, 1999).

4.3.2 Is it a problem of subunit assembly?

One could argue that the substituted sequences contain amino acids involved in the subunits assembly, and therefore, mutating them will inhibit the proper formation of the nAChR pentamer. Studies performed in the muscle nAChRs have shown that the extracellular domains of the subunits controls the specificity of subunit oligomerization, and that the first transmembrane domain is required to orient the subunits to assemble (Keller & Taylor, 1999). The amino acids shown to important for the subunit assembly do

not involve the residues in position 184 to 191. Moreover, the fact that the α-BTX sensitive chimeric α3 subunit constructs give rise to functional nAChRs when coexpressed with β2 or β4 in *Xenopus* oocytes proves α3/α1- and α3/α7-containing nAChRs assemble properly (Levandoski *et al.*, 1999).

4.3.3 Do the neurons from α3 -/- mice lack a protein essential for the formation of functional nAChRs?

We already mentioned that Xu and colleagues did not report the nAChR subunit expression pattern in the α3 -/- mouse SCG neurons.

Another unlikely explanation would be that the neurons from α3 -/- mice do not express a protein, other than the nAChRs subunits, also required for functional nAChRs expression.

A number of proteins have now been identified that interact with various receptors and appear to play roles in the membrane targeting process. These include gephryn for glycinergic receptors, the Glutamate Receptor Interacting Protein (GRIP) for AMPA-type glutamate receptors, Homer for metabotropic glutamate receptors, and rapsyn for muscle nAChRs (Feng *et al.*, 1998). Although, such protein has not been identified yet for the targeting of neuronal nAChRs, it is possible that its expression is disrupted in α3 -/- mouse neurons.

Our results suggest that it is unlikely since the infection of α3 -/- neurons with Ad α3 gives rise to functional nAChRs that have similar ACh-evoked current than wild-type.

4.3.4 Is it a problem with the folding of the α3 protein?

The difference in the expression of the α-BTX sensitive α3/α1 subunits between expression in *Xenopus* oocytes and expression in neurons suggests that it might be a protein folding problem.

a) Effect of the temperature

A number of studies have showed that expression of some mutated proteins was temperature sensitive. Such cases have been reported for the mutation ΔF508 in the CFTR, causing cystic fibrosis, and also for the mutant N470D of HERG, responsible for the congenital long QT syndrome

For example, Denning and coworkers tested the effect of temperature on the processing of CFTRΔF508 (Denning *et al.*, 1992). They showed that the processing of CFTRΔF508 reverts toward that of wild-type as the incubation temperature is reduced in transfected 3T3 fibroblasts, and that this process was time-dependant and reversible. Applied cAMP agonists stimulated large chloride-selective currents, when the whole-cell patch-clamp measurements were performed on CFTRΔF508 transfected fibroblasts incubated at 30°C for two days (Denning *et al.*, 1992). Similar results were obtained with expression of the HERG protein in which an asparagine is replaced with an aspartic acid in position 470 (N470D). Zhou and colleagues demonstrated that the N470D LQT2 is trafficking-deficient when expressed at 37°C in HEK 293 cells, whereas at 27°C its trafficking to the plasma membrane and channel function are markedly improved (Zhou *et al.*, 1999).

We tested the possibility that temperature was inhibiting the expression of the α-BTX sensitive chimeric α3/α1 subunit constructs by incubating the infected neurons at room temperature for 24 to 48 hours before electrophysiological analysis. Our results show that lowering the temperature does not allow functional nAChRs to be expressed at the neuronal membrane. Cotransfecting α3/α1 or α3/α7 together with β4 in HEK 293 cells would be a good test to confirm the data we obtained in neurons. Electrophysiological recording on the transfected cells should not detect ACh sensitivity when the cells have been incubated at 37°C. It would be interesting to see if ACh-evoked currents are present on transfected HEK cells incubated at room temperature.

The folding of the proteins targeted to the cell surface requires proper glycosylation which takes place in the endoplasmic reticulum (ER) and continues in the Golgi apparatus. It is well established that the most important glycosylation required for protein cell surface expression, the N-glycosylation, occurs at a consensus sites N-X-S/T in the newly synthesized protein (Wickner *et al.*, 1999). Careful examination of the sequence 184-191 in α1, α3 and α7 reveals that no N-glycosylation sites are present in any of these subunits amino acid sequences. The closest N-glycosylation site in the α3 subunit is located upstream the substituted region at the asparagine in position 141 (N141) which is conserved in α1 and α7 subunits. Therefore, the sequence substitution did not remove *per se* an important N-glycosylation site in the α3 184-191 amino acid sequence. However, it is conceivable that the sequence substitution modified the conformation of the chimeric subunit protein which hindered the glycosylation of the residue N141 and led to a misfolded protein that was retained and/or degraded in the ER.

b) Future experiments

A number of biochemical experiments could allow us to locate the chimeric subunit in the cell. We would first need a way to follow the chimeric α3 subunit. The simplest would be to test the binding of labeled α-BTX on neurons from α3 -/- mice infected with Ad α3/α1 or Ad α3/α7. This labeling experiment would determine if the absence of current in response to ACh is due to the expression of nonfunctional nAChRs on the membrane. The same labeling experiment performed on permeabelized neurons should show if the chimeric subunit protein is retained in the ER.

If the α-BTX does not bind to the chimeric α3 subunit, we could get some of the monoclonal antibody raised against the rat α3 nAChR subunit recently developed by Yeh and colleagues (Yeh *et al.*, 2001). Another alternative is to add an epitope tag to the extra cellular C-terminus of the α-BTX sensitive chimeric α3 subunits.

Western blot performed on proteins extracted from Ad α3/α1 infected neurons from α3 -/- mice would show us if the chimeric protein is expressed, and what its glycosylation state is, since the mature form of a protein migrates at a higher molecular weight than partially glycosylated forms.

Immunocytochemistry on permeabelized neurons from α3 -/- mice infected with Ad α3/α1 should show that the chimeric protein is retained in the ER. Then, we might be able to do mutational analysis on the 7 amino acids sequence to refine the lack of functionality to one or two critical amino acids.

Conclusion

My findings indicate that SCG sympathetic neurons from α3 -/- mice lack functional nAChRs. Furthermore, I demonstrate that the expression of the α3 subunit is crucial for the appearance of functional nAChRs in mouse sympathetic SCG neurons. I also show that SCG neurons from α3 -/- mice combined with the use of adenoviruses is an appropriate model to study the domains in the α3 subunit involved in nAChRs function in sympathetic neurons. Using this model system, I confirmed the loss of nAChRs rectification consecutive to the mutation a single amino acid in the α3 subunit M2 domain, previously demonstrated in *Xenopus* oocytes. To the contrary, expression of α-BTX sensitive chimeric α3 subunits in this system did not form functional nAChRs, although these chimeric subunits were giving rise to functional nAChRs in *Xenopus* oocytes.

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