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Trans-ganglionic neurotransmission in the autonomic nervous system: Lessons from knock-out mice

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“Non est ad astra mollis e terris via”

“A rough road leads from earth to the stars”

Lucius Annaeus Seneca (Hercules Furens, 437)

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChBP	Acetylcholine binding protein
ADP	After-depolarisation
AHP	After-hyperpolarisation
ANS	Autonomic nervous system
α -BgTx	α -Bungarotoxin
ChAT	Choline acetyltransferase
CHT	Choline transporter
CNS	Central nervous system
DNA	Deoxyribonucleic acid
d-TC	d-Tubocurarine
EPI	Epibatidine
ENS	Enteric nervous system
EPSP	Excitatory postsynaptic potential
GABA	γ -aminobutyric acid
Gly	Glycine
HMB	Hexamethonium bromide
IC ₅₀	Half maximal inhibitory concentration
IP	Immunoprecipitation
IPSP	Inhibitory postsynaptic potential
KO	Knockout

mAChR/s	Muscarinic acetylcholine receptor/s
nAChR/s	Nicotinic acetylcholine receptor/s
NA	Noradrenaline
NIC	Nicotine
NMJ	Neuromuscular junction
NS	Nervous system
PNS	Parasympathetic nervous system
PNU	1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl) urea
PPF	Paired-pulse facilitation
PSP	Postsynaptic potential
RNA	Ribonucleic acid
RT	Room temperature
SCG	Superior cervical ganglion
SEM	Standard error of the mean
SNS	Sympathetic nervous system
TM	Transmembrane domain
TTX	Tetrodotoxin
VACht	Vesicular acetylcholine transporter
WT	Wild type

ZUSAMMENFASSUNG

In meiner Dissertationsarbeit habe ich die synaptische Transmission im oberen Halsganglion (superior cervical ganglion, SCG) in Mäusen untersucht. Das SCG innerviert diverse Bereiche in Kopf und Hals und dient als Schaltstelle des sympathischen Nervensystems, wo nikotinische Acetylcholinrezeptoren (nAChRs) für eine rasche exzitatorische Neurotransmission verantwortlich sind. Untersucht wurden Wildtyp- (WT) Mäuse und Knockout- (KO) Mäuse, bei denen gezielt die Gene ausgeschaltet wurden, welche für die Untereinheiten $\alpha 5$, $\alpha 7$, $\beta 2$ und $\beta 4$ des nAChR kodieren. Konkret handelt es sich hier um $\alpha 5$ KO, $\alpha 5\beta 2$ KO, $\alpha 5\beta 4$ KO, $\alpha 5\alpha 7\beta 2$ KO und $\alpha 5\alpha 7\beta 4$ KO Tiere. Heteromere nAChRs im SCG von WT Mäusen bestehen aus den Untereinheiten $\alpha 3\beta 4$ (55%), $\alpha 3\beta 4\alpha 5$ (24%) und $\alpha 3\beta 4\beta 2$ (21%).

$\alpha 5\beta 4$ KO Mäuse wuchsen langsamer heran, und ihre SCGs weisen morphologische Unterschiede auf, die auf eine verzögerte Reifung deuten. Wie durch Immunpräzipitation (IP) bestimmt, findet man in diesem Genotyp nur 15% nAChRs, verglichen mit WT und $\alpha 5\beta 2$ KO Mäusen. $\alpha 5\beta 4$ KO Mäuse exprimieren außerdem nur $\alpha 3\beta 2$ heteromere Rezeptoren, die sich hinsichtlich ihrer biophysikalischen Eigenschaften deutlich von den Rezeptoren in WT und $\alpha 5\beta 2$ KO (die lediglich $\alpha 3\beta 4$ heteromere Rezeptoren enthalten) unterscheiden, wie frühere Zellkulturexperimente belegen. Dementsprechend sind die durch Acetylcholin (ACh) ausgelösten Ströme in $\alpha 5\beta 4$ KO Mäusen fünfmal kleiner, sie fallen während der Aktivierung durch ACh schneller ab, und sie desensitivieren bei chronischer Applikation von Nikotin bereits bei Konzentrationen, die zehnmal geringer als in WT oder $\alpha 5\beta 2$ KO Tieren sind.

Ziel meiner Arbeit war, mittels extrazellulärer Ableitungen vom efferenten Nerven (als compound action potential, CAP) sowie intrazellulärer EPSP-Aufzeichnungen, die transganglionäre Neurotransmission im intakten SCG der erwähnten Genotypen, insbesondere aber in $\alpha 5\beta 4$ KO Mäusen zu untersuchen. Aufgrund der sehr unterschiedlichen biochemischen und elektrophysiologischen Eigenschaften der nAChR in diesen Mäusen hatten wir erwartet, dass sich auch Unterschiede in der synaptischen Transmission zeigen würden. Die Versuche haben allerdings gezeigt, dass die Unterschiede vergleichsweise gering sind: supramaximale Reizungen erzeugten auch in $\alpha 5\beta 4$ KO Mäusen CAPs mit vergleichbarer Amplitude, auch wenn fast doppelt so hohe

Reizstärken zur Auslösung von Schwellenwert-CAPs im Vergleich zu WT oder $\alpha 5\beta 2$ KO notwendig waren.

Durch den Vergleich von $\alpha 5\beta 2$ Doppel-KO mit $\alpha 5$ Einzel-KO Tieren konnte ich die Auswirkung der $\alpha 5$ Untereinheit auf die Funktion von $\alpha 3\beta 4$ Rezeptoren abschätzen. So war der Ganglienblocker Hexamethonium in Rezeptoren von Genotypen ohne $\alpha 5$ signifikant potenter als in $\alpha 5$ -hältigen Rezeptoren. Modellrechnungen auf Basis dieser Beobachtungen zeigten, dass $\alpha 5$ -hältige Rezeptoren sowohl in der Synapse des SCG als auch auf der Zelloberfläche weit mehr verbreitet zu sein scheinen als unsere IP Experimente vermuten ließen. Demzufolge könnten $\alpha 3\beta 4\alpha 5$ Rezeptoren der Zelloberfläche 2-4 mal häufiger vorkommen als $\alpha 3\beta 4$ Rezeptoren, während unsere IPs auf ein umgekehrtes Verhältnis deuteten, bei dem die $\alpha 3\beta 4$ Rezeptoren die $\alpha 3\beta 4\alpha 5$ Rezeptoren im Verhältnis 3:1 übertrafen.

Im Gegensatz zu früheren Beobachtungen im SCG von Ratten und Meerschweinchen konnte ich in keinem der getesteten Genotypen eine Langzeitpotenzierung nachweisen. Interessanterweise trat im WT eine paired-pulse facilitation auf (verstärkte postsynaptische Antwort nach zweimaliger Reizung des präsynaptischen Eingangs), welche bei Fehlen der Untereinheiten $\alpha 5$ oder $\beta 4$ nicht erfolgte.

Intrazelluläre EPSP Ableitungen lassen vermuten, dass das SCG in der Maus keine „strong inputs“ enthält: Die synaptische Antwort war bei Neuronen, deren Membranpotential nicht durch Strominjektion manipuliert wurde, stets mit einem Aktionspotential kombiniert. Um somit EPSPs zu messen musste das Membranpotential durch Strominjektion auf -100 mV verringert werden. Diese Versuche zeigten keine signifikanten Unterschiede in den EPSP Amplituden der verschiedenen Genotypen. Allerdings fielen die EPSPs in WT oder $\alpha 5\beta 2$ KO Mäusen im Vergleich zu $\alpha 5\beta 4$ KO Mäusen mehr als doppelt so schnell ab.

Zusammengefasst weisen meine Daten darauf hin, dass die synaptische Transmission im SCG mit einer großen Zuverlässigkeit (high safety factor) arbeitet, und dass $\beta 2$ (die klassische β -Untereinheit des zentralen Nervensystems) $\beta 4$ (die klassische β -Untereinheit des autonomen Nervensystems) effizient ersetzen kann. Außerdem sprechen wir der akzessorischen Untereinheit $\alpha 5$ größere modulatorische Fähigkeiten zu als bisher beschrieben, da sie nicht nur die Pharmakologie und Affinität des nikotinischen Acetylcholinrezeptors beeinflusst, sondern auch für die Kurzzeitplastizität des SCG eine Rolle zu spielen scheint.

ABSTRACT

I studied transganglionic neurotransmission in the superior cervical ganglion (SCG) of wild type (WT) mice and of mice with targeted deletions of the nicotinic acetylcholine receptor (nAChR) subunit genes $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$. Nicotinic AChRs mediate fast excitatory transmission in the SCG, which serves as a relay station of the sympathetic nervous system and innervates parts of the head and neck. Heteromeric receptors that occur in the wild type mouse SCG consist of $\alpha 3\beta 4$ (55%), $\alpha 3\beta 4\alpha 5$ (24%), and $\alpha 3\beta 4\beta 2$ (21%), as previously resolved by our lab. The knockout (KO) mice I used were $\alpha 5$ KO, $\alpha 5\beta 2$ KO, $\alpha 5\beta 4$ KO, $\alpha 5\alpha 7\beta 2$ KO and $\alpha 5\alpha 7\beta 4$ KO.

$\alpha 5\beta 4$ KO mice showed a reduced body growth and a different SCG morphology, possibly indicating a slower maturation. The overall number of nAChRs in the SCG is dramatically reduced (to 15% of nAChRs found in WT mice) in these mice. $\alpha 5\beta 4$ KO mice furthermore express just $\alpha 3\beta 2$ nAChRs, which greatly differ in their biophysical properties from receptors found in WT and $\alpha 5\beta 2$ KO animals. Previous cell culture experiments have shown that in $\alpha 5\beta 4$ KO mice, peak currents in response to ACh were five times smaller and declined much more rapidly than in WT or $\alpha 5\beta 2$ KO mice. Additionally, $\alpha 3\beta 2$ receptors were about ten times more sensitive to desensitisation by chronically applied nicotine. Having assessed the biochemical and electrophysiological properties of these receptors we now wanted to learn whether transganglionic transmission - measured with extracellular CAP as well as with intracellular EPSP recordings - in the $\alpha 5\beta 4$ KO might also be impaired.

Despite all the alterations mentioned above, neurotransmission in $\alpha 5\beta 4$ KO mice was nearly normal: upon supramaximal stimulation, the amplitude of CAPs was not reduced in $\alpha 5\beta 4$ KO mice, although threshold CAPs required nearly two times the stimulation strength compared to WT or $\alpha 5\beta 2$ KO mice.

By comparing $\alpha 5\beta 2$ double-KO with $\alpha 5$ single-KO, I was able to assess the impact of the $\alpha 5$ subunit on the function of $\alpha 3\beta 4$ receptors. I thus found that the potency of the ganglionic blocker hexamethonium was significantly increased in mice lacking the $\alpha 5$ subunit. Additionally, the modelling of my data suggests that $\alpha 5$ -containing receptors are present on the cell surface and in synapses of the SCG to a greater extent than predicted from our previous immunoprecipitation (IP) experiments. Hence, when looking at cell surface receptors with electrophysiological and pharmacological tools, $\alpha 3\beta 4\alpha 5$ appeared

to exceed $\alpha 3\beta 4$ receptors about 2-4 times, while previous IP experiments had shown that $\alpha 3\beta 4$ outnumber $\alpha 3\beta 4\alpha 5$ receptors with a 3:1 ratio.

Contrary to previous observations in the SCG of rats and guinea pigs, I was unable to induce long term potentiation in any of the mouse genotypes. Still, paired-pulse facilitation occurred in WT mice but not in mice lacking the $\alpha 5$ or the $\beta 4$ subunit.

Intracellular EPSP recordings implied that mouse SCGs only contain strong inputs, since all neurons produced an action potential as the first response when tested at resting membrane potential. The underlying EPSPs measured at -100mV showed similar amplitudes between all genotypes, but the EPSP decay in $\alpha 5\beta 4$ KO mice was more than two times less than in WT or $\alpha 5\beta 2$ KO mice.

Taken together, my data suggest that synaptic transmission in the SCG functions with a high safety factor, and that $\beta 2$ can efficiently replace $\beta 4$ as the classical β -subunit in the autonomic nervous system. Major modulatory effects than expected are assigned to the accessory subunit $\alpha 5$, which not only alters receptor pharmacology and antagonist affinity, but also plays a role in short time plasticity of the SCG.

1. INTRODUCTION

1.1 The autonomic nervous system and its function

The autonomic nervous system (ANS) regulates basic visceral processes that are usually independent of voluntary control (Langley, 1903). It controls visceral organs as heart, lungs, kidneys, liver, spleen, digestive organs, urinary bladder and internal genital organs. The ANS additionally controls exocrine and endocrine glands, blood vessels throughout the body and the pupil size.

Depending on their output regions within the spinal cord, the executing ANS pathways were historically divided into two divisions. Thoracic and lumbar outflows of the spinal cord (making their first synaptic contacts within paravertebral ganglia of the sympathetic chain) were believed to control and maintain the “sympathies” between organs and were thus called sympathetic nervous system (SNS) (Langley, 1916) (Fig. 1.1). On the other side, cranial and sacral outflows of the spinal cord (making their first synaptic contacts within ganglia lying close to their target organs) were found to exert effects on the cardiovascular system opposite to the SNS and were thus named parasympathetic nervous system (PNS) (Langley, 1916). Later on, the enteric nervous system (ENS) was introduced as a third division of the ANS (Müller, 1920; Langley, 1921), as it became clear that this part not only contained histologically different neurons but was also capable to operate without any connection to the CNS (Langley, 1921; Furness, 2006a) (Fig. 1.1).

The SNS is activated during states of arousal by physical or psychological stress and causes complex neuro-hormonal adjustments as the augmentation of cardiac output, the promotion of blood supply to skeletal muscles or the inhibition of the digestive system. On the contrary, the PNS regulates activities that occur when the body is at rest, including digestion, salivation, lacrimation, sexual arousal, urination and defecation. For this reason, the SNS is simplistically said to be in charge of the “fight or flight” response, whereas the PNS controls the “rest and digest” reactions. The ENS is the intrinsic nervous system of the gastrointestinal tract. It contains reflex circuits that detect the physiological state of the gastrointestinal tract and provides outputs to control gut movement and local blood flow (Gershon, 2005; Furness, 2006a, b). In contrast to SNS and PNS, the ENS contains extensive neural circuits with local autonomous function, a circumstance that

conveyed the ENS the title “second brain”. Additionally, the ANS includes so called mixed ganglia that contain cell bodies of both sympathetic and parasympathetic postganglionic neurons. Mixed properties are prominent in ganglia that supply the urinary bladder, internal genital organs and blood vessels of the pelvic region (Dail *et al.*, 1975; Jänig & McLachlan, 1987).

The actual function of SNS and PNS depends on particular temporary requirements of organs and tissues and it is aimed at maintaining the homeostasis of the body interacting with the environment. Homeostasis is usually achieved through reflex mechanisms which are predominantly involuntary (Jänig, 2006). They include particularly viscerovisceral reflexes (in which the stimulation of visceral receptors results in activation of visceral motor phenomena), as well as somato-autonomic reflexes (where the stimulation of the musculoskeletal system or the dermis of the skin leads to an alteration in autonomic nervous system function) (Sato *et al.*, 1997).

Most target tissues are innervated by only one part of the autonomic system. Few target tissues are innervated by both the sympathetic and parasympathetic NS (e.g. urinary bladder, pacemaker cells and atria of the heart, iris, and some blood vessels). When the activation of SNS and PNS causes opposite actions of the innervated organs, most responses are excitatory rather than inhibitory (e.g. decreased secretion, relaxation of muscles) (Furness, 2006b). For example, the opposite actions of sympathetic and parasympathetic systems on the pupil size is a result of the distinct target muscles (dilator pupillae for sympathetic, sphincter pupillae for parasympathetic) provided by each system. The heart rate is an example that shows how the same effect may be due to the function of the SNS or the PNS. Actually, in larger mammals (e.g. dogs, goats, horses), fast changes of heart rate, e.g., during changes of body position and emotional stress, are generated via activity changes in the parasympathetic neurons to the pacemaker cells by means of parasympathetic modulation of sympathetic effects (Levy & Zieske, 1969a, b). Due to these sympatho-vagal interactions, the sympathetic enhancement of the heart rate is less effective the higher the background level of vagal activity is (Mace & Levy, 1983a, b), a phenomenon termed “accentuated antagonism” (Levy, 1971; Levy, 1977; Mizuno *et al.*, 2008), also reviewed by Coote (2013). On the other hand, the sustained increase of heart rate during exercise is mainly generated by activation of sympathetic neurons supplying the heart. It is probable that some organs which can be affected by both systems under experimental conditions are primarily under the control of only one system under physiological conditions *in vivo* (Jänig, 2006).

The ANS not only consists of outflows from the CNS that traverse ganglionic relays to finally innervate effector tissues, but it also contains organ-to-organ connections that bypass the CNS. One example is the intestinofugal neuron that projects from the intestine to prevertebral ganglia (Fig. 1.1) and thus is an afferent neuron serving reflexes between gut regions. Such entero-enteric reflexes act back on the gastrointestinal tract to finally inhibit its movements (Kuntz, 1938; Kuntz & Van Biskirk, 1941; Kuntz & Saccomanno, 1944). These intrinsic connections that provide an integration of sympathetic or parasympathetic signals have been also found in cardiac ganglia (Adams & Cuevas, 2004) as well as in bronchial and tracheal ganglia (Fisher, 1964).

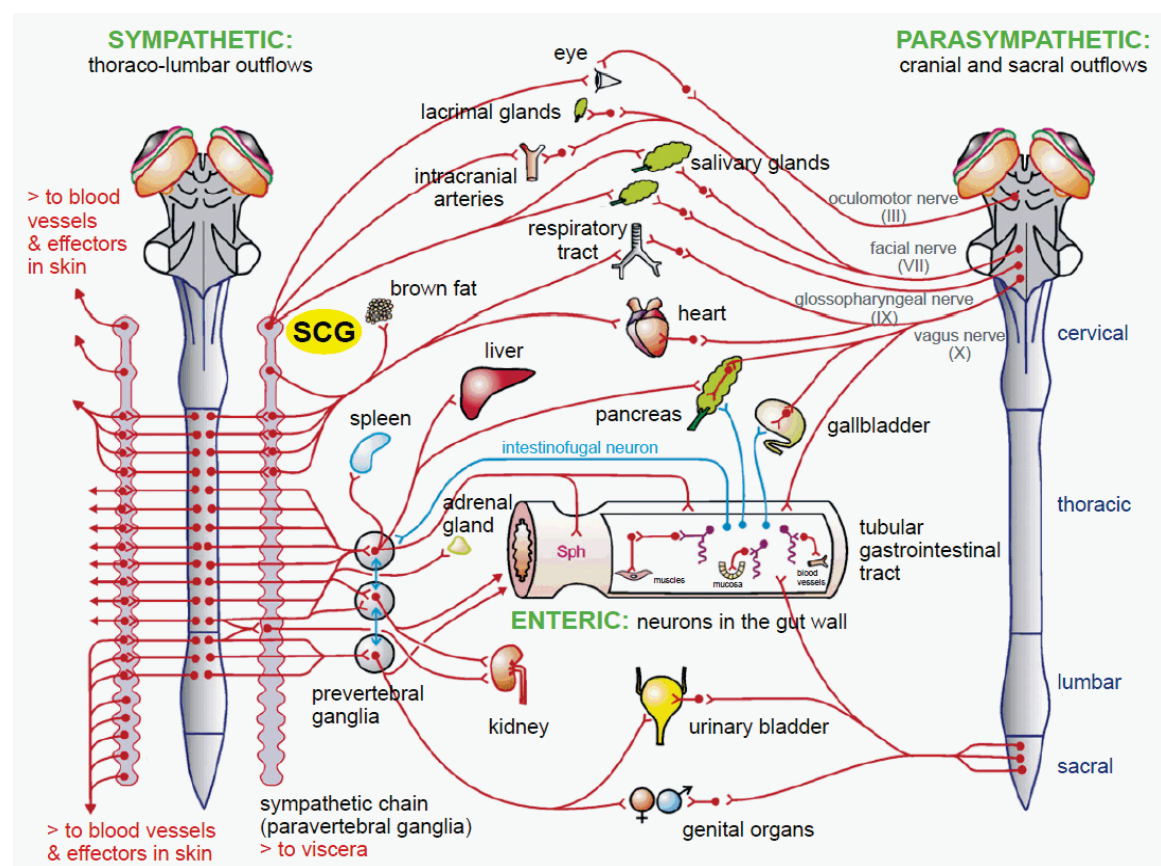


Figure 1.1: Scheme of the efferent (motor) pathways of the autonomic nervous system (ANS). The ANS is organised in three divisions: sympathetic (left), parasympathetic (right) and enteric (centre). Spinal cord and brain stem are depicted twice to show both the sympathetic and the parasympathetic outflows to the major organs. The enteric division is represented by neurons of the gut wall. The sympathetic chain is formed by paravertebral ganglia that supply blood vessels throughout the body and effectors in the skin (sweat glands, pilomotor muscles). Some sympathetic spinal cord efferent neurons pass first through the sympathetic chain and then through prevertebral ganglia and plexuses to supply visceral organs. The superior cervical ganglion (SCG) lies on top of the sympathetic chain. It supplies parts of the head and neck and intracranial arteries. Pathways that run rostrally and caudally within the sympathetic chain as well as visceral afferents that supply all organs are omitted to simplify the figure. Picture modified from Furness (2006b), with permission.

Much autonomic function, such as the blood pressure, is additionally dependent on circadian rhythms, showing daily rhythms that correspond to the sleep-wake cycle (Giles, 2006). In controlling internal as well as surface organs, the ANS acts in synergy with the endocrine system.

Although Langley defined the ANS as a purely efferent system (Langley, 1921), the discovery of reflex circles entirely contained within the wall of the intestine and the identification of afferent neurons of these reflex circuits (Kirchgessner *et al.*, 1992), has obliged the scientific community to reconsider the inclusion of autonomic afferents as being part of the ANS. Nevertheless, visceral afferent neurons are usually not included in the definition of the ANS.

1.2 Structural organisation of the sympathetic nervous system

1.2.1 The sympathetic system: thoraco-lumbar outflows

The cell bodies of the sympathetic preganglionic neurons lie in the spinal cord and extend from segments T1 to the rostral part of L3 in humans. They are usually clustered in clumps at the edge of the intermediate grey matter, where they form the intermediolateral columns. Neurons receive inputs from interneurons in the dorsal horn and intermediate zone, as well as direct projections from the medulla, pons, hypothalamus, and other spinal segments (propriospinal inputs) (Jänig & McLachlan, 2002).

Ganglia are arranged like a ladder throughout the thoracolumbar segments: preganglionic axons project ipsilaterally from the cord via the segmental ventral root and spinal nerve, and thence in the white ramus to the paravertebral chain, where they synapse with postganglionic neurons in the same and several adjacent segmental ganglia (Fig. 1.1). In rats, preganglionic SCG axons grow into the paravertebral chain between days 12 and 13 of gestation (E12-E13) (Rubin, 1985b). Most of the functional connections occur segmentally (Langley, 1921). Neurons in the upper thoracic segments project rostrally to innervate targets in the head, whereas many lumbar preganglionic neurons project caudally to reach targets in the lower trunk and hind limbs. Some preganglionic axons traverse the chain into the splanchnic nerves (major, minor, lumbar) and synapse in the prevertebral ganglia (coeliac, superior mesenteric, inferior mesenteric), which supply the abdominal and pelvic viscera (Jänig & McLachlan, 2002; Jänig, 2006).

The paravertebral ganglia are interconnected and form a chain on each side of the vertebral column, extending from the base of the skull to the sacrum. There is usually one pair of ganglia per spinal segment, except for the superior cervical ganglion (SCG) at the rostral end of the cervical sympathetic chain (Fig. 1.1) and the stellate ganglion at the rostral end of the thoracic sympathetic chain. These two ganglia represent several sympathetic ganglia fused together.

The prevertebral ganglia lie around the base of the large arterial branches from the abdominal aorta and contain vasoconstrictor neurons, as well as neurons that regulate motility and secretomotor. They receive preganglionic inputs from the mid to lower thoracic and upper lumbar segments and project to the abdominal and pelvic organs. Besides pre- and postganglionic neurons, prevertebral ganglia also contain the terminals of axons from enteric afferent neurons (Szurszewski *et al.*, 2002).

Paravertebral ganglia contain only preganglionic terminals, postganglionic neurons and associated supporting glia. They additionally may contain “paraganglion cells” which have no axon but release catecholamines (especially DA or NA and rarely adrenaline) and serotonin into the vasculature (Verhofstad *et al.*, 1981; Prud'homme *et al.*, 1999). These cells are called “small intensively fluorescent” (SIF) cells, corresponding to small granule-containing cells in the electron microscope. Due to a high variability in structure and transmitter content, their function is still not clear; they might act as chemoreceptors for substances in the blood, as interneurons between preganglionic innervation and primary postganglionic neurons, or have para-endocrine roles (Taxi *et al.*, 1983). SIF cells within the SCG are regarded as modulators of ganglionic transmission.

1.2.2 The superior cervical ganglion (SCG)

The SCG is the uppermost ganglion of the paravertebral sympathetic chain. Anatomically, it is believed to represent the fusion of the four upper ganglia corresponding to the upper four cervical nerves and is therefore the biggest ganglion of the chain. The superior cervical ganglion is situated opposite C2 and C3 vertebrae, behind the internal carotid artery and jugular vein and in front of longus capitis muscle. Its shape is often fusiform.

As all paravertebral ganglia, the SCG in humans receives preganglionic axons via a white ramus. Actually, most preganglionic axons are myelinated. All SCG inputs come ordinarily from the upper thoracic segments of the spinal cord. In general, in progressively larger animals (from mice to rabbits), the cells projecting to the SCG arise

from a larger number of segments, spreading principally in the caudal direction. Thus in the mouse, the cells innervating the SCG are distributed across the five segments T1-T5, in rats C8-T5, in guinea pigs C8-T6 and in rabbits C8-T7 (Nja & Purves, 1977; Purves & Hume, 1981; Purves *et al.*, 1986).

Most postganglionic neurons within the SCG send their axons via the internal carotid nerve (ICN) to target organs in the head; some postganglionic neurons in the SCG project to the upper two or three cervical segments. Contrary to preganglionic neurons, postganglionic axons are not myelinated. More than 50% of the SCG neurons are vasoconstrictor and innervate various vascular beds (skin, salivary and mucosal glands, skeletal muscle, intracranial). The rest is mainly pilomotor, sudomotor or secretomotor to salivary glands and mucosal glands. A subpopulation of SCG neurons supplies targets in and around the eye, and a few innervate the pineal gland (Jänig & McLachlan, 2002).

1.3 Synaptic transmission within the ANS

1.3.1 The cholinergic synapse

Neurotransmission describes the process by which electric signals (action potentials, APs) are passed from one neuron to another neuron or muscle cell. APs are generated at the axon hillock and conducted along the axon until they reach synapses, specialized contact sites between the neurons where the information is transferred to the next neuron or muscle cell. Synapses typically occur at swellings along the axon terminal termed varicosities. Once an AP reaches a varicosity, its signal can be passed on to the following cell by the release of neurotransmitters. This type of neurotransmission is called “chemical transmission” and it is generally opposed to “electrical transmission” mediated by Gap junctions, protein channels that tightly couple two adjacent cells and permit direct diffusion of ions between them. In the mouse SCG, Gap junctions are only relevant for transmission in satellite glia cells (Hanani *et al.*, 2010).

In the following I will focus on chemical transmission and as such on the structure and function of chemical synapses. Here, presynaptic and postsynaptic membranes are separated by a synaptic cleft that is about 20–50 nm wide. The membranes adhere to each other thanks to a matrix of fibrous extracellular protein that fills the synaptic cleft. The presynaptic side of the synapse contains several synaptic vesicles, small membrane-surrounded spheres, each about 50 nm in diameter. These vesicles store neurotransmitters. Some axon terminals also comprise secretory granules, which are larger vesicles (about

100 nm in diameter) that contain densely packed proteins, so-called neuropeptides, which in part function as co-transmitters. Both on the pre- and on the postsynaptic site, proteins are thickly packed in and around the membrane. The presynaptic membrane contains the active zone, where synaptic vesicles are clustered in the cytoplasm and where transmitter release actually takes place. At the postsynaptic site, proteins are also thickly accumulated in and just under the postsynaptic membrane to form postsynaptic densities. They contain the neurotransmitter receptors, which in case of nerve-nerve connections convert the chemical signal (neurotransmitter) into an electrical signal (membrane polarisation change).

Postsynaptic responses vary depending on the type of receptor activated by the neurotransmitter. Postsynaptic receptors can be divided into metabotropic and ionotropic receptors. Metabotropic receptors are proteins that activate G-proteins, themselves acting on effector proteins (G-protein gated ion channels, enzymes). Enzymes synthesise second messengers that cause signalling cascades aimed at regulating the function of the ion channel and altering the cellular metabolism.

The focus of my work is on ionotropic receptors, membrane spanning proteins usually composed of four to five pore forming subunits. This pore is closed in the absence of neurotransmitter and opened when transmitters bind to the receptor, causing a conformational change of the protein to allow the passage of ions. Depending on the distribution of ions between the extra- and intracellular space, ions will flow along the electrochemical gradient and cause the postsynaptic cell to depolarise from the resting membrane potential (RMP) or to hyperpolarise. Depolarisations may bring the membrane close to the threshold for the generation of an AP (excitatory effect), whereas hyperpolarisations exert inhibitory effects by reducing the probability of the generation of an AP. Transient depolarisations of the postsynaptic membrane are therefore called excitatory postsynaptic potentials (EPSPs), whereas transient hyperpolarisations are called inhibitory postsynaptic potentials (IPSPs). EPSPs are induced by receptors permeable to cations like Na^+ (e.g. ACh-receptors), and IPSPs are induced by receptors permeable to Cl^- (e.g. GABA, glycine receptors) (Fig. 1.2). In the prolonged presence of an agonist, receptors may furthermore enter a state of desensitisation where channels are closed.

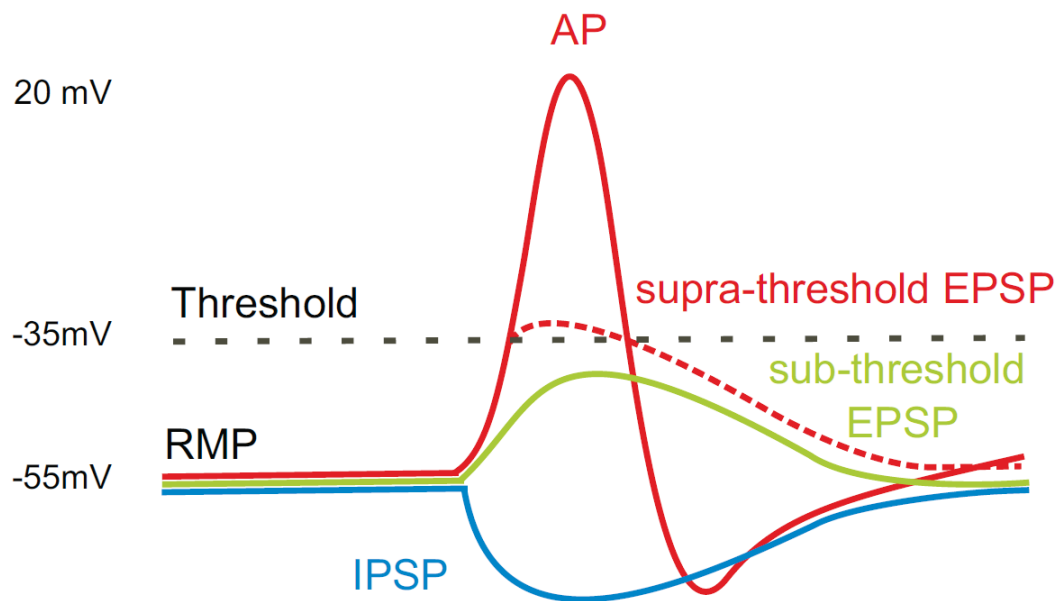


Figure 1.2: Schematic drawing of inhibitory (IPSP) and excitatory (EPSP) postsynaptic potentials. Amplitudes may vary. Once an EPSP reaches the threshold for activating voltage-gated sodium channels, an action potential (AP) is fired. RMP = resting membrane potential.

Neurotransmitter receptors not only exist on the postsynaptic side but also on the presynaptic axon terminal, where they function as auto- or hetero-receptors. Presynaptic metabotropic autoreceptors usually activate second messenger cascades that lastly inhibit neurotransmitter release or even its synthesis (Bitran & Bustos, 1982). For this reason, they play a key role in the regulation of the presynaptic terminal and are thought to be sort of a safety valve, curbing (over)activity of this particular type of neurotransmission (Bear *et al.*, 2007). However, (the ionotropic) nicotinic receptors at presynaptic sites play a key role in the central nervous system by facilitating the release of various neurotransmitters, including ACh itself (Role & Berg, 1996).

In the autonomic NS synaptic transmission is chemical (Fig. 1.3) and occurs between pre- and postganglionic neurons (ionotropic receptors), as well as between postganglionic neurons and their effector cells (metabotropic receptors). Preganglionic neurons within the sympathetic and parasympathetic NS are cholinergic. They use acetylcholine (ACh) as principal neurotransmitter that acts on nicotinic acetylcholine receptors (nAChRs) (Figs. 1.3 and 1.4) (Langley, 1903, 1921). The operating mode of the cholinergic synapse is cursory described in Figure 1.3.

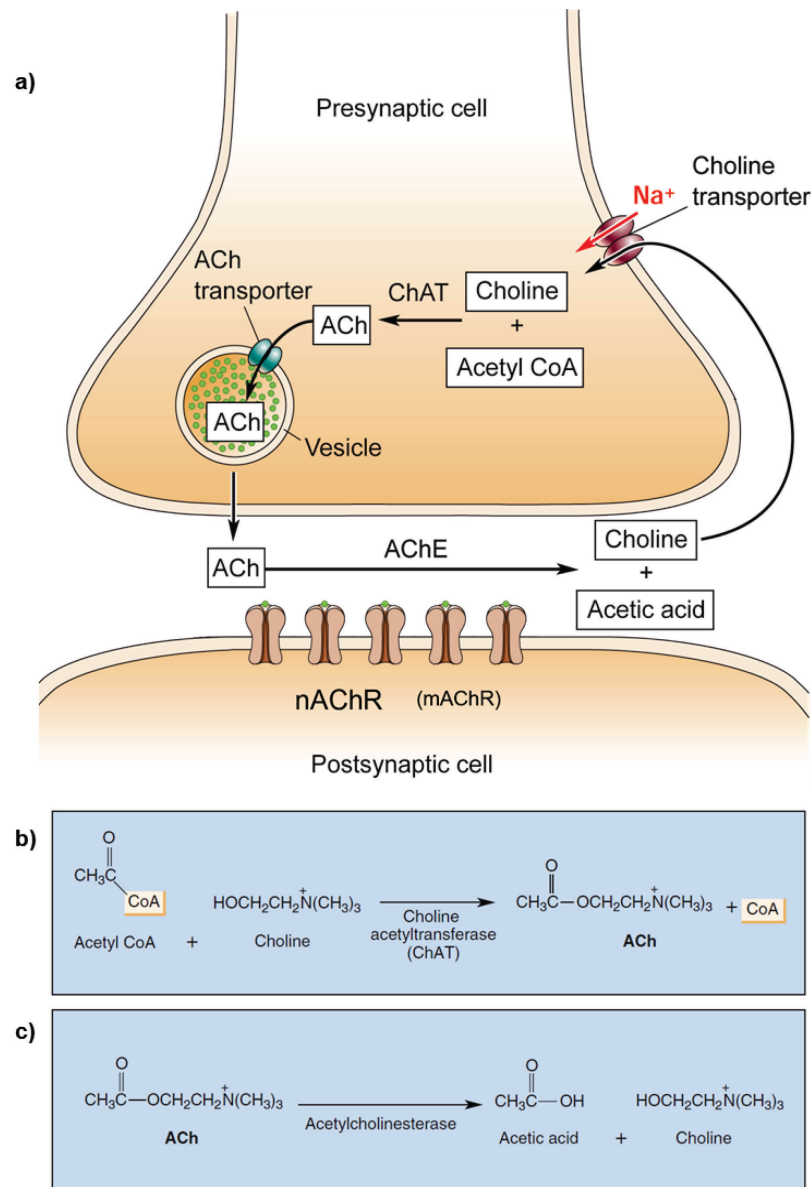


Figure 1.3: The life cycle of acetylcholine (ACh). **a)** Simplified model of the cholinergic synapse: ACh is synthesized from choline and acetyl coenzyme A (Acetyl CoA) by the enzyme choline acetyltransferase (ChAT) in the cytosol of the axon terminal. The neurotransmitter is then concentrated in synaptic vesicles by the vesicular ACh transporter (vAChT). Neurotransmitter release is triggered by the arrival of an action potential (AP) in the axon terminal. The following depolarisation of the terminal membrane causes voltage-gated calcium channels in the active zones to open. Due to its large inward driving force, Ca^{2+} will enter into the cytoplasm to trigger exocytosis and thus the release ACh into the synaptic cleft. In the giant axon of the squid, the process from Ca^{2+} influx to exocytosis lasts about 200 μs , in mammalian synapses that operate at higher temperatures it is even quicker. ACh released into the synaptic cleft binds to ionotropic nicotinic ACh receptors (nAChRs) (and to metabotropic muscarinic receptors, mAChRs). Binding to nicotinic receptors leads to a conformational change and the opening of the receptor pore to allow the passage of the cations Na^+ , K^+ , and Ca^{2+} . The influx of Na^+ is primarily responsible for the depolarisation of the postsynaptic cell (EPSP), which then may trigger an action potential (AP) once the threshold for activating voltage-gated Na^+ channels is reached. The ACh degrading enzyme acetylcholinesterase (AChE), which is also manufactured by cholinergic neurons and released into the synaptic cleft, very rapidly cleaves ACh into choline and acetic acid. Much of the resulting choline is taken up by cholinergic axon terminals via the specific choline transporter ChT1 and reused for ACh synthesis. Since the presence of choline limits how much ACh can be synthesized in the axon terminal, the transport of choline into the neuron is a crucial step in ACh synthesis. **b)** ACh synthesis from Acetyl-CoA and choline. **c)** ACh degradation to acetic acid and choline. Picture modified from the book “Neuroscience – Exploring the brain”, Bear *et al.* (2007), with permission.

1.3.2 Co-transmitters in the ANS

All preganglionic autonomic neurons (both sympathetic and parasympathetic) use ACh as their fast excitatory neurotransmitter (Langley, 1903, 1921). In addition, many preganglionic autonomic neurons also contain neuropeptides acting as co-transmitters. They mediate slow excitatory post-synaptic potentials and thus facilitate cholinergic transmission, as reviewed by Gibbins (1995). In sympathetic and parasympathetic preganglionic neurons these peptides comprise the vasoactive intestinal polypeptide (VIP) and VIP-related molecules substance P, the calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptides (PACAP) neurotensin – all of which can depolarise the postsynaptic sympathetic neurons – and enkephalin, which may mediate presynaptic inhibition of postganglionic transmission [see Gibbins (1992), Morris and Gibbins (1992), Forsgren *et al.* (1992), Goadsby and Edvinsson (1996), Gibbins (1995), Ip *et al.* (1982), Ip *et al.* (1984), Ip *et al.* (1985), Lai *et al.* (1997), Beaudet *et al.* (1998), Beaudet *et al.* (2000)].

All postganglionic parasympathetic neurons are potentially cholinergic, meaning that they contain the enzyme choline-acetyltransferase (ChAT) and that ACh acts on muscarinic acetylcholine receptors (mAChRs). Like for preganglionic transmission, postganglionic parasympathetic synapses also seem to use other substances as co-transmitter. These substances are nitric oxide (NO) and VIP, both synthesized in the same parasympathetic neurons [see Gibbins (1995); Jänig (2006)], and in the bladder also ATP (Fowler *et al.*, 2008).

Instead, most postganglionic sympathetic neurons are adrenergic and use either the catecholamine noradrenaline (NA) (in mammals) or adrenaline (ADR) (in reptiles and amphibians) as transmitter, acting on both alpha- and beta-adrenoceptors (α/β adrenoR). These synapses sometimes use the co-transmitters adenosine triphosphate (ATP) and neuropeptide Y (NPY) (Lundberg *et al.*, 1983; von Kugelgen & Starke, 1991; Stjarne, 1998; Burnstock, 1999; Stjarne, 2001). Only sympathetic postganglionic neurons innervating sweat glands are cholinergic and sometimes use NO and VIP as co-transmitters (Jänig, 2006; Burnstock, 2013) (Fig. 1.4).

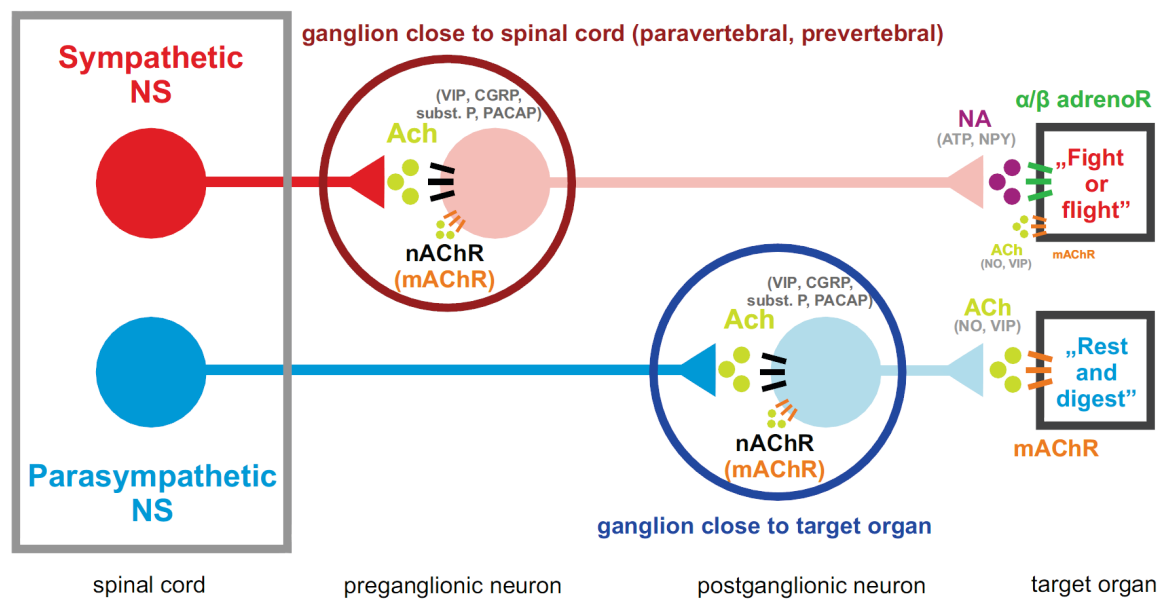


Figure 1.4: Comparative diagram of neurotransmitters and receptors in mammalian sympathetic and parasympathetic pathways. Ganglia of the sympathetic nervous system lie close to the spinal cord (paravertebral or prevertebral). Ganglia of the parasympathetic nervous system lie close to the effector organs. All preganglionic fibres are cholinergic and release acetylcholine (ACh) into the synaptic cleft of sympathetic and parasympathetic ganglia. There, ACh acts on nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). In both, the sympathetic and parasympathetic pathways co-transmitter like the vasoactive intestinal polypeptide (VIP), substance P, the calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating polypeptides (PACAP) may be involved in neurotransmission. ACh, together with the co-transmitters nitric oxide (NO) and VIP is the main neurotransmitter between postganglionic parasympathetic neurons and effector organs. There, ACh acts on mAChRs. Instead, postganglionic sympathetic synapses use mainly noradrenaline (NA) acting on adrenoceptors (type α and β) together with the co-transmitters adenosine triphosphate (ATP) and neuropeptide Y (NPY). Sympathetic postganglionic neurons that contact sweat gland are cholinergic and use ACh (with NO and VIP).

1.3.3 Quantal release of ACh

ACh is thought to be released in quanta into the synaptic cleft. The quantal theory of synaptic transmission was originally proposed for the neuromuscular junction (NMJ) (Del Castillo & Katz, 1954b, a; Katz, 1969), where small, spontaneously occurring post synaptic potentials (PSP), and evoked endplate potentials (ePSP) were observed. PSPs have peak amplitudes that follow a unimodal distribution, whereas nerve evoked ePSPs amplitudes follow a multimodal (Poisson) distribution in which each peak occurs in integral multiples of the mean amplitude of spontaneous miniature potentials (Martin, 1977; McLachlan, 1978; Walmsley, 1993). Thus, it was suggested that the spontaneous miniature potential represents the basic unit, or “quantum” (q), from which the evoked

response is composed. Furthermore, electron microscopic studies of membrane-docked vesicles led to the hypothesis that a spontaneous miniature endplate potential represents the postsynaptic response to the presynaptic release of the neurotransmitter contents of a single vesicle. Therefore, one quantum corresponds to the neurotransmitter content of one vesicle (“quantal size”).

When an AP reaches the presynaptic terminal, quanta of transmitters are secreted in a probabilistic, intermittent manner. The release occurs from a pool of available membrane bound vesicles that contain the neurotransmitter. Thus, the EPSP represents the sum of the probabilistic release at all release sites. The term “quantal content” (not to be confused with the quantal size q) was additionally introduced by Katz and colleagues to describe the “mean number of quanta simultaneously released”. The quantal content m can be calculated as the product of n and p ($m = np$), where p is the probability of a vesicle to be released and n is the total number of docked vesicles (or the number of release sites if one vesicle is pre-docked in one release site).

Quantal analysis of transmission at the neuromuscular junction revealed that one single AP in the presynaptic terminal triggers the exocytosis of about 200 vesicles and generated an EPSP of 40 mV or more. In contrast, at many synapses of the CNS, the content of only one single vesicle is released in response to a presynaptic AP and causes an EPSP which is hundred times smaller than at the NMJ (Bear *et al.*, 2007).

Therefore, the relationship between the amount of released transmitter and the quantal postsynaptic potential (or current) is still unclear. One hypothesis suggests that the amount of transmitter is directly related to the amplitude of the postsynaptic potential. This would mean that if more transmitters are contained in - and released from - a vesicle, then the area and number of activated receptors will be larger, and the PSP will be greater. An alternative hypothesis proposes that enough transmitters are always released from a single vesicle to saturate all of the postsynaptic receptors. Under this hypothesis, the PSP would represent the (fixed) total number of available receptor-channel complexes that would be insensitive to the amount of transmitter quanta released by the vesicles [reviewed by McLachlan (1978); Walmsley (1993); Frerking and Wilson (1996) for the CNS].

As further studies have shown, the quantal theory not only applies to the NMJ, but also to synapses of the CNS (Isaacson & Walmsley, 1995; Walmsley *et al.*, 1998) and PNS (Blackman *et al.*, 1963; Bennett, 1994; Lin & Bennett, 1994; Bennett *et al.*, 2001). Actually, the mechanisms suggested by the quantal theory are likely to be common to all

fast chemical synapses, including the cholinergic synapse in the SCG (McLachlan, 1975a, b).

1.4 Connectivity within the SCG

In both autonomic divisions, every preganglionic axon innervates a certain number of ganglion cells (divergence), and at the same time most ganglion cells receive synaptic inputs from a variety of preganglionic axons (convergence) (Fig. 1.5).

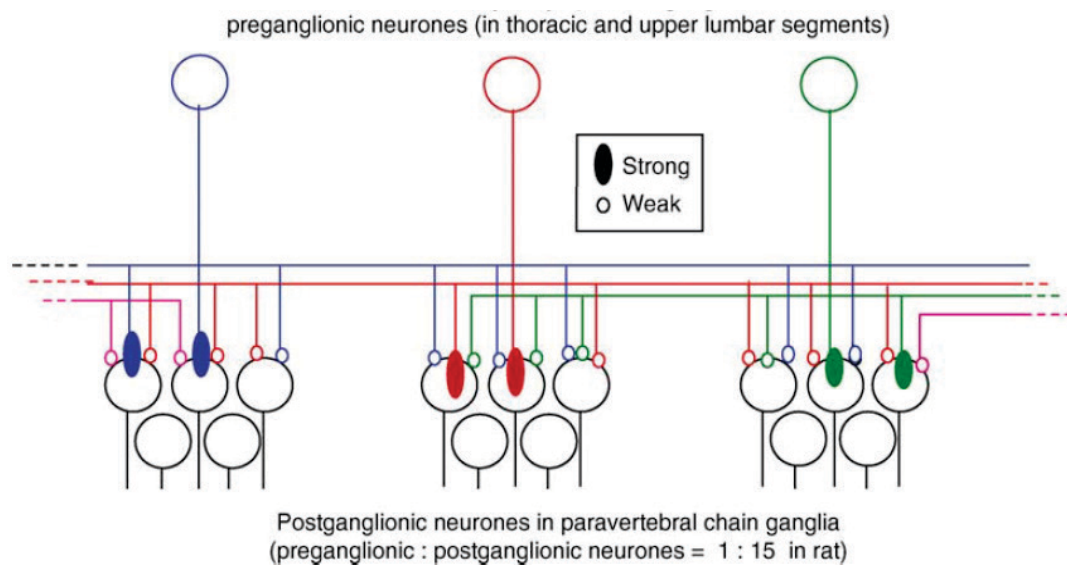


Figure 1.5: Connectivity in sympathetic ganglia of the thoracic paravertebral chain. Preganglionic axons arising in every spinal segment enter the sympathetic chain where they form synapses with postganglionic neurons. Synapses can be either suprathreshold (strong) or sub-threshold (weak). For definition of strong and weak synapses see chapter 1.4.3. Usually, axons tend to form strong synapses in the corresponding segmental ganglion. Additionally, they send collateral branches to adjacent segmental ganglia where they generally form weak synapses. Weak synapses do not contribute to the postganglionic AP in normal conditions, but they can develop and take over the connection if the strong input is damaged or missing. Picture taken from the book “Encyclopaedia of Neuroscience”, Binder *et al.* (2009), with permission.

1.4.1 Amplification and integration of signals

Anatomical studies have indicated that there is a predominance of postganglionic neurons in sympathetic pathways, showing a high degree of divergence. For example, in the mouse, each preganglionic neuron innervates 64 ganglion cells and the ratio of pre- to postganglionic neurons equals about 1:14 (in the rat, guinea pig and rabbit is about 1:27) (Purves *et al.*, 1986). Even though these are rough estimations and do probably underestimate the actual number of postganglionic neurons, they clearly show that the

main function of ganglia is to amplify the signals coming from the CNS (McLachlan, 2003). If there were no ganglia but direct connections from the spinal cord to the target organs (as for skeletal muscle), there would be the necessity of much more outputs from the cord (= preganglionic neurons).

Convergence plays also a big role in the SCG. Purves and colleagues (1986) showed that in mice every SCG receives 4-5 inputs (innervating axons) and the number increases with the body weight (8-9 in rats, 12 in guinea pigs, 15-16 in rabbits, at least 20 in humans). In general, postganglionic cells that lack dendrites (as in amphibian sympathetic and parasympathetic ganglia) are generally innervated by a single axon, whereas neurons with higher numbers of primary dendrites (present in larger mammals) receive information from a proportionally greater number of different axons (Purves & Hume, 1981; Purves & Lichtman, 1985).

1.4.2 The synaptic organisation of ganglia

By serially sectioning dye-filled sympathetic neurons in the lumbar sympathetic chain of guinea pigs, Gibbins and colleagues (1998) gained interesting insights in the synaptic organisation of ganglia. The three main results were as follows:

1. Synapses are rare and cover only 1-2% of the surface of the postsynaptic neurons. The rest of the surface is covered by Schwann cells.
2. Synapses are randomly distributed over the soma and the dendrites. The number of pre-ganglionic inputs for individual lumbar sympathetic ganglia of guinea pigs ranges between 5 and 15, corresponding with the size of both the dendritic and the peripheral target fields (Gibbins & Matthew, 1996). By attempting to discriminate between (postganglionic) somatic and dendritic synapses, Forehand (1985) found that in mice the mean number of somatic synapses per ganglion cell (mean surface area = $1593 \mu\text{m}^2$) amounts 24, while the mean number of dendritic synapses per ganglion cell amounts 264. For comparison, in rats there are 11 somatic and 319 dendritic synapses though the mean somatic surface area of the rat SCG neuron equals $1750 \mu\text{m}^2$ (Forehand, 1985). In a careful electronmicroscopic study using serial sections, Gibbins et al (1998) reported for guinea pig lumbar sympathetic ganglia about 35 boutons on the soma and 100 axodendritic boutons on a neuron with a larger dendritic tree, whereas a neuron with a smaller dendritic tree had 50 boutons at the soma and just 60 at dendrites. In cells with a reduced or lacking dendritic arbour, synaptic contacts are made

only on the soma. (Forehand, 1985; Smolen & Beaston-Wimmer, 1986). The larger number of synapses on the dendrites actually only applies to adult neurons. Hence, during development, neurotransmission begins prior to the elaboration of ganglion cell dendrites and therefore the first synapses are axosomatic. As dendrites appear (from day E14 in rats) (Rubin, 1985a), synaptogenesis becomes focused upon these processes (Rubin, 1985c), and the density of dendritic innervation becomes greater than the density of synapses on the soma. Experiments in mice, hamsters, guinea pigs and rabbits confirmed Rubin's results from rats (Forehand, 1985).

3. Most preganglionic endings do not contact the target cells, but are surrounded by thin lamellae of Schwann cells. Among the endings that do contact the target neurons and contain varicosities with vesicles, about 50% lack conventional pre- and/or postsynaptic specialisations, suggesting that only a minority of preganglionic varicosities participate in fast synaptic nicotinic transmission.

1.4.3 Strong and weak inputs

Preganglionic axons that converge on a postganglionic neuron may elicit excitatory postsynaptic potentials of different size and duration. Size and shape of the EPSP depend on several factors (Jänig & McLachlan, 1987; Gibbins *et al.*, 1998; Jänig, 2006):

1. The number of transmitter release sites. This relates to the number of varicosities of the preganglionic axon that synapse on the postganglionic neuron.
2. The number of quanta and the probability of release at each site (McLachlan, 1975b, c). The probability of release is “a consequence of the inherently stochastic nature of the molecular and cellular processes that drive vesicle exocytosis” (Del Castillo & Katz, 1954a). For each action potential (AP), neurotransmitter release has a certain likelihood of occurrence (Jänig, 2006).
3. The membrane properties of the postganglionic neuron, e.g. membrane conductance (influence on EPSP duration). While the membrane conductance may vary along the soma-dendritic arbour, the properties of the soma and proximal dendrites dominate in determining the size of the potential change (Jänig & McLachlan, 2002). Hence, most sympathetic neurons are considered electronically compact (Redman *et al.*, 1987; Gola & Niel, 1993), and synaptic signals generated on a distal dendrite up to 120 μm long (the average length for

these neurons) probably will reach the soma with relatively little attenuation (Gibbins *et al.*, 1998; Jamieson *et al.*, 2003).

4. The number and properties of the postsynaptic receptors [see Walmsley *et al.* (1998)]

Postganglionic neurons are typically innervated by one (sometimes two, very rarely three) preganglionic axon that, when stimulated, always evokes an EPSP that reaches the threshold for the generation of an AP. These inputs have a very large safety factor, as every incoming AP is translated into an outgoing one. Such suprathreshold inputs are called “**strong**” inputs. They have been profoundly studied *in vitro* in rats and guinea pigs (Holman & Hirst, 1977; McLachlan, 1978; Hirst & McLachlan, 1984; Cassell & McLachlan, 1986; Hirst & McLachlan, 1986), as well as *in vivo* in the cat (Janig, 1985, 1988).

In addition to this (usually) unique strong input, postganglionic neurons receive up to 15 inputs (depending on the on the type of neuron, the ganglion and the animal species) (Purves *et al.*, 1986; Ivanov & Purves, 1989) that always produce subthreshold EPSPs. These inputs are called “**weak**” as they never produce APs on their own (Holman & Hirst, 1977; Hirst & McLachlan, 1984, 1986; Gibbins *et al.*, 1998) Most of these observations have been made in rat, guinea pig, or rabbit, so similar experiments in mice have been rare. Hence, an early study in mice suggests that many SCG neurons also receive one strong and a few weak inputs, but due to the similar voltage threshold for stimulating the two classes of inputs, it is difficult to determine the exact number of weak inputs (Jobling & Gibbins, 1999).

Strong and weak inputs are schematically depicted in Figure 1.6.

Strong inputs are characterised by a high number of ACh quanta released (more than 20), which generate currents up to several nA in amplitude. This is reflected by big EPSPs that can reach up to 100 mV in amplitude, as can be measured by hyperpolarising the cell to prevent generation of APs. Hirst and McLachlan (1986) suggested that the synaptic contacts of a strong preganglionic input are associated with a single dendrite and particularly with an area of the postsynaptic membrane that contains a cluster of voltage-dependent calcium channels. Instead, Gibbins and colleagues (1998) proposed that strong preganglionic inputs make a large number of axosomatic contacts, whereas weak inputs make a small number of axodendritic contacts. This model is supported by the fact that in

simple neurons without dendrites axosomatic inputs are strong (Streichert & Sargent, 1989; Callister & Walmsley, 1996) and by the higher number of boutons (15-50) for every axosomatic input compared to axodendritic boutons (5-10) in lumbar sympathetic ganglia of guinea pigs. Hence, a single axosomatic input could have up to ten times more release sites than axodendritic inputs (Gibbins *et al.*, 1998).

Weak inputs have a low quantal content even at normal levels of extracellular Ca^{2+} . Interestingly, weak inputs hardly ever contribute to the postsynaptic discharge. This happens only when the strong axon is disrupted (after e.g., injury to the spinal cord) and neurons are temporarily left without strong input (McLachlan, 1974), or in the unlikely case that several weak inputs impinging on a neuron are synchronously activated. In the second case, synchronous activation results in spatial summation and synaptic integration (Fig. 1.6) (Eccles, 1955; Blackman, 1974; Skok & Ivanov, 1987; Jänig, 2006; Rimmer & Horn, 2010).

“Weak” inputs have a low number of release sites for ACh, and the probability of neurotransmitter release is very low (0.1-0.6) (Hirst & McLachlan, 1986; McLachlan, 2003). On the contrary, “strong” inputs are characterized by a high probability of release (1), related to a greater number of release sites and a high quantal content (Holman & Hirst, 1977; McLachlan, 1978; Hirst & McLachlan, 1986; Gibbins *et al.*, 1998).

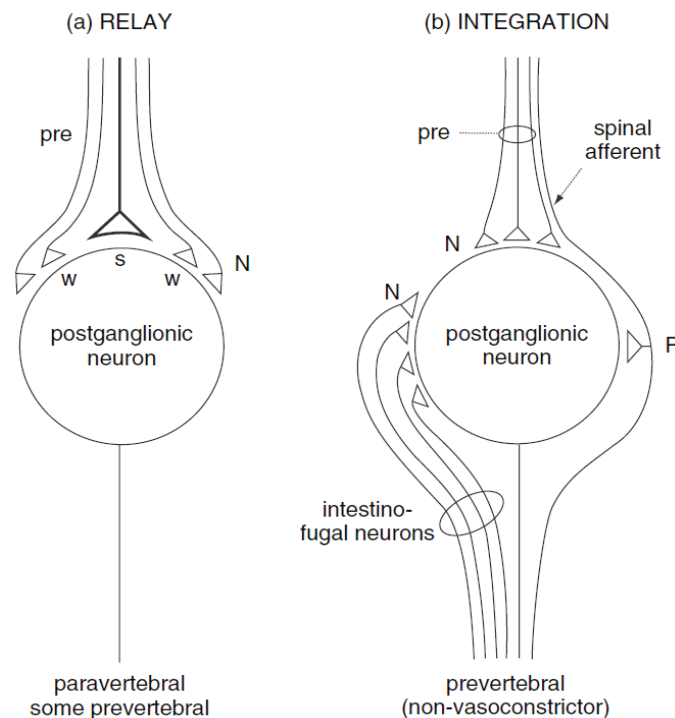


Figure 1.6: Function of autonomic ganglia and input types. (a) Relay function: most paravertebral, some prevertebral sympathetic neurons and most parasympathetic neurons have usually one (seldom two) strong (S, suprathreshold) preganglionic synaptic inputs and several weak (W, subthreshold) synaptic inputs. **(b) Integration function:** postganglionic neurons of the enteric nervous system contain synaptic inputs from both preganglionic neurons and intestinofugal neurons of the enteric nervous system and also from collaterals of spinal visceral afferents. The first two are cholinergic and nicotinic (N) but subthreshold (weak), while intestinofugal afferences also contain peptides that function as neurotransmitters (e.g., VIP). The collaterals of spinal visceral afferent neurons release substance P (P) and produce a slow depolarisation. Enteric neurons fire only after integration of several subthreshold inputs, with or without the peptide-induced slow depolarization. Picture taken from the book “The integrative action of the autonomic nervous system – Neurobiology of Homeostasis”, Jänig (2006), with permission.

Another distinction between strong and weak synapses can be made concerning the role of voltage-sensitive Ca^{2+} -channels of the presynaptic site for the release of ACh, as experiments performed in guinea pigs showed (Ireland *et al.*, 1999). While both synapses utilise N-type channels (transmitter release was reduced by ~34-39% through the specific blocker ω -conotoxin GVIA), only weak synapses additionally use P-type channels (reduction by ~42% through the specific blocker ω -agatoxin IVA in weak inputs, nearly no effect in strong inputs). Blockers of Q-, L-, and T-type channels had no significant effect on ACh transmission at either synapses.

1.5 Nicotinic acetylcholine receptors (nAChRs) in the SCG

1.5.1 nAChR structure

Within the SCG, fast excitatory synaptic transmission is mediated by ACh acting on nicotinic acetylcholine receptors (nAChRs) [for review see (Jänig, 2006)].

The nicotinic ACh receptor is a transmembrane pentameric glycoprotein of about 300000 Da. By electron microscopy it appears as a cylinder of about 16nm length and 8nm diameter, formed by 5 subunits, each spanning the membrane four times (Fig. 1.7). Seen from the synaptic cleft, the nAChR appears as a pseudo-symmetrical pentameric rosette (Fig. 1.7 B), each petal corresponding to a single nAChR subunit.

Every subunit is 300-600 residues in length and contains a globular extracellular NH₂-terminal domain, transmembrane domains made of a bundle of four α -helices (M1-M4), a large cytoplasmic domain inserted between M3 and M4, and short extracellular COOH-terminal domain. As the cytoplasmic domain is missing in the prokaryotic homologue of nAChRs, it seems not essential for signal transduction. In their extracellular portion of the NH₂-terminus, nAChRs form a so called “cys loop” (Fig. 1.7 C) due to a pair of disulphide-bonded cysteine residues separated by 13 aminoacids (Kao & Karlin, 1986; Tsunoyama & Gojobori, 1998). The cys-loop gave the name to the cys-loop superfamily of receptors that also includes 5-hydroxytryptamine type 3 (5-HT₃) receptors, γ -aminobutyric acid type A (GABA_A), GABA_C receptors, glycine receptors, invertebrate glutamate and histidine receptors (Karlin, 2002).

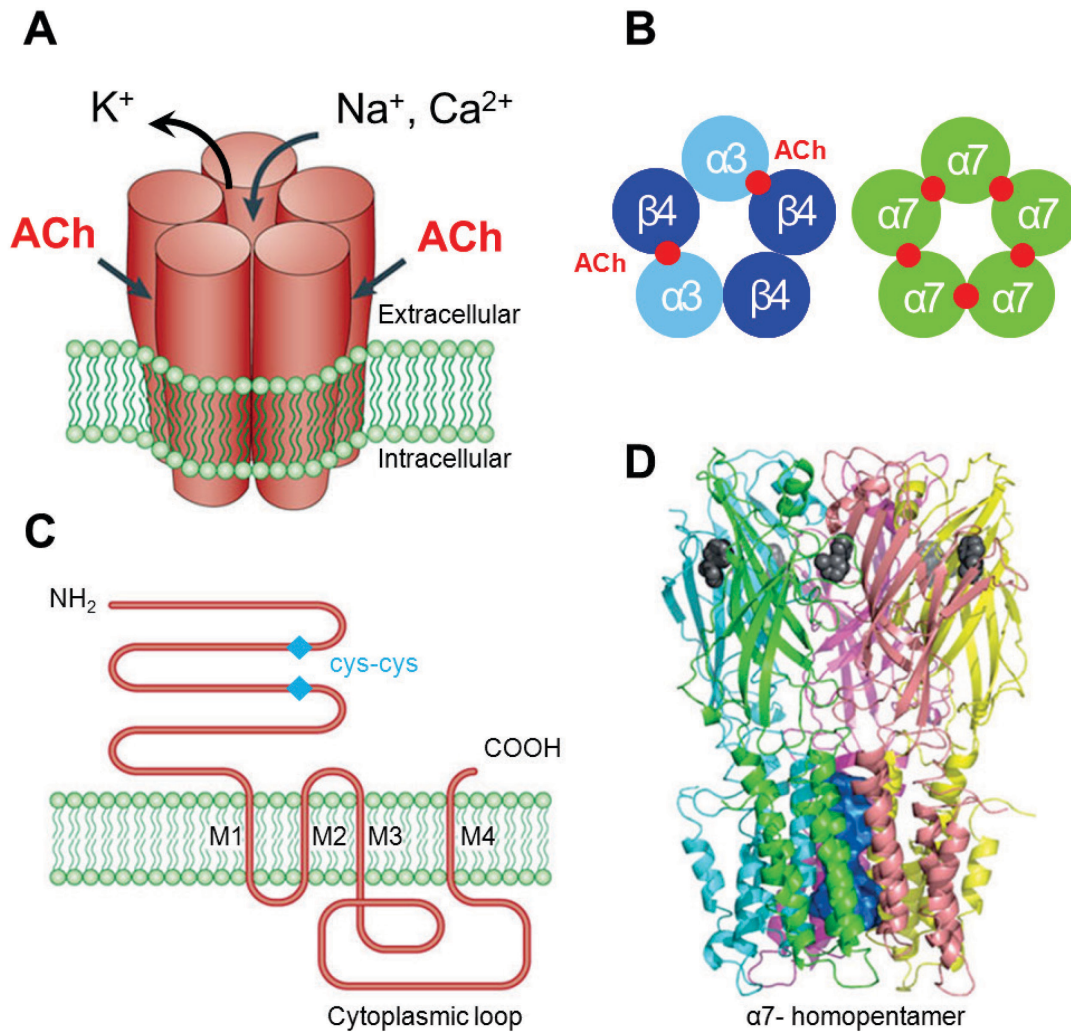


Figure 1.7: Structure of the neuronal nicotinic acetylcholine receptor. **A)** The transmembrane oligomer consisting of five subunits. The channel is permeable for Na^+ , K^+ and Ca^{2+} . **B)** The receptor rosette seen from the synaptic cleft. The two main types of nAChRs in ganglia are the $\alpha 3\beta 4$ hetero-oligomer and the $\alpha 7$ homo-oligomer. The number of agonist binding sites per pentamer ranges from two (in heteropentameric receptors) to five (in homopentameric receptors). Heteropentamers in the SCG may additionally contain the subunits $\alpha 5$ and $\beta 2$. **C)** Each subunit consists of a large NH_2 -terminal extracellular domain (carrying the ACh-binding cys-loop cysteines and the boundary between subunits), a transmembrane domain (M1-M4), a variable cytoplasmic domain and an extracellular COOH -terminal domain. **D)** Side view of a model of the $\alpha 7$ nAChR, showing five nicotine molecules (dark grey) in the binding sites and the ionic channel (dark blue). Parts A, C and D have been modified from Changeux (2010) and Taly *et al.* (2009), with permission.

The nAChR contains the binding site for the neurotransmitter ACh and an intrinsic cationic channel that is permeable for Na^+ , K^+ and Ca^{2+} ions. The ion channel is delineated by a water pore with low electron density that runs through the entire length of the protein. Upon agonist binding in the extracellular domain, the M2 helices lining the pore start rotating, and nAChRs undergo an allosteric transition from the closed resting

conformation to an open state. When nAChRs are open (the pore is opened by $\sim 3\text{\AA}$), under normal physiological conditions they allow an influx of Na^+ and Ca^{2+} (to a lesser extent than Na^+) and an efflux of K^+ . In the closed state, the nAChR is occluded by a “hydrophobic girdle” that constitutes a barrier to ion permeation (Galzi & Changeux, 1995; Karlin, 2002).

In the prolonged presence of an agonist, the nAChR closes within milliseconds to minutes and enters a so called “desensitised state”, in which the channel cannot be activated again. In this state the receptor produces a reduced response to the neurotransmitter or agonist drug. Desensitisation is believed to be related to a structural change of the binding site due to an affinity shift of the agonist (Changeux *et al.*, 1984; Buisson & Bertrand, 1998). This results in a non-conducting closed conformation of the channel pore, that is distinct from the non-conducting resting conformation (Wilson & Karlin, 2001). For the nAChR, two desensitised states (with a fast and slow onset) have been proposed to exist between the open and the closed state (Changeux & Edelstein, 2005). When no agonist is bound, the resting state is the most stable conformation, whereas the desensitised state is the most stable conformation when an agonist is bound. Desensitisation is particularly interesting for the study of neuronal nAChRs, since it may regulate the activity of the brain in cases where cholinergic ligands are present over large time periods (Jones & Westbrook, 1996).

1.5.2 nAChR subunit composition

nAChRs are widely expressed both in the CNS and in the ANS, as well as at the neuromuscular junction in skeletal muscles.

Muscle-type nAChRs are made of $\alpha 1$, $\beta 1$, δ , and either γ (embryonic) or ϵ (adult) subunits (Reynolds & Karlin, 1978), whereas vertebrate neuronal nAChRs are composed of α and β (*i.e.* non- α) subunits.

α subunits are defined by the presence of a pair of vicinal cysteines close to which the agonist binds (principal binding site). β -subunits lack these cysteines and provide the complementary binding site in $\alpha\beta$ -pairs. In sum, for the assembly of neuronal nAChRs, 9 α -subunits ($\alpha 2$ – $\alpha 10$) and 3 β -subunits ($\beta 2$ – $\beta 4$) have been identified in mammals and avian species (Schoepfer *et al.*, 1990; Jensen *et al.*, 2005; Gotti *et al.*, 2006). While $\alpha 7$, $\alpha 8$ and $\alpha 9$ can form functional homopentamers providing both principal and complementary faces of the agonist binding site (Gotti *et al.*, 2006), all other α -subunits (except of $\alpha 5$,

see below) form functional complexes only when co-expressed with either $\beta 2$ or $\beta 4$ (Vernallis *et al.*, 1993; Karlin, 2002).

Out of the 12 identified neuronal nAChR subunits, autonomic neurons typically express only the subunits $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ (Wang *et al.*, 2002b; Mao *et al.*, 2006; Putz *et al.*, 2008; David *et al.*, 2010). $\alpha 3$ and $\beta 4$ constitute the “backbone subunits” of the ANS, as they are present in every peripheral heteromeric nAChR. Heteromers may additionally contain the subunits $\alpha 5$ and $\beta 2$, whereas $\alpha 7$ forms homoligomers, as described above. Different receptor subtypes may reflect diverse stoichiometries and subunit arrangements (McGehee & Role, 1995). The actual subunit composition of heteromeric nAChRs in the mouse SCG (see Fig. 1.10) has been revealed by our group (David *et al.*, 2010). Additionally to $\alpha 3$, $\alpha 5$, $\beta 2$ and $\beta 4$, SCGs in early postnatal mice also contain the subunit $\alpha 4$, which is downregulated to very low levels during development (Putz *et al.*, 2008; David *et al.*, 2010; Scholze *et al.*, 2011).

1.5.3 Knock-out (KO) mice lacking nAChR subunits

A valuable tool to study the function of distinct receptors is provided by knock-out (KO) mice (Marubio & Changeux, 2000; Champtiaux & Changeux, 2002). Compared to the expression of specific subunits in heterologous systems (e.g. *Xenopus laevis* oocytes or HEK cells), KO-mice that lack one or more receptor subunits give the chance to investigate the (pharmacological and biophysical) characteristics of receptors in their native environment.

So far, the following nAChR subunit KO-mouse-models have been generated: $\alpha 2$ KO, $\alpha 3$ KO, $\alpha 4$ KO, $\alpha 5$ KO, $\alpha 6$ KO, $\alpha 7$ KO, $\alpha 9$ KO, $\alpha 10$ KO, $\beta 2$ KO, $\beta 3$ KO, $\beta 4$ KO, $\alpha 5\beta 4$ KO, $\alpha 5\beta 2$ KO, $\alpha 5\alpha 7\beta 4$ KO, $\alpha 5\alpha 7\beta 2$ KO and $\beta 2\beta 4$ KO (Picciotto *et al.*, 1995; Orr-Urtreger *et al.*, 1997; Marubio *et al.*, 1999; Vetter *et al.*, 1999; Xu *et al.*, 1999a; Xu *et al.*, 1999b; Cordero-Erausquin *et al.*, 2000; Champtiaux *et al.*, 2002; Wang *et al.*, 2002a; Cui *et al.*, 2003; Salas *et al.*, 2003; Kedmi *et al.*, 2004; Vetter *et al.*, 2007; Taranda *et al.*, 2009; David *et al.*, 2010; Lotfipour *et al.*, 2013).

All mouse strains except of $\beta 2\beta 4$ KO and $\alpha 3$ KO (see below for exception) appear grossly normal and healthy. $\alpha 3$ KO and $\beta 2\beta 4$ KO mice survive to birth but have impaired growth and increased perinatal mortality within the first week of life. They present enlarged bladders with dribbling urination and develop urinary infection and bladder stones. The ocular pupils are widely dilated and do not constrict in response to light (Xu *et al.*, 1999a; Xu *et al.*, 1999b; De Biasi, 2002). The phenotype of $\alpha 3$ KO and $\beta 2\beta 4$ KO

mice resembles the rare human genetic disorder of megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS), a disorder of smooth muscles in the abdomen and digestive system. Patients usually die in infancy from septicemia.

These severe phenotypes are readily explained by observations that all nicotinic receptors in the sympathetic and parasympathetic nervous system contain the subunits $\alpha 3$ and $\beta 4$ (Mao *et al.*, 2006; David *et al.*, 2010). As shown by David *et al.* (2010) and in this thesis report, the $\beta 2$ subunit can partially replace $\beta 4$ in the respective KO to restore almost normal autonomic function.

The group of Ellis Cooper furthermore observed that in $\alpha 3$ KO mice, not only fast synaptic transmission (and currents in response to directly applied ACh) in the SCG was missing, but that ACh output was also strongly impaired (Rassadi *et al.*, 2005). Data shown in this 2005 paper were based on short-lived mice generated by Avi Orr-Urtreger (Xu *et al.*, 1999a), whereas for their 2009 report, outbred $\alpha 3$ KO mice on a mixed C57BL/6/J - CD+ background (Rassadi *et al.*, 2005) were used. These animals live for several months in good health. Interestingly, the preganglionic nerve terminals in these mice formed morphologically normal synapses with sympathetic neurons that persisted in postnatal animals despite the absence of any fast synaptic transmission. The strongly impaired ACh output was explained by the absence of functional high-affinity choline transporters. Hence, developing presynaptic terminals require an activity-dependent retrograde signal (conferred by functional receptors) that induces the expression of high-affinity choline transporters (Krishnaswamy & Cooper, 2009).

The most important phenotypical characteristics of the single KO-mice used in our lab are described below.

- **$\beta 2$ KO mice:**

The $\beta 2$ subunit is the most widely expressed subunit in the brain (Wada *et al.*, 1989; Hill *et al.*, 1993; Zoli *et al.*, 1995) and was historically the first one to be knocked out (Picciotto *et al.*, 1995). $\beta 2$ KO mice lose the majority of high affinity binding sites for the agonist epibatidine in several brain areas, while still expressing unaltered levels of homopentameric $\alpha 7$ nAChRs (Picciotto *et al.*, 1995). Histological studies on aged mice showed that the $\beta 2$ subunit is implicated in neurodegeneration (cerebral cortex alterations, loss of hippocampal neurons, astro- and microgliosis). At the behavioural level, the $\beta 2$ subunit was

found to be involved in learning and memory (in a passive avoidance paradigm KO mice perform better in learning negative associations, but perform worse in the acquisition of spatial information than WT mice), nociception, nicotine addiction (nicotine stimulates dopamine release in the ventral striatum of WT but not KO mice), and the organisation of sleep (Picciotto *et al.*, 1995; Zoli *et al.*, 1999; Rossi *et al.*, 2001; Cohen *et al.*, 2002; Laudénbach *et al.*, 2002; Picciotto & Corrigall, 2002; Champtiaux *et al.*, 2003; Lena *et al.*, 2004).

- **β4KO mice:**

As Wang and colleagues described, mice lacking the subunit β4 show a weakened bradycardic response to vagal stimulation and an increased sensitivity to hexamethonium, a ganglion-blocking quaternary ammonium compound (see 2.3.7). Additionally, they observed a strong reduction in ileal contractile responses to the nicotinic agonists cytisine, dimethylphenylpiperazinium iodide (DMPP), nicotine (10 mg/kg) and epibatidine (0.1 mg/kg) (Wang *et al.*, 2003). The group also showed that β4KO mice (as well as α5β4KO mice) are highly resistant to nicotine induced clonic-tonic seizures (Kedmi *et al.*, 2004).

In the past years, interesting insights were gained in our laboratory on the contribution of β4 both at the biochemical and the physiological level. Accordingly, immunoprecipitation experiments revealed that the total number of nAChRs present in the pre-pubertal mouse SCG is dramatically reduced in genotypes lacking the backbone subunit β4 (David *et al.*, 2010). As shown in Figure 1.8, the number of receptors is reduced to 13% and 8% in β4KO and α5β4KO mice, respectively, compared to WT mice and all other β4-containing genotypes (David *et al.*, 2010). Although no data exist on the number of receptors within α5α7β4KO mice, they likely contain similar (little) amounts of heteromeric nAChRs as the other two β4-missing genotypes (Scholze, personal communication). Furthermore, α7 receptors neither contribute to synaptic transmission in the SCG (as shown in my thesis), nor do they contribute to nicotine-induced currents in cultured α5β2KO and α5β4KO SCG cells (David *et al.*, 2010; Ciuraszkiewicz *et al.*, 2013).

Our second principal findings came from electrophysiological experiments (patch clamp recordings) comparing the pharmacological and biophysical properties of α3β2 receptors (present in α5β4KO mice) with α3β4 receptors

(present in $\alpha 5\beta 2$ KO mice). $\alpha 3\beta 4$ receptors not only produced ACh-induced currents with about 2.5 amplitudes, but did also desensitise much slower than $\alpha 3\beta 2$ receptors (Fig. 1.9). Given that the overall number of receptors (determined by immunoprecipitation) is reduced by about a factor of 5, whereas currents for $\alpha 3\beta 2$ receptors are just 2.5 times smaller, this implies that nAChRs are more efficiently targeted to cell membranes of $\alpha 5\beta 4$ KO mice.

Whereas nicotine was just moderately more potent an agonist on $\alpha 3\beta 2$ receptors compared to $\alpha 3\beta 4$ receptors, it much more potently desensitised $\alpha 3\beta 2$ receptors when pre-treating SCG cell cultures at low (nicotine) concentrations (Fig. 1.10). Differences in desensitisation have previously been observed for nAChRs receptors heterologously expressed in *Xenopus laevis* oocytes (Papke & Heinemann, 1994; Fenster *et al.*, 1997; Gerzanich *et al.*, 1998; Fenster *et al.*, 1999) as well as in HEK 293 cells (Wang *et al.*, 1998).

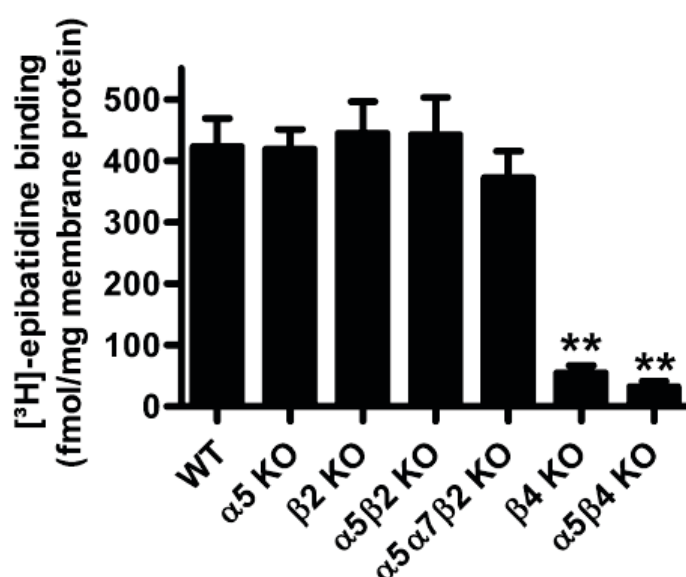


Figure 1.8: Total amount of nAChRs in the mouse SCG. The number of nAChRs in the SCG was established by binding essays with 1nm [³H]-epibatidine. nAChRs are significantly reduced in $\beta 4$ KO and $\alpha 5\beta 4$ KO mice, but not in $\beta 4$ -containing genotypes. The additional absence of the homopentamer $\alpha 7$, as shown for $\alpha 5\alpha 7\beta 2$ KO mice, has no impact on the expression of normal numbers of nAChRs. Note that by this method both surface and intracellular receptors are detected. Figure taken from David *et al.* (2010), with permission.

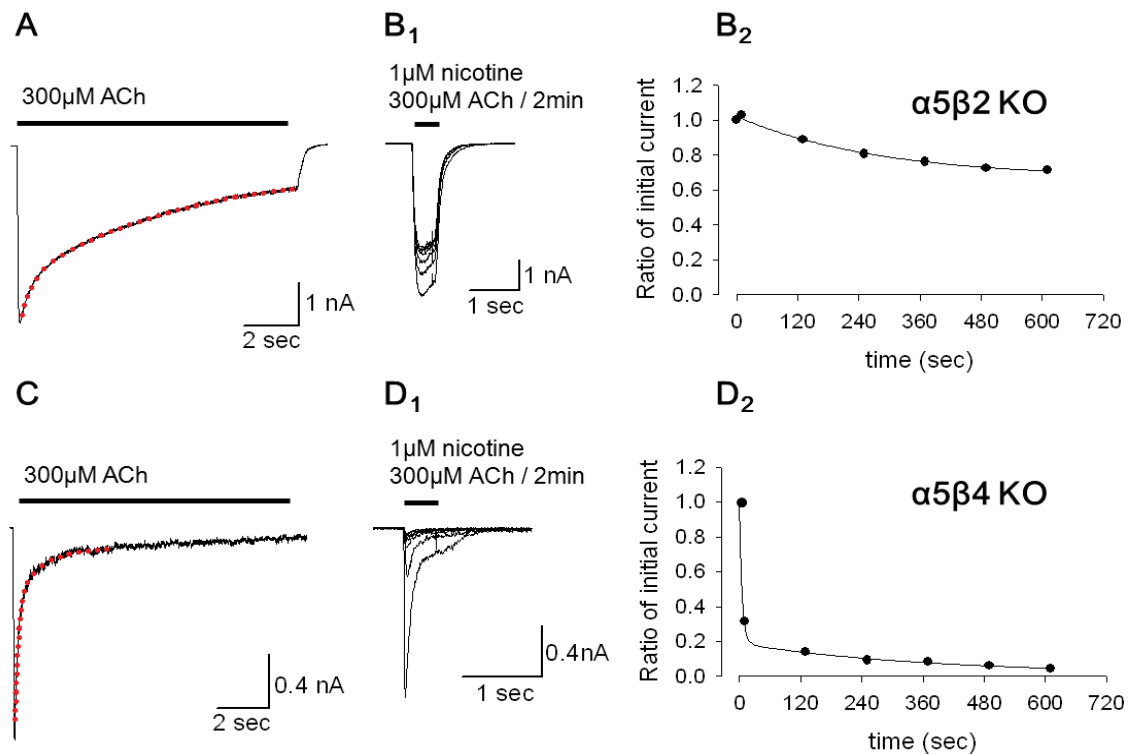


Figure 1.9: Patch clamp measurements of SCG neurons in $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice. A and C: fast (“classical”) desensitisation of the receptor induced by pulses of 300 μ M ACh: the pure $\alpha 3\beta 2$ receptors present in $\alpha 5\beta 4$ KO desensitise much faster than $\alpha 3\beta 4$ receptors present in $\alpha 5\beta 2$ KO mice. Notice the different scaling. Receptors lacking the backbone subunit $\beta 4$ thus desensitise faster and produce smaller currents. B–D: slow (“high-affinity”) desensitisation of receptors induced by the continuous presence of 1 μ M nicotine. When neurons were pre-treated with nicotine, amplitudes of currents induced by 300 μ M ACh decrease to 80% in $\alpha 5\beta 2$ KO mice and to only 15% in $\alpha 5\beta 4$ KO mice within 10 min. Nicotine IC_{50} values were approximately 0.1 μ M for $\alpha 3\beta 2$ and 1 μ M for $\alpha 3\beta 4$ receptors (not shown). Data were generously provided by Sigismund Huck and partially published in David *et al.* (2010).

- **$\alpha 5$ KO mice:**

$\alpha 5$ is considered an accessory subunit, as it cannot form functional receptors without the essential subunits and as it does not participate in forming (principal or secondary) ACh binding sites (Ramirez-Latorre *et al.*, 1996; Wang *et al.*, 1996; Groot-Kormelink *et al.*, 1998). However, the $\alpha 5$ KO genotype revealed altered cardiac parasympathetic ganglionic transmission and increased sensitivity to hexamethonium (Wang *et al.*, 2002a). In SCG neurons, where $\alpha 5$ -containing nAChRs reach about 25% (Mao *et al.*, 2006; David *et al.*, 2010), the deletion of this subunit dramatically enhanced the efficacy of presynaptic receptors (Fischer *et al.*, 2005). On the other hand, in the same neurons, David *et al.* (2010) found no contribution of $\alpha 5$ on the current decay time constants in either $\beta 2$ KO mice (i.e. $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ receptors) or $\alpha 5\beta 2$ KO mice (i.e. pure $\alpha 3\beta 4$ receptors; both

compared to receptors in WT mice). Likewise, decay time constants and agonist potencies did not differ significantly between $\alpha 3\beta 4$ and $\alpha 3\alpha 5\beta 4$ receptors expressed in HEK cells (Nelson *et al.*, 2001). When expressed in *Xenopus* oocytes, the presence of $\alpha 5$ largely increased the ACh sensitivity and the efficacies of nicotine and DMPP in $\alpha 3\beta 2$ receptors but not in $\alpha 3\beta 4$ receptors (Gerzanich *et al.*, 1998). However, both Ca^{2+} -permeability and desensitisation were enhanced when $\alpha 5$ was co-expressed with $\alpha 3\beta 4$ or $\alpha 3\beta 2$ in oocytes (Gerzanich *et al.*, 1998).

Nicotine-induced hypo-locomotion and tonic-clonic seizures (possibly arising in the hippocampus) were dramatically reduced in $\alpha 5\text{KO}$ mice compared to WT animals (Salas *et al.*, 2003). $\alpha 5$ -containing receptors associated with $\alpha 3$ and $\beta 4$ in the habenula-interpeduncular system have recently become a focus of attention as key players of tobacco addiction (Fowler *et al.*, 2011; Frahm *et al.*, 2011; Scholze *et al.*, 2012; Beiranvand *et al.*, 2014).

- **$\alpha 7\text{KO}$ mice:**

Since $\alpha 7$ homopentameric receptors are widely distributed in neuronal and non-neuronal tissue, it was surprising that $\alpha 7\text{KO}$ mice are viable and procreate normally. KO mice have normal [^3H] nicotine affinity but lack α -bungarotoxin binding, which is prominent in the hippocampus, amygdala and neocortex of control mice. Moreover, $\alpha 7\text{KO}$ mice lack rapidly desensitising, methyllycaconitine-sensitive, nicotinic currents that are present in hippocampal neurons (Orr-Urtreger *et al.*, 1997), they have a decreased sperm motility (Bray *et al.*, 2005) and asynchronous oestrous cycles (Morley & Rodriguez-Sierra, 2004). Furthermore, deletion of $\alpha 7$ decreased baroreflex-mediated tachycardia, suggesting that $\alpha 7$ receptors participate in the autonomic reflex that maintains blood pressure homeostasis (Franceschini *et al.*, 2000). Lastly, $\alpha 7$ lacking animals display corrupted synaptic plasticity and memory (Dougherty *et al.*, 2003; Puzzo *et al.*, 2008) and show brain dysfunctions in schizophrenia (Freedman & Goldowitz, 2010) and Alzheimer's disease (Wang *et al.*, 2000).

Studies based on fluorescence and electron microscopy have shown that in the chick ciliary ganglion, $\alpha 7$ nAChRs are excluded from synapses *proper* but are clustered around synapses on spines engulfed by presynaptic structures which are packed with synaptic vesicles (Jacob & Berg, 1983; Horch & Sargent, 1995).

This lead to the question whether $\alpha 7$ receptor would be or not required for synaptic transmission. Through whole cell patch clamp recordings *in situ*, Chang and Berg (1999) were able to show that in the chick ciliary ganglion $\alpha 7$ nAChRs are actually necessary for reliable synaptic transmission and for tightly synchronized firing early in development, since $\alpha 7$ blockade by α -bungarotoxin caused transmission failures.

On the contrary, other investigations showed that in the mouse, $\alpha 7$ is not required for parasympathetic control of the heart (Deck *et al.*, 2005), and that $\alpha 7$ does not contribute to trans-ganglionic transmission in the SCG (Brown & Fumagalli, 1977; Chiappinelli & Zigmond, 1978). Similarly, in patch clamp recordings of cultured mouse SCG neurons, David *et al.* (2010) found no critical contribution of $\alpha 7$ to nicotine-induced currents, as shown by $\alpha 7$ -specific agonists / antagonists as well as by the use of mouse genotypes lacking the subunit $\alpha 7$.

Finally, the $\alpha 7$ subunit was proposed to be involved in not only in enhancing synaptic transmission (McGehee *et al.*, 1995), but also to support long term potentiation (LTP) in several rodent brain regions of the hippocampus, as the dentate gyrus and the CA1 (Sawada *et al.*, 1994; Ji *et al.*, 2001; Mann & Greenfield, 2003; Welsby *et al.*, 2006). The facilitating action of nicotine on LTP was mediated via $\alpha 7$ receptors since it was simulated by the selective $\alpha 7$ agonist choline *in vivo* (Matsuyama *et al.*, 2000) and the selective $\alpha 7$ agonist 2,4-dimethoxybenzylidene anabaseine *in vitro* (Hunter *et al.*, 1994).

1.5.4 Subunit composition of nAChR in the mouse SCG

As shown by immunoprecipitation and Western blots, David and colleagues found that Wild type (WT) mice express 55% pure $\alpha 3\beta 4$ receptors, 24% $\alpha 3\beta 4\alpha 5$ receptors and 21% $\alpha 3\beta 4\beta 2$ receptors. These data are almost identical to what has been observed in the rat SCG (Mao *et al.*, 2006). In the mouse SCG, the subunits $\alpha 5$ and $\beta 2$ were never found together in the same receptor but only co-assembled with $\alpha 3$ and $\beta 4$ (see Fig. 1.10). By using KO mice, it was also shown that the two subunits $\beta 2$ and $\alpha 5$ were not up-regulated when the other one is missing (in either $\alpha 5$ KO or $\beta 2$ KO mice) and thus reached levels similar to WT mice. Deletion of $\beta 4$ would also eliminate $\alpha 5$ -containing receptors in the SCG. Hence, mice lacking either just $\beta 4$ or double-KO lacking $\alpha 5$ as well as $\beta 4$ expressed pure $\alpha 3\beta 2$ nAChRs. Since all nAChRs in the SCG consist of $\alpha 3$ and $\beta 4$, pure $\alpha 3\beta 2$ receptors only occur in the SCG of KO but not of WT mice (David *et al.*, 2010). Based on

these observations, the subunit composition within the SCG of WT mice and mice lacking distinct nAChR subunits is summarised in Figure 1.10.

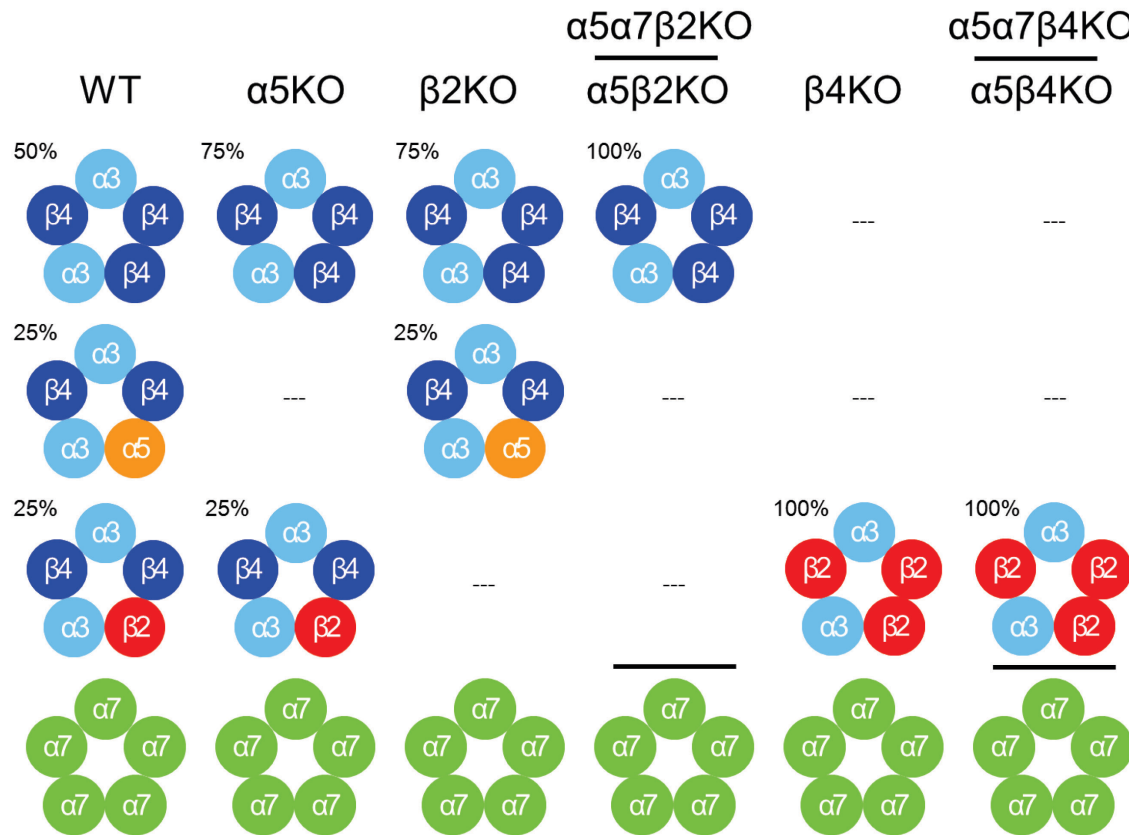


Figure 1.10: nAChRs subunit composition and occurrence in the mouse SCG. Heteropentameric receptors are composed of the “backbone subunits” $\alpha 3$ and $\beta 4$ and may additionally contain the subunits $\beta 2$ or $\alpha 5$ (never expressed together in the same receptor). Homopentameric receptors are formed by the subunit $\alpha 7$. The percentages express the approximate relative frequency of the corresponding receptor compositions within all heteropentameric nAChRs expressed in every genotype. All genotypes contain $\alpha 7$ -receptors unless knocked out (KO), as in the triple-knock out mice. Wild type (WT) mice additionally contain approximately 50% of pure $\alpha 3\beta 4$ -receptors, 25% of $\alpha 3\beta 4\alpha 5$ -receptors and 25% of $\alpha 3\beta 4\beta 2$ -receptors. $\alpha 5$ KO mice additionally contain approximately 75% of pure $\alpha 3\beta 4$ -receptors and 25% of $\alpha 3\beta 4\beta 2$ -receptors. $\beta 2$ KO mice additionally contain approximately 75% of pure $\alpha 3\beta 4$ -receptors and 25% of $\alpha 3\beta 4\alpha 5$ -receptors. $\alpha 5\beta 2$ KO mice express 100% $\alpha 3\beta 4$ -receptors, whereas $\beta 4$ KO and $\alpha 5\beta 4$ KO mice contain 100% $\alpha 3\beta 2$ -receptors. $\alpha 5\alpha 7\beta 2$ KO and $\alpha 5\alpha 7\beta 4$ KO mice only contain the heteropentameric receptors $\alpha 3\beta 4$ and $\alpha 3\beta 2$, respectively. This scheme was created based on data taken from David *et al.* (2010).

1.6 Goal of the present work

Previous findings which prompted this study are summarised as follows:

1. In the mouse SCG, deletion of the nAChR subunit $\beta 4$ reduces the overall number of nAChRs and leads to the expression of $\alpha 3\beta 2$ receptors. Application of an agonist produces smaller currents in cultured SCG neurons by activating these receptors, which are also more susceptible to desensitisation.
2. $\alpha 3\beta 2$ receptors may be of interest in the CNS [reviewed by Gotti *et al.* (2009)]
3. Receptors containing $\alpha 5$ more effectively confer synaptic transmission in intracardiac (parasympathetic) ganglia than receptors lacking this subunit in $\alpha 5$ KO mice.
4. Hexamethonium more potently inhibits nicotinic receptors lacking $\alpha 5$ in intracardiac (parasympathetic) ganglia than receptors of WT mice which contain this subunit.
5. Receptors containing $\alpha 5$ assembling with $\alpha 3$ and $\beta 4$ are of major interest in nicotine dependence and withdrawal (Gangitano *et al.*, 2009; Salas *et al.*, 2009; Fowler *et al.*, 2011; Frahm *et al.*, 2011).
6. Long-term potentiation has been described in the rat SCG.

These observations raise several questions:

- Is transganglionic/synaptic transmission impaired if only 15% of (overall) receptors are expressed in the SCG of $\beta 4$ or $\alpha 5\beta 4$ KO mice?
- How is transganglionic/synaptic transmission affected if only mediated by $\alpha 3\beta 2$ receptors?
- Is there a relationship between the genotype/number of expressed nAChRs and synaptic strength as determined by strong and weak inputs?
- How does the presence/absence of $\alpha 5$ affect transganglionic/synaptic transmission in the mouse SCG?
- What is the IC_{50} for hexamethonium for $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 2$ receptors?
- Can LTP be induced in the mouse SCG? And are $\alpha 7$ receptors involved in inducing and maintaining LTP due to their high Ca^{2+} -permeability?
- What can our experimental model tell about the desensitisation properties of $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 3\beta 2$ receptors?

My goal was to study the transganglionic/synaptic neurotransmission in the intact SCG of the mouse, with the purpose to find out how distinct nicotinic receptors (especially within the two double-KO models $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO) affect the function of the synapse.

The methods initially used were extracellular recordings of the compound action potential (CAP), recorded from the efferent (internal carotid) nerve upon stimulating the afferent (cervical sympathetic) nerve.

Following our main hypothesis, ganglia of $\alpha 5\beta 4$ KO mice (or $\alpha 5\alpha 7\beta 4$ KO mice) might show an impaired synaptic transmission simply because the number of postsynaptic nAChRs would be insufficient. Biochemical data (dramatically reduced number of overall nicotinic receptors) and electrophysiological data (small currents, fast desensitisation observed in cultured SCG neurons upon application of agonists) supported this idea. This hypothesis presupposes that the reduction of nAChRs observed by our IP-studies also affects receptors at postsynaptic sites. The availability of the respective KO-mouse models also gave us the chance to study the actual role of the subunits $\alpha 5$ and $\alpha 7$ for synaptic transmission in the SCG.

In a second approach (intracellular recordings of the excitatory postsynaptic potential, EPSP) we wanted to verify whether synaptic strength would be reflected by the number of receptors in different genotypes, thus hypothesising that $\alpha 5\beta 4$ KO mice, due to their reduced number of nAChRs, would form less suprathreshold “strong” synapses than control animals. This hypothesis presupposed that not only the quantal content of ACh (at presynaptic sites), but also a significant reduction of receptors at the postsynaptic site determines the size of an EPSP.

The last (minor) part of the work focused on the possibility to induce LTP in the mouse SCG. Though less studied, LTP does exist not only in the CNS but also in several parts of the ANS. To date, ganglionic LTP within the SCG has only been found in rats, cats and guinea pigs, but not in mice (Briggs & McAfee, 1988; Bachoo *et al.*, 1992; Morales *et al.*, 1994; Weinreich *et al.*, 1995; Heppner & Fiekers, 2003; Alzoubi *et al.*, 2008, 2010). If present also in mice, I planned to study the role of $\alpha 7$ receptors, known to be highly permeable for Ca^{2+} , which plays a key role for the induction of LTP.

2. MATERIAL AND METHODS

2.1 Animals

Experiments were performed on C57BL/6 mice, a widely used inbred laboratory mouse strain (Fig. 2.1). Wild Type (WT) as well as transgenic mice, carrying deletions of specific nAChR subunits, served as test animals. In this work, only the following genotypes besides WT mice were used: $\alpha 5$ -single-knock-out ($\alpha 5$ KO), $\alpha 5\beta 2$ -double-knock-out ($\alpha 5\beta 2$ KO), $\alpha 5\beta 4$ -double-knock-out ($\alpha 5\beta 4$ KO), $\alpha 5\alpha 7\beta 2$ -triple-knock-out ($\alpha 5\alpha 7\beta 2$ KO) and $\alpha 5\alpha 7\beta 4$ -triple-knock-out ($\alpha 5\alpha 7\beta 4$ KO).



Figure 2.1: Male 6 weeks old WT C57BL/6 mouse (<http://jaxmice.jax.org/strain/000664.html>, 1.12.2014).

$\alpha 5$ KO (Salas *et al.*, 2003) $\beta 4$ KO mice (Kedmi *et al.*, 2004) and $\alpha 5\beta 4$ KO mice were provided by Avi Orr-Utreger, Genetic Institute, Tel-Aviv Sourasky Medical Center, Tel-Aviv University, Israel. $\alpha 7$ KO mice (Orr-Utreger *et al.*, 1997) were purchased from Jackson Laboratories, Maine, USA. $\beta 2$ KO mice (Picciotto *et al.*, 1995) were provided by J.P. Changeux, Laboratoire de Neurobiologie Moléculaire, Institut Pasteur, Paris, France.

Double KO mice lacking the subunits $\alpha 5$ and $\beta 2$ ($\alpha 5\beta 2$ KO) were generated by our lab by crossing the respective single KO lines. Triple KO animals lacking $\alpha 5$, $\beta 2$ and $\alpha 7$ ($\alpha 5\alpha 7\beta 2$ KO) or $\alpha 5$, $\beta 4$ and $\alpha 7$ ($\alpha 5\alpha 7\beta 4$ KO) were similarly obtained by crossing the

respective double KO lines with $\alpha 7$ single KO animals. All mice used in this study were backcrossed onto a C57BL/6 background for six ($\beta 4$ KO), seven ($\alpha 5$ KO and $\alpha 7$ KO), or twelve ($\beta 2$ KO) generations after germ-line transmission.

Mice were housed in group cages containing 2-6 animals at a stable room temperature of 21 °C on a 10 h / 14 h light/dark schedule. Water and food were freely accessible. Only male mice aged 4-6 weeks (CAP recordings) or 6-10 weeks (EPSP recordings) were used. Females were not tested in order to exclude possible hormone interactions. Animal care and experiments were in compliance with the European Communities Council directive (86/609/EEC) and the Austrian federal law governing animal experiments (Tierversuchsgesetz 2012 – TVG 2012, BGBl. I Nr. 114/2012).

2.2 Dissection of the intact SCG

Mice were anaesthetised with CO₂ and quickly decapitated with scissors. The head was immediately immersed in ~ 100 ml oxygenated Locke's solution, pinned down to the Sylgard coated polystyrene preparation receptacle with four syringe needles (0.4 x 20 mm, 25G, Gr.20, B Braun, Melsungen, Germany) and carefully rinsed with fresh physiological solution until the liquid was completely transparent (Fig. 2.2A). The following dissection was done with the help of a stereo microscope (Wild Heerbrugg M8 zoom stereo microscope, Leica, Wetzlar, Germany). During the entire dissection, the tissue was frequently rinsed with Locke's solution. The stumps of trachea and oesophagus were removed together with surrounding musculature and connective tissue so that the two superior cervical ganglia were clearly identifiable (Fig. 2.2B). By grasping only the internal carotid artery and the internal jugular vein (vein and artery run parallel to the cervical sympathetic nerve) with forceps (Dumont #5 forceps, 0.08 x 0.04 mm, FST, Heidelberg, Germany) the innervating cervical sympathetic nerve was separated from the surrounding tissue (Fig. 2.2C). The aim was to leave as much connective tissue as possible adherent to the cervical sympathetic nerve. Attention was made not to touch, stretch, squeeze, or cut the cervical sympathetic nerve. It was important to keep sufficient connective tissue around the nerve to be able to tightly suck it into the suction electrode later on. As a second step, the postganglionic internal carotid nerve was cut as far as possible from the body of the SCG with micro scissors (Moria Dowell spring scissors, 10 mm blade; Vannas-Tübingen spring scissors, 5 mm blade, FST, Heidelberg, Germany). Again, attention was paid not to damage the nerve. The last step was to

remove the entire SCG with its freed nerves attached. By grasping the connective tissue surrounding the SCG body and cutting the tissue behind the SCG, the ganglion was extracted from the head and placed into a petri dish (Nunc, 35 x 10 mm, Sigma, St. Louis, Missouri, USA) filled with fresh oxygenated Locke's solution. The subsequent preparation of the SCG diverged depending on its designation for Compound Action Potential (CAP) or Excitatory Postsynaptic Potential (EPSP) recordings.

For extracellular recordings of the CAP, the ganglion was only roughly cleaned from excess fatty and connective tissue that would disturb the recording when flooding around the superfused ganglion (Fig. 2.2D).

For intracellular (EPSP) recordings, ganglia had to be carefully cleaned from protruding muscle and connective tissue and were tightly pinned down the Sylgard-coated recording chamber. The pinning of the ganglion was the most risky part of the preparation and would last 20-30 min. First, 4 pinning tungsten needles were put diametrically opposed at the ganglion edges, then others followed one by one. Fifteen to twenty-five needles were necessary per ganglion. The needles were only pierced through the fine sheath of connective tissue surrounding the ganglion (Fig. 2.2E), while the ganglion itself had not to be punctured and damaged. The aim was to pin down the ganglion as flat and tight as possible, until the preparation resembled a tent pitched down to the floor. This would achieve a stable hold during superfusion and a high probability of a durable cell contact. The tighter the ganglion was pinned down the recording chamber, the more likely it was to succeed in penetrating the capsule without obstructing the recording electrode with loose connective tissue.

Both for intracellular and extracellular recordings some piece of fat or connective tissue was set aside at the end of the preparation to better tighten the nerve fit within the suction electrode.

Forceps were periodically sharpened using a black arcansas stone, as described by the sharpening kit.

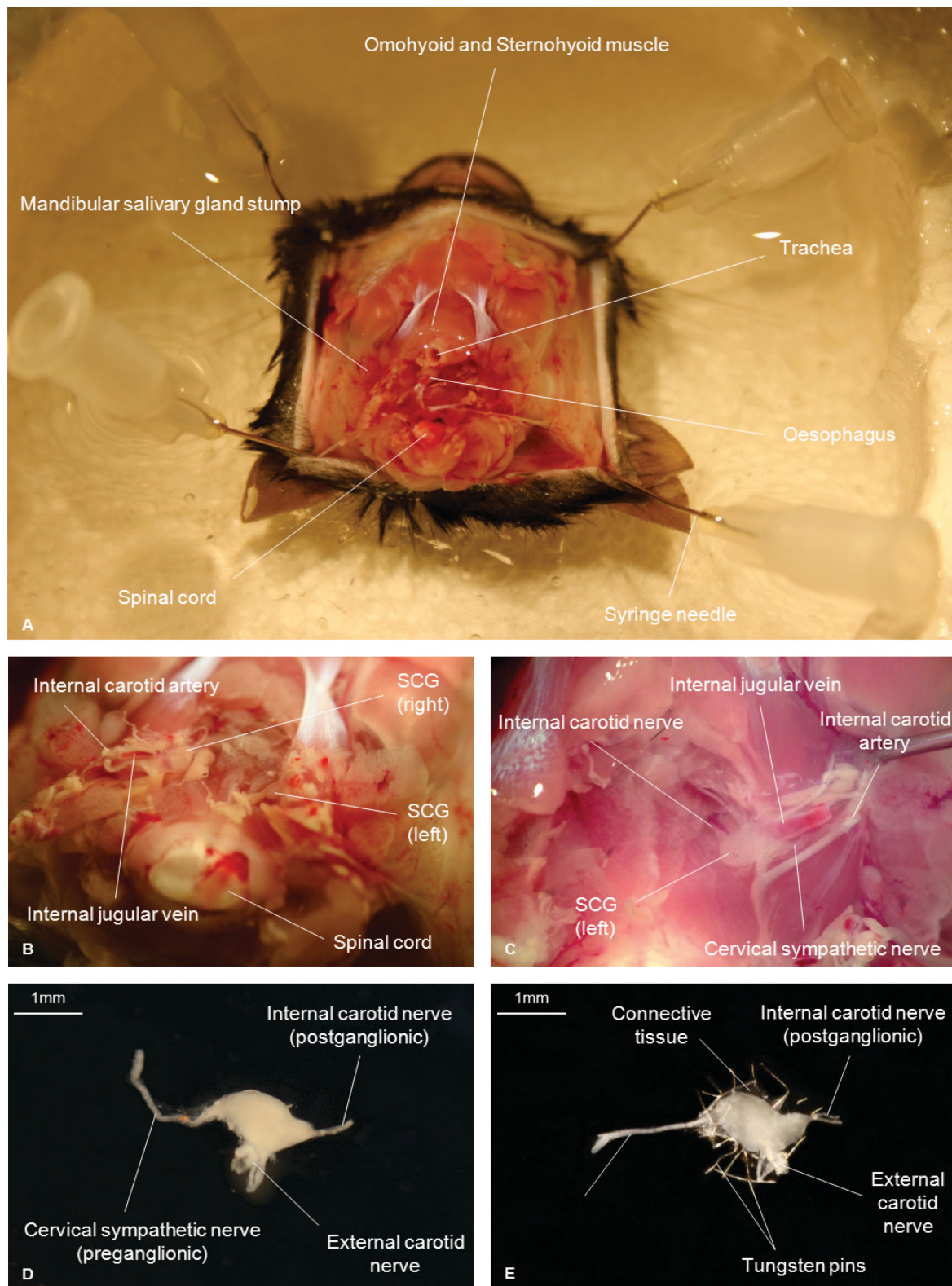


Figure 2.2: Dissection steps of a male 6 weeks old WT C57BL/6 mouse. **A)** Mouse head pinned down to the Sylgard coated polystyrene preparation receptacle. **B)** Left and right SCG become visible after cutting away protruding parts of trachea, oesophagus, spinal cord and muscles. **C)** Left SCG with its cervical sympathetic nerve already separated from the surrounding tissue **D)** Isolated left SCG ready for extracellular CAP recordings. **E)** Left SCG tightly pinned down the recording chamber and ready for intracellular (EPSP) recordings.

2.3 Preparatory steps

2.3.1 Fabrication and usage of suction electrodes

Suction electrodes were created by holding the ending of a thick-walled borosilicate glass pipette (Science Products, GB150-8P) over a flame of a butane filled pencil torch (1523, Central Pneumatic-Harbor Freight Tools, USA). As soon as the pipette end bended down forming an angle of approximately 135° , the pipette was retracted from the fire source. The edge of the pipette was then heated again to set the pore diameter: shorter melting periods would produce bigger pores, and vice versa. A series of ten suction electrodes with increasing pore diameters was subsequently produced. Too long melting periods would lead to an occlusion of the pipette. Pore diameters were measured with the aid of a stereo microscope. Pipettes were then adjusted in length and the posterior ends were fire-polished. The pipettes that best fitted for the pre- and the postganglionic nerves were chosen and used for all experiments.

To create functional suction electrodes, pipettes were inserted into electrode holders (Microelectrode Holder Str male wire 1.5 mm, MEH7W15, WPI, Sarasota, Florida, USA) which were equipped with a pressure port and connected by a tube to a 5 ml-syringe (Omnifix Syringe 5 ml LS, B. Braun, Melsungen, Germany).

By drawing up the syringe, liquids and/or the nerves could be sucked into the glass pipette. The suction electrode holder designed for preganglionic nerve stimulation was further equipped with a reference electrode, consisting in a silver wire (Ag 99%, diameter = 0.25 mm) closely wrapped around the pipette in tight loops (Fig. 2.4). Electrical pulses were generated between outer and inner silver wire.

2.3.2 Fabrication of coated petri dishes

Nunc petri dishes (35 mm) intended for intracellular recordings were coated with Sylgard silicone elastomer (SYLGARD® 184 Silicone Elastomer Kit 10:1, Dow Corning, Midland, USA). The amount of Sylgard was chosen in order to cover the bottom of the petri dish forming a ~ 1 mm thick layer. Usually 2 ml of Sylgard were sufficient. The thickness of the Sylgard floor was constraint by the need of transparency on one hand, and a stable pin fixation on the other hand. The Sylgard was prepared at a ratio A:B = 10:1.3 (instead of 10:1 as recommended) to ensure a harder consistency of the layer and thus a better fixing of the tungsten pins. Coated petri dishes were given 12h to get dry. The Sylgard coating was then treated as follows to reduce surface tension and avoid

beading of liquids. Some Locke's solution was poured into the dish and let dry up until it crystallized. The back of forceps was then used to scratch the crystal-covered Sylgard coating. This action was repeated two or three times until water drops and clusters stopped forming on the Sylgard surface. The Sylgard surface was not visibly damaged by this operation.

2.3.3 Fabrication of pinning needles

Pinning needles were prepared freshly before each dissection by cutting a piece of tungsten wire (diameter 25 μ M) into 20-40 pins of about 2 mm length. The cutting procedure occurred with the aid of a stereo microscope and the wire was kept under water to prevent pins from shooting around while cutting. Pins that remained straight could be reused several times.

2.3.4 Fabrication and filling of sharp electrodes

Sharp electrodes were made of thick-walled borosilicate glass pipettes with filament (0.86 x 1.5 x 80mm GB150F-8P, Science Products), using a Flaming Brown puller P-97. Pulling parameters were set in a way to produce electrodes with 120-130 M Ω resistance and about 8 mm taper. Electrodes should not be too elastic in order to penetrate the connective tissue sheath rather than denting it. Puller parameters had to be adapted after each filament change, as well as due to occasional indoor climate changes (temperature, humidity) (Tab. 2.1).

Electrodes were backfilled with sterile 0.5M KCl using a 5 ml syringe equipped with a filter (Sterile Syringe Filter 0.20 μ M, md. 25 mm, Corning Inc, New York, USA) and a microloader tip (PipetteTip Microloader 0.5-20 μ l, Eppendorf, Hamburg, Germany). First, a small drop of KCl was put on the back end of the glass pipette. By placing the pipette vertically, the tip facing downwards, after about 5-10 min the drop of KCl filled the tip of the electrode due to capillary force. To achieve this, pipettes were fixed with some modelling clay to a beaker. For a faster filling of the electrodes, the humidity inside the beaker had to be kept high. Therefore, the bottom was always covered with some water and the beaker was shut by a lid. However, in about 10% of the cases, the pipette tips still contained air bubbles that sometimes could be removed with the aid of a cat whisker. Only bubble-free pipettes were used. The rest of the pipette was then backfilled by injecting KCl with the microloader, and the pipette was given another 5-10 min hanging vertically.

Table 2.1: Puller parameters used to pull sharp electrodes

Date	Pressure	Heat	Pull	Velocity	Delay
2.10.2013	485	485	160	100	120
3.5.2012	485	495	170	100	100
12.3.2012	485	480	160	100	100
21.4.2011	250	480	100	100	60

2.3.5 Chlorination and cleansing of silver electrodes

Silver electrodes were chlorinated by the electroplating technique, making the wires positive with respect to the surrounding HCl solution. Silver wires destined to be electrodes were connected with the positive pole of a 9V-battery. A much thicker Ag-wire was connected to the negative pole of the battery. Both Ag wires were then immersed in a 1N HCl solution. By closing the electric circuit, oxygen bubbles were generated around the thick Ag-wire, while the thin wire (the future electrode) was covered with a white AgCl surface coating. The procedure lasted a few seconds and was repeated if needed (approximately once a month, when the baseline started drifting during experiments). When electroplating a previously plated wire, it was usually necessary to carefully remove residual AgCl mechanically with the help of some abrasive paper. An occasional pollution with fat was removed with acetone.

After every working day, silver electrodes and electrode holders were rinsed with distilled water and carefully dried.

2.3.6 Physiological Locke's solution and the superfusion system

Physiological Locke's solution was prepared according to Briggs and McAfee (1988), as shown in Table 2.2. A ten-fold concentrated stock solution was prepared about twice a year. Since Ca^{2+} and Mg^{2+} would react with the phosphate and precipitate at this high 10x concentration, the stock solution was prepared without CaCl_2 and MgCl_2 . These substances were added only to the final 1x Locke's solution, prepared freshly before an experiment. Locke's 10x was filtered, filled in a 1L autoclaved glass bottle and stored at room temperature (21-23 °C). Locke's 1x was filled in a 1L glass bottle and bubbled with 95% O_2 / 5% CO_2 for at least 15-30 min before an experiment. At room temperature, the oxygenated 1x Locke's solution had pH 7.2. Locke's 1x was stored at -4 °C for up to one month.

Table 2.2: Physiological Locke's solution

LOCKE'S 10x stock solution (lacking CaCl₂ and MgCl₂)			
Substance	MW (g/mol)	Final 1x concentration (mM)	Amount for 1L 10x-Locke (g)
NaCl	58.44	136.0	79.500
NaHCO ₃	84.01	20.0	16.802
Glucose	180.20	8.0	14.416
KCl	74.55	5.6	4.175 (= 56 ml 1M stock)
NaH ₂ PO ₄	137.99	1.2	1.656 (= 12 ml 1M stock)
Addendum for LOCKE'S 1x final solution			
Substance	MW (g/mol)	Final 1x concentration (mM)	Amount for 1L 1x-Locke (g)
Locke 10x	---	---	100 ml
CaCl ₂ • 2H ₂ O	147.02	2.2	3.234
MgCl ₂ • 6H ₂ O	203.30	1.2	2.440

The superfusion system consisted of silicone tubes (inner diameter 1 mm) connected to two pumps (2120 Varioperpex II peristaltic pump, LKB, Bromma, Sweden), one for the inflow and one for the outflow of the physiological solution. To avoid electrical noise, a 2 cm long syringe needle connected to ground was intercalated in each tube. The superfusion flow rate measured approximately 1 ml/min.

All CAP- and EPSP-recordings took place at room temperature (21-23 °C). Locke's solution was equally kept at room temperature during experiments. When needed, during CAP recordings, drugs as nicotine (NIC), hexamethonium bromide (HMB), or PNU-120596 (PNU) were added to the oxygenated Locke's solution. Drugs were applied until no further change of the CAP amplitude was visible (15-30 min). During experiments, all solutions were kept in 50 ml centrifuge tubes (50 ml polypropylene Conical Tube 30 x 115 mm, FalconTM, Corning, Inc, New York, USA) and were continuously bubbled therein with 95% O₂ / 5% CO₂.

The level of superfusing solution inside the recording chamber was set as low as possible in order to minimize contact with the recording electrode and thus electric noise, while completely covering the ganglion.

2.3.7 Drugs

- **Nicotine (NIC): C₁₀H₁₄N₂**

Nicotine, an alkaloid from the tobacco plant, is a naturally occurring agonist of nAChRs. It was the ligand originally used to classify the receptors (Langley, 1907). It activates all subtypes of nAChRs, except for $\alpha 9$ and $\alpha 9\alpha 10$ channels (where it acts as an antagonist; IC₅₀ values: 31 μ M and 4 μ M, respectively) (Elgoyhen *et al.*, 1994; Elgoyhen *et al.*, 2001) and binds with high affinity to $\alpha 4\beta 2^*$ nAChR (K_i: 0.6-10 nM) and less affinity to $\alpha 3\beta 4$ (K_i: 290-476 nM) and $\alpha 7$ nAChRs (K_i: 400-15000 nM) (Marks *et al.*, 1986; Anand *et al.*, 1993; Marks *et al.*, 1996; Kem *et al.*, 1997; Xiao *et al.*, 1998; Xiao & Kellar, 2004; Rueter *et al.*, 2006). Being a widespread drug of abuse, NIC is the most frequently used reference substance in studies on nAChRs.

In my study, NIC was added to the superfusing Locke's solution for desensitisation purpose during CAP recordings. The doses used ranged from 0.03 μ M to 10 μ M and were applied for 15-20 min, until no further decrease of the CAP amplitude could be observed.

- **Hexamethonium bromide (HMB): C₁₂H₃₀Br₂N₂**

Hexamethonium bromide is a nonselective and non-competitive open channel antagonist of nAChRs. It was introduced in the 1950s as a ganglionic blocker to treat hypertension (Smith *et al.*, 1954), but the sympatholytic and parasympatholytic side effects of HMB treatment (e.g. orthostatic hypotension, sexual dysfunction, constipation, urinary retention, glaucoma, blurry vision, decreased lacrimal gland secretion) led to discontinuing its use. Due to its hydrophilic nature, HMB cannot cross the blood brain barrier (Paton & Zaimis, 1949). It blocks all $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nicotinic receptors with or without $\alpha 5$ subunits (Nelson & Lindstrom, 1999). In vitro, concentrations of 1-100 μ M are used to inhibit nAChRs (Wang *et al.*, 2002a; Eaton *et al.*, 2003; Deck *et al.*, 2005; Wang *et al.*, 2005).

In my study, HMB was added to the superfusing Locke's solution to block transganglionic transmission by nAChRs. The doses used ranged from 12.5 μ M to 3.2 mM and were applied for 20-30 min until no further decrease of the CAP amplitude could be observed.

- **PNU-120596 (PNU): $C_{13}H_{14}ClN_3O_4$**

PNU is a type II positive allosteric modulator of nAChRs. It binds at sites distinct from the agonist binding site and decreases the energy barrier between the open and desensitised state of the receptor, thus enhancing the agonist-evoked response along with a decreased desensitisation of the agonist response (Bertrand & Gopalakrishnan, 2007). PNU is one of the better characterised urea analogs (Hurst *et al.*, 2005). It potentiates ACh-evoked $\alpha 7$ nAChR currents by increasing several fold a maximal amplitude and slowing down receptor's desensitisation (David *et al.*, 2010) and improves sensory gating deficits induced by amphetamine in rat-models for schizophrenia, learning and memory (Bertrand & Gopalakrishnan, 2007).

In my study, PNU (10 μ M) was added to the superfusing Locke's solution during CAP recordings to test the involvement of $\alpha 7$ receptors in ganglionic transmission.

2.4 Electrophysiology

2.4.1 The recording equipment

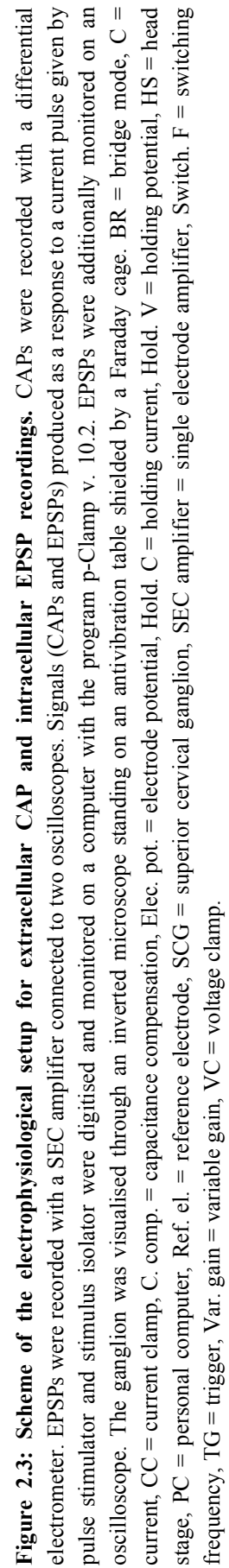
A phase contrast inverted microscope (Nikon, Diaphot 300) allowed visualisation of ganglia, nerves, and electrode tips. To achieve stable recordings, the microscope was positioned on an antivibration table (Benchmate 2212, Kinetic Systems, Inc) and was shielded by a Faraday cage (Fig. 2.3).

Ganglia were stimulated with current pulses generated by channel 2 or 6+7 (10 V output) of a pulse stimulator (Master -8, AMPI, Jerusalem, Israel) connected to a stimulus isolator (Isoflex, AMPI, Jerusalem, Israel). Pulses were given through a suction electrode to the preganglionic nerve (Figs. 2.3 - 2.6).

For extracellular CAP recordings, CAPs were recorded from the internal carotid nerve with a suction electrode connected to a differential electrometer (MetaMetrics AK47).

For intracellular EPSP recordings, EPSPs were recorded from the ganglion body with a sharp electrode positioned with a xyz-stage micromanipulator (SM LN 1, Luigs Neumann, Ratingen, Germany). The signal was pre-amplified by a headstage (npi, Tamm, Germany) and amplified by a single electrode amplifier (SEC 05 LX, npi, Tamm, Germany). The signals and the electrode potential were additionally monitored by two storage oscilloscopes (HM 205-3, HAMEG, Mainhausen, Germany). Data were digitized

by a Digidata 1200 interface digitizer (Axon Instruments/Molecular Devices, Sunnyvale, California, USA) and stored on a personal computer by means of the p-Clamp software (Clampex, v.10.2, Axon Instruments/Molecular Devices, Sunnyvale, California, USA).



2.4.2 Experimental procedure

Before starting a CAP or EPSP recording, the ganglion was given at least 30 min to adapt to the superfusion flow of oxygenated Locke' solution. During this adaptation period, both the pre- and the postganglionic nerve (CAP recordings), or only the preganglionic nerve (EPSP recordings) were already pulled into the stimulating suction electrode (Fig. 2.5, Fig. 2.6). Some fat particle or connective tissue was pulled into the suction electrodes together with the nerve to stabilize the contact and to ensure a tight fit between nerve and pipette.

1. CAP recordings

Ganglia were stimulated with current pulses (duration 50 μ s, variable amplitude) at 0.033 Hz, unless otherwise mentioned. The stimulus was set 5 ms after sweep start. For LTP-recordings, ganglia were first adapted to pulses at 0.033 Hz for 30 min and then a 20 Hz pulse train (400 pulses / 20 s) was given manually. After the train, pulses were set back to 0.033 Hz. Stimulating parameters and recording protocols are reported in Table 2.3.

Starting with the smallest current amplitude that the stimulus isolator could produce, the stimulus intensity was slowly increased until the first discernible CAP appeared just after the stimulus artefact. Since the CAP is the algebraic sum of all individual fibre APs, by increasing the stimulus further, one recruits more and more presynaptic fibres, and therefore more and more postsynaptic APs add up to produce a bell-shaped curve (Fig. 2.4). The onset of the CAP-curve is due to the APs generated by the fastest fibres (bigger diameter, lower threshold), while slower fibres (smaller diameter, higher threshold) will contribute APs to the tail section of the CAP.

Experiments were done at supramaximal intensity (electrometer gain 500), meaning that the stimulus amplitude was increased by 10-15% above the stimulus strength that produced maximal CAPs. This was done to ensure that all possible fibres were activated.

Figure 2.5A schematically elucidates the signal transduction pathway and CAP recording setup.

Before starting a recording, ganglia were given 20 min to check for CAP amplitude stability. Unstable CAPs and diminishing amplitudes were a sign of a loose fit between nerve(s) and suction electrode(s). In such cases, nerves had to be sucked in again together with some more fat/connective tissue.

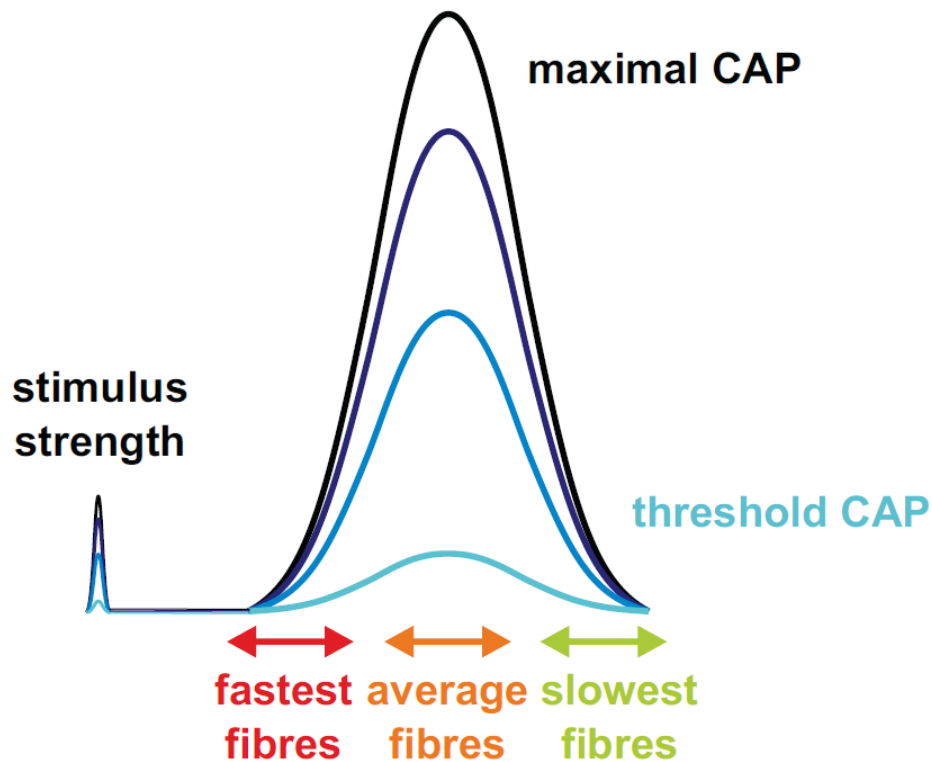


Figure 2.4: The bell-shaped compound action potential (CAP). By increasing the stimulus strength step by step the CAP gradually increases from its threshold to the maximal amplitude. Fast and slow presynaptic fibres contribute differently to the shape of the postsynaptic CAP.

Table 2.3: Stimulating parameters Master-8

Stimulating Parameters Master-8						
	CAP recordings			EPSP recordings		
Setting	CH 1	CH 2	CH 6+7	CH 1	CH 2	CH 6+7
Mode	FR	TG	TR	FR	TG	TG
Duration	D1: 2 ms	D3: 50 μ s	D3: 50 μ s	D1: 100 μ s	D3: 20 ms	D3: 50 μ s
Delay	L1: ---	L3: 5 ms	L3: ---	L1: ---	L3: 350 ms	L3: 100 ms
Interval	I1: 30s	I3: ---	I3: 50 ms	I1: 3,002s	I3: ---	I3: ---
n Pulses	M1: ---	M3: ---	M3: 400	M1: ---	M3: ---	M3: ---
Connections	CH 1 $\rightarrow$ CH 2 CH 6+7: triggered manually			CH 1 $\rightarrow$ CH 2 CH 1 $\rightarrow$ CH 6+7		

CH = channel, D = duration, FR = free run, I = interval, L = delay, M = number of pulses, TG = trigger, TR = train

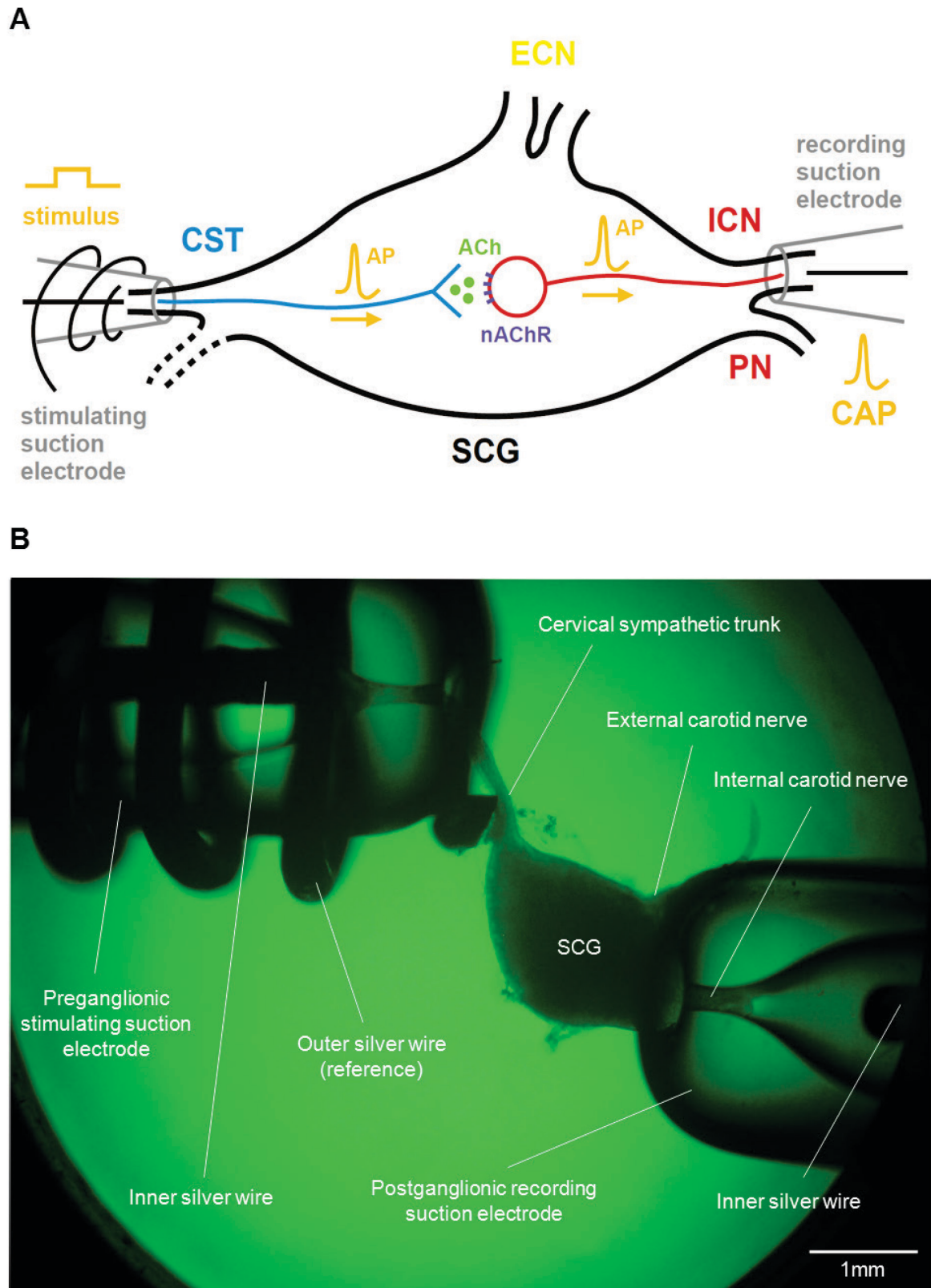


Figure 2.5: Recording a CAP. **A)** Schematic drawing of CAP generation within the mouse SCG. The dotted line represents a nerve trunk sometimes observed in mice (and rats: Bowers and Zigmond, 1979). **B)** View into the inverse microscope of the mouse SCG during a CAP recording. AP = action potential, CST = cervical sympathetic trunk (i.e. cervical sympathetic nerve), ECN = external carotid nerve, ICN = internal carotid nerve, PN = pterygopalatine nerve.

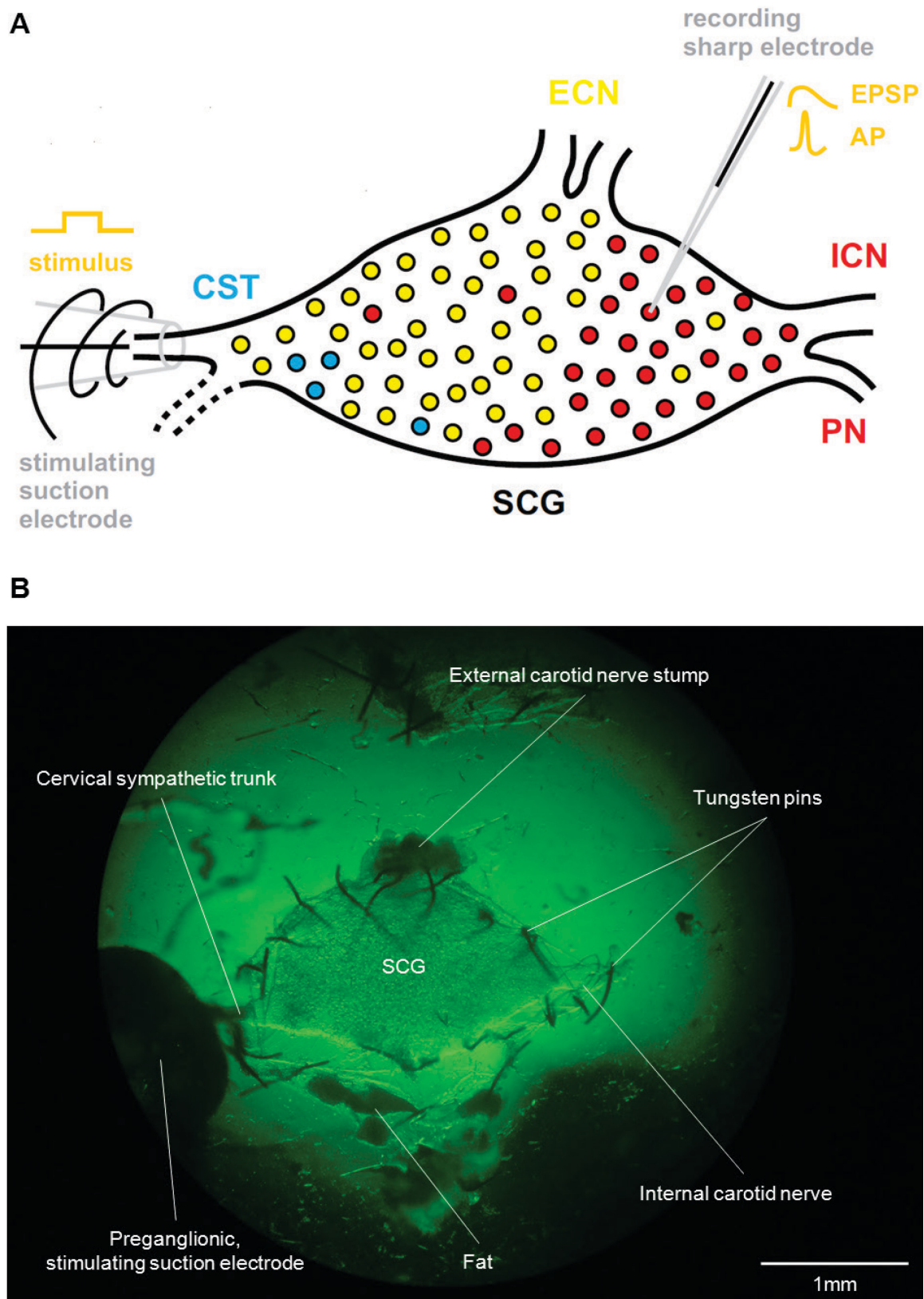


Figure 2.6: Recording an EPSP. **A)** Schematic drawing of EPSP recordings and of the localisation of neurons within the rat SCG. The dotted line represents a nerve trunk sometimes observed in mice (and rats: Bowers and Zigmond, 1979). **B)** View into the inverse microscope of the mouse SCG pinned down for EPSP recordings. CST = cervical sympathetic trunk (i.e. cervical sympathetic nerve), ECN = external carotid nerve, ICN = internal carotid nerve, PN = pterygopalatine nerve, SCG = superior cervical ganglion.

2. EPSP recordings

Since I used an inverted microscope, the positioning of the recording sharp electrode required some time. First, the focus of the microscope was set to the cervical sympathetic trunk sucked into the stimulating electrode. The tip of the sharp electrode was positioned at the same focus level (same horizontal plane) on the right side of the SCG. Then the sharp electrode was slightly retracted (up to $\sim 200\ \mu\text{M}$), in order to lie above the ganglion. Afterwards, the sharp electrode was shifted to the left until it was lying over the SCG (the tip could not be seen any more) and slowly advanced to the SCG. The aim was to record from cells lying at the rostral part of the SCG, since this is the localisation of neurons innervating the ICN (Bowers & Zigmond, 1979). As soon as the electrode resistance started increasing (indicating that the tip was nearly contacting the SCG), the approach was set to $20\ \mu\text{m}$ steps. A sudden depolarization to $-45/-65\ \text{mV}$ indicated the impalement of a cell. Only if the resting membrane potential (RMP) remained constantly between -45 and $-65\ \text{mV}$ for 10 min, this cell was used for an experiment.

For EPSP recordings ganglia were stimulated with current pulses (duration $50\ \mu\text{s}$, variable amplitude) at $0.33\ \text{Hz}$. The stimulus was set 100 ms after start of the recording. In order to monitor the stability of the membrane resistance, a depolarizing current pulse (duration 20 ms) was set 350 ms from sweep start (after membrane recovery from AP or EPSP to resting membrane potential). Stimulating parameters and recording protocols are reported in Table 2.3.

Similarly to CAP recordings, also for EPSP recordings I started with the smallest current amplitude that the stimulus isolator could produce and increased it until the first discernible neuronal response appeared on the screen. The first response could theoretically be either a subthreshold (weak) EPSP of varying amplitude, or a suprathreshold (strong) EPSP, that directly generated an AP (Li & Horn, 2006). To obtain comparable EPSP amplitudes between data sets, neurons were hyperpolarised to $-100\ \text{mV}$, a polarity at which no AP should be generated anymore, and even an underlying strong EPSP could be visualised (Fig. 2.7).

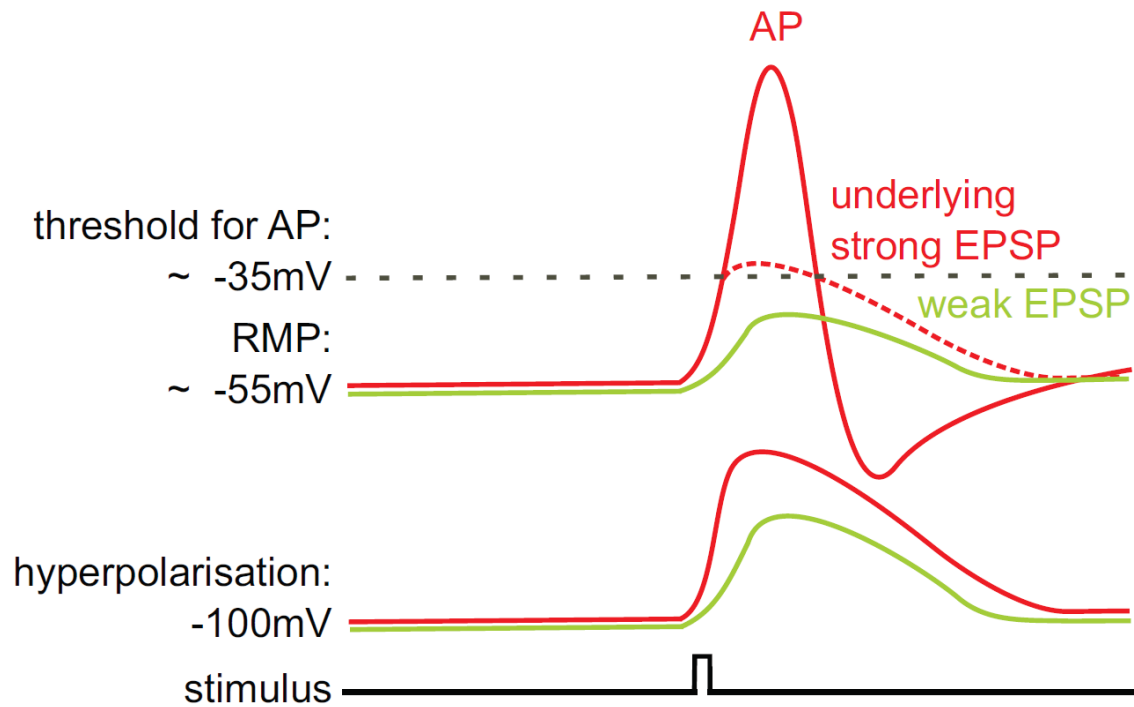


Figure 2.7: Hyperpolarisation method. To obtain comparable amplitudes of subthreshold (weak) and suprathreshold (strong) EPSPs, neurons were hyperpolarised down to -100 mV.

2.5 Data analysis

All data were recorded with Clampex 10.2 and analysed with Clampfit 10.2 (pClamp 10 electrophysiology data acquisition and analysis software, Molecular Devices, USA).

For determining CAP amplitudes, 30 traces were averaged and filtered with a lowpass (Gaussian, cutoff at 2000 Hz). CAP amplitudes were measured between a cursor set just before the onset of the stimulus artefact and a cursor set at the maximal peak.

For determining EPSP amplitudes, membrane potentials were hyperpolarised to -100 mV, 20 traces were averaged and filtered with a lowpass (Gaussian, cutoff at 2000 Hz). EPSP amplitudes were measured between a cursor set just before the onset of the stimulus artefact and a cursor set at the maximal peak. The slope of the EPSP decay was measured between a cursor set 4 ms after the maximal peak (corresponding to about 90% of the amplitude) and a cursor set around 350 ms from the beginning, just before the onset of the depolarising step necessary to monitor the membrane resistance.

The decay phases were fitted to a double exponential using a least-squares algorithm:

$$f(t) = \sum_{i=1}^n A_i e^{-t/\tau_i} + C$$

The fit solves for the amplitude A , the time constant τ , and the constant y-offset C for each component i . The fitting method used was Levenberg-Marquardt.

In general, neurons with a resting membrane potential of < -45 mV, as well as neurons with an overshoot of < 0 mV were excluded (Rimmer & Horn, 2010). All recordings were considered stable if the baseline did not move more than $\pm 3\%$.

Statistics and curve fittings were done with GraphPad Prism (version 6). Data were then exported into SigmaPlot (version 11) to draw plots. CAP and EPSP amplitudes were analysed using a one-way independent measures ANOVA followed by a Bonferroni's multiple comparison post-hoc test to assess the effects of genotypes and or treatment. For the CAP experiments with increasing pulse frequencies, data were analysed by a two-way repeated measures ANOVA (with pulse number as repeated factor) followed by a Bonferroni's multiple comparison post-hoc test. All p -values higher than 0.05 were considered as not significant (n.s. $p > 0.05$). In the figures, significant differences are marked with asterisks (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

Table 2.4: Chemicals

Substance	MW (g/mol)	Company	Order No.
Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	147.02	Fluka	21101
Glucose ($\text{C}_6\text{H}_{12}\text{O}_6$)	180.2	Merck	1.08337.1000
Hexamethonium bromide ($\text{C}_{12}\text{H}_{30}\text{Br}_2\text{N}_2$)	362.2	Sigma	H-0879
Hydrochloric acid, for analysis 37% (1.19) (HCl)	36.46	Merck	1.00317.2500
Magnesium chloride hexahydrate ($\text{Mg Cl}_2 \cdot 6\text{H}_2\text{O}$)	203.31	Fluka	63072
Nicotine ($\text{C}_{10}\text{H}_{14}\text{N}_2$)	162.2	Sigma	N-3876
PNU-120596 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl) urea ($\text{C}_{13}\text{H}_{14}\text{ClN}_3\text{O}_4$)	269.25	Tocris	2498
Potassium chloride (KCl)	74.55	Riedel-de-Haen	12636
Sodium bicarbonate (NaHCO_3)	84.01	Fluka	71.630
Sodium chloride (NaCl)	58.44	Merck	1.06404.1000
Sodium dihydrogen phosphate dihydrate (NaH_2PO_4)	137.99	Merck	1.06346.0500
SYLGARD® 184 Silicone Elastomer Kit 10:1	---	Dow Corning	---

Table 2.5: Material

	Product	Company	Order No.
For dissection	Moria MC26 Dowell Spring Scissors – 10 mm Blades	FST	15370-26
	Vannas-Tübingen Spring Scissors – 5 mm Blades	FST	15005-08
	Dumont #5 Forceps – Standard - 0.08 mm x 0.04 mm	FST	11251-10
	Dumont Forceps Repair Kit	FST	29000-00
	Tungsten Wire: diameter 0.025 mm	Good Fellow	W 005130
	Nunc Petri Dish: diameter × height 35 mm × 10 mm	Sigma	153066
	Disposable hypodermic needle 0.4 x 20 mm, 27G, Gr. 20	B. Braun	4657705
	Wild – Heerbrugg discussion zoom stereomicroscope, M8	Leica	---
Electrophysiology setup	Antivibration Table for Microscopy, Benchmate 2212	Kinetic Systems	---
	Diaphot 300 Phase Contrast Inverted Microscope	Nikon	---
	Digidata 1200 Interface Digitizer	Axon Instruments	---
	Electrometer MetaMetrics AK47 UU	---	---
	Faraday Cage	---	---
	Flexible Stimulus Isolator - Isoflex	A.M.P.I.	---
	Master-8 Pulse Stimulator	A.M.P.I.	---
	Single Electrode Amplifier SEC 05 LX	npi	---
	Storage Oscilloscope HM 205-3 (2)	HAMEG	---
For electrodes	Borosilicate glass pipettes with fire-polished ends with filament 0.86 x 1.5 x 80mm GB150F-8P	Science Products	---
	Flaming Brown Micropipette Puller P-97	Sutter Instruments	---
	Microelectrode Holder Str Male Wire 1.5 mm	WPI	MEH7W15
	Pencil Torch 1523	Central Pneumatic	---
	PipetteTip Microloader 0.5-20 µl	Eppendorf	5242956003
	Sterile Syringe Filter 0.20 µM, membrane diam. 25 mm	Corning Inc	431219
	XYZ Stage Micromanipulator SM LN 1	Luigs Neumann	---
Superfusion	Omnifix Syringe 5 ml LS	B. Braun	8502350N
	2120 Varioperpex II Peristaltic Pump (2)	LKB Bromma	---
	50 ml polypropylene Conical Tube 30 x 115 mm, Falcon™	Corning Inc	352070
	Silicone Tubing id 1 mm, od 2 mm	---	---

3. RESULTS AND DISCUSSION

PART I:

SCG MORPHOLOGY

3.1 Mouse age and connective tissue quality

Even though mice used for CAP recordings had all the same age (ranging from four to six weeks), their body weight fluctuated strongly. While all $\beta 4$ -containing genotypes weighed approximately 25g, $\beta 4$ -lacking genotypes weighed about 20.5g, which is a reduction of almost 20% (Tab. 3.1). The animal weight had no influence on the SCG size, usually ranging between 1.9-2.2 mm length and 1.4-1.6 mm width. The only difference between smaller and bigger mice consisted in the amount and composition of the connective tissue surrounding the SCG: in bigger mice the layers of connective tissue were thicker and more tightly packed. While this difference had no impact on the recording of CAPs, where the only attention was directed to a tight fit of the suction electrodes, EPSP recordings were affected by the texture of the connective tissue. Ganglia with a thicker and tighter connective tissue were easier to stably pin down, the most important prerequisite for good intracellular recordings. Additionally, tight and less elastic connective tissues were easier to be penetrated by sharp electrodes, which occluded less often. For this reason, for EPSP recordings, we decided to use $\alpha 5\beta 4$ KO mice aged 8-10 weeks, when their body weight and ganglionic connective tissue properties were comparable to the ones of WT and $\alpha 5\beta 2$ KO mice aged 4-6 weeks.

3.2 SCG shape

An additional morphological difference was observed concerning the shape of SCGs in mature animals. In fact, sometimes we found malformed SCGs characterised by a reduced ganglion body and an enlarged preganglionic nerve (Fig. 3.1). This enlargement was filled by neuronal cell bodies (visible with transmitted light) that apparently had not reached their end position. Ganglia with this malformation were found in all genotypes and at any age, but the incidence was 6-8-fold higher in $\beta 4$ -lacking genotypes.

Even though this study includes only data from normally shaped ganglia, it is worth mentioning that abnormally shaped ganglia (when technically recordable) gave comparable results to normally shaped SCGs concerning EPSP recordings.

Table 3.1: Mouse weight and incidence of abnormally shaped SCGs

	WT	a5b2KO	a5b4KO	a5a7b2KO	a5a7b4KO	a5KO	Hetero
N animals	56	52	51	18	18	8	10
Mean weight	25.8	24.7	20.9	24.8	20.1	24.9	22.0
N abnormal SCGs	1	1	8	0	2	0	0
% abnormal SCGs	1.8	1.9	15.7	0.0	11.1	0.0	0.0

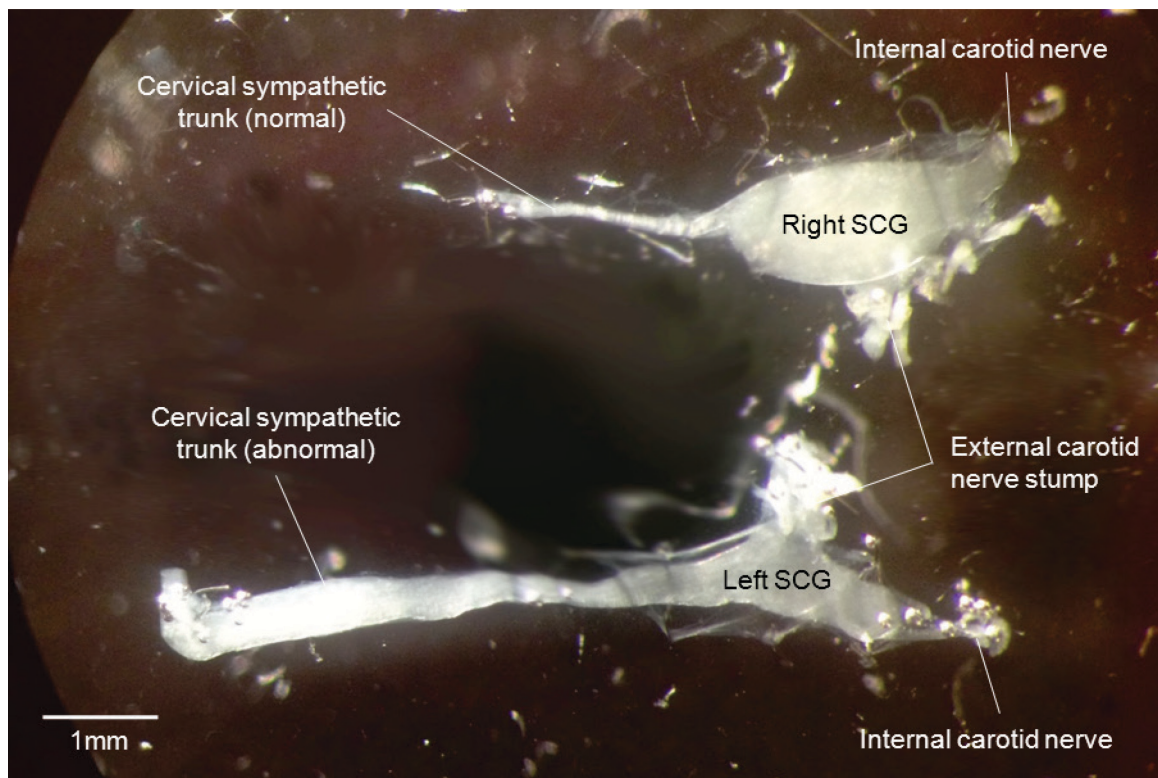


Figure 3.1: Picture of normal and abnormal SCGs of one WT mouse. The bottom SCG is abnormally shaped, being smaller and thinner than the normal (top) SCG. Its cervical sympathetic trunk is 5-10 times thicker than normally. The passage between nerve and ganglion body is gradual and hardly discernible.

PART II:

EXTRACELLULAR RECORDINGS OF THE CAP

3.3 Compound action potentials (CAPs)

The first set of experiments consisted in the recording of compound action potentials (CAPs) generated by the internal carotid nerve of the SCG. These CAP recordings were very stable and allowed experiments lasting for several hours. After drug application (e.g. HMB, Nic), CAPs could completely recover to their initial amplitudes.

CAPs reached their maximal amplitudes (ranging between 300 μ V and 600 μ V) 12-18 ms after the afferent nerve had been stimulated in all tested animals (Fig. 3.2).

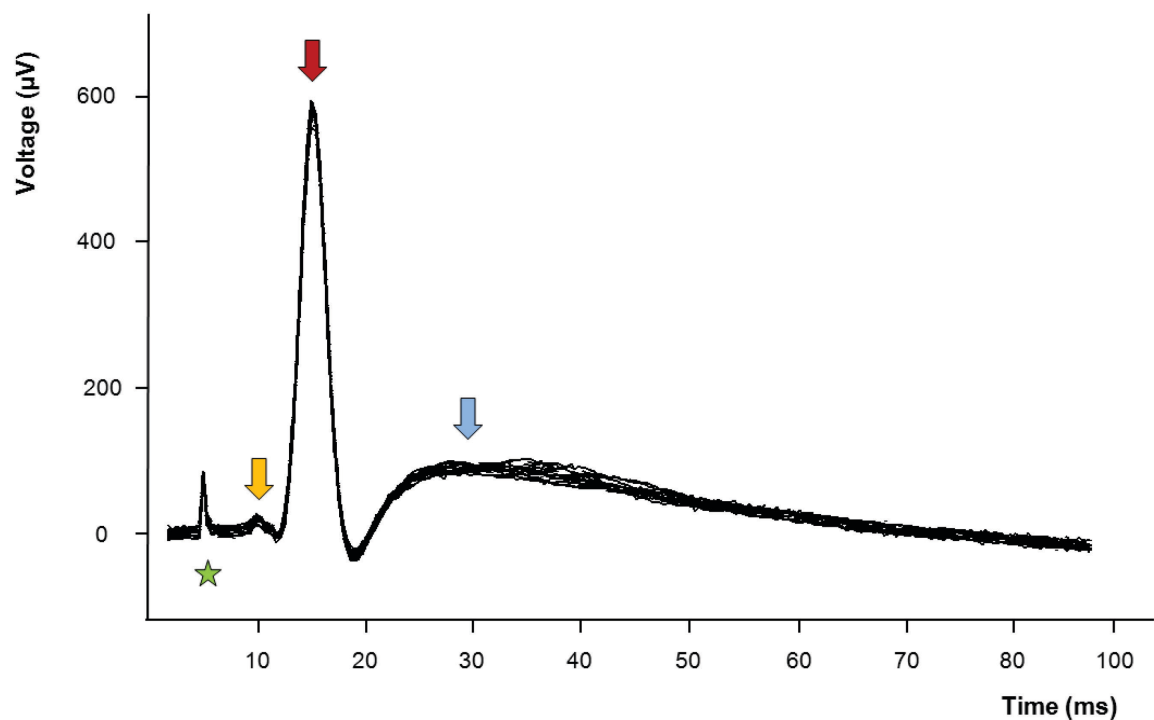


Figure 3.2: Typical recording of postganglionic compound action potentials (CAPs). CAPs (20 superimposed traces) were recorded from the internal carotid nerve of the SCG of a 5 week old WT-mouse. Electrical stimuli were given at supramaximal intensity at 0.5 Hz. The red arrow indicates the CAP, the green star the stimulus artefact. The yellow arrow points at presynaptic volley, whereas the blue arrow shows postsynaptic APs resulting from temporal summation (Modified from David et al, 2010).

The first signal in the records shows the stimulus artefact, followed by a small peak, the CAP itself, and finally a long lasting and slowly declining elevation (Fig. 3.2). The small peak probably represents the sum of preganglionic Aps. The CAP starts 2-3 ms thereafter, the delay being caused by the synaptic transmission. For comparison, synaptic delay in the rat SCG has been shown to range between 2-6 ms (Eccles, 1955, 1956; Skok & Heeroog, 1975).

Our explanation for the pre- and the postsynaptic deflection is supported by the fact that, when blocked by the ganglion blocker HMB, the long-lasting peak diminished in amplitude in parallel with the CAP, whereas the small presynaptic volley remained stable (Fig. 3.3). Instead, Li and Horn (2006) described the first peak as part of the postsynaptic CAP, since it was reversibly blocked by 100 μM Cd^{2+} (an antagonist of voltage-dependent Ca^{2+} channels) and by 10 μM dihydro- β -erythroidin (a nicotinic receptor antagonist).

As described in chapter 2.4.2, ganglia were stimulated with slowly increasing current pulses starting from the smallest stimulus possible. The threshold stimulus intensity was defined as the current at which the first CAP was just discernible, i.e. when the CAP reached 15-20 μV amplitude. By increasing the stimulus further, more and more axons (requiring stronger stimuli) were activated, and this resulted in CAPs with increasing amplitudes. The maximal CAP was reached when all available preganglionic axons were activated.

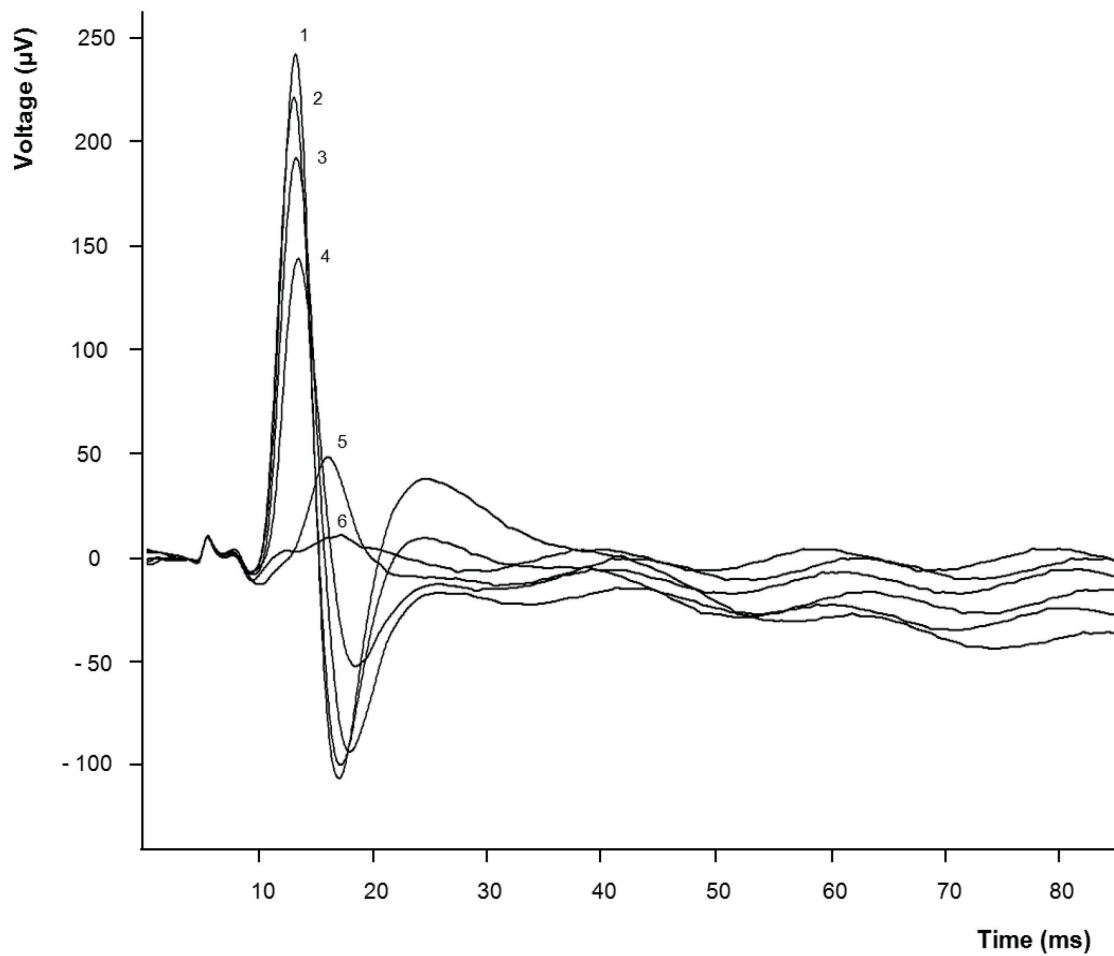


Figure 3.3: CAPs blocked by hexamethonium bromide (HMB). CAPs recorded from the internal carotid nerve of the SCG of a 6 week old WT-mouse were blocked with increasing concentrations of HMB. Electrical stimuli were given at supramaximal intensity at 0.5 Hz. Only six superimposed traces are shown. Notice how the peaks occurring after the CAP get blocked by HMB as the CAPs do, whereas the small preganglionic non-synaptic peaks remain stable. Trace 1: no treatment. Trace 2: +100 μ M HMB. Trace 3: + 200 μ M HMB. Trace 4: + 400 μ M HMB. Trace 5: + 800 μ M HMB. Trace 6: + 1.6 mM HMB.

3.4 Similar CAP amplitudes, different stimulus thresholds

The first three genotypes tested were WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO. In these genotypes, CAPs following supramaximal stimulation reached similar amplitudes around 335-365 μ V (Fig. 3.4, panel A; Tab. 3.2). On the contrary, threshold stimulus intensities necessary to elicit CAPs of 15-20 μ V amplitude differed between the genotypes, showing a significant increase of the mean stimulus threshold in $\alpha 5\beta 4$ KO (54.83 μ A), as compared to WT (31.17 μ A) and $\alpha 5\beta 2$ KO mice (31.06 μ A) (Fig. 3.4, panel B; Tab. 3.2). Stimuli necessary for supramaximal responses did not differ between genotypes ($F = 1.068$; $p = 0.3837$).

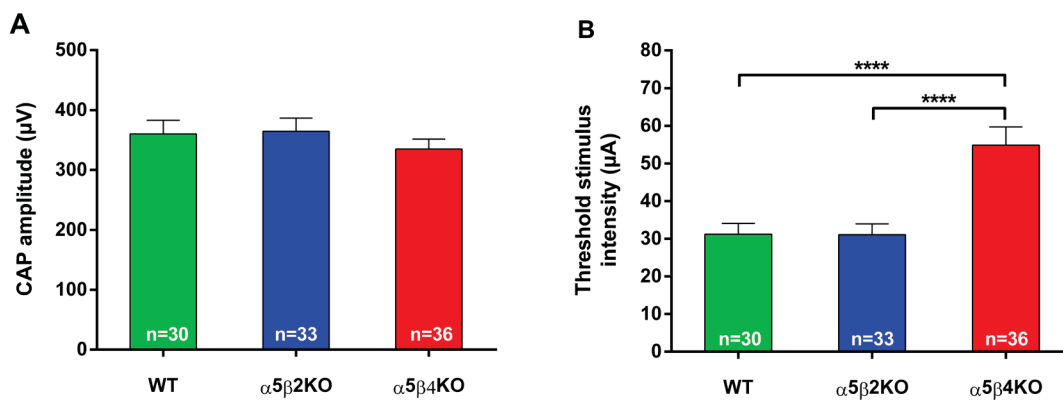


Figure 3.4: CAP amplitudes and threshold stimulus intensities of WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice.
A) CAPs following supramaximal stimulation did not differ between genotypes ($F = 0.75$; $p = 0.48$).
B) Threshold stimulus intensities necessary to elicit CAPs of 15-20 μ V amplitude were significantly higher in $\alpha 5\beta 4$ KO mice ($F = 13.46$; $\alpha 5\beta 4$ KO vs. WT: $p < 0.0001$; $\alpha 5\beta 4$ KO vs. $\alpha 5\beta 2$ KO: $p < 0.0001$). Data were compared using one way ANOVA, followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM.

Against our initial assumption, the maximal CAP amplitude was not reduced in $\alpha 5\beta 4$ KO mice, even though the overall number of nAChRs in this genotype (pure $\alpha 3\beta 2$ receptors) reaches only 15% of the receptors present in WT or $\alpha 5\beta 2$ KO mice (David *et al.*, 2010). This finding suggests that either the remaining 15% $\alpha 3\beta 2$ receptors are concentrated at normal levels at the postsynaptic site, or that synapses operate with such a high safety factor that, despite the considerable reduction, they still allow unimpaired trans-ganglionic transmission.

The first hypothesis could have been probed by the means of confocal fluorescence microscopy. Here we should be able to see a high ratio of fluorescence at synapses, compared to the fluorescence in the cytoplasm (and possibly to the fluorescence at

extrasynaptic membrane areas). However, due to the lack of proven subunit-specific (anti- $\alpha 3$) antibodies suitable for immunohistochemistry, I was unable to follow this approach.

The second hypothesis has been proposed before (see Gibbins et al., 1998): they explained that the quantum of released transmitter (i.e. the quantum size) is the limiting factor which determines the size of an EPSP (and thus the efficacy of synaptic transmission) while postganglionic nAChRs are present in surplus and are thus not saturated (see also Walmsley et al., 1998). Given such a high safety factor under normal conditions, even a reduced number of postsynaptic nAChRs (as might be the case in $\alpha 5\beta 4$ KO mice) could still be sufficient to facilitate normal transganglionic transmission.

However, compared with WT and $\alpha 5\beta 2$ KO mice, it was necessary to apply nearly twice the amplitude of electrical stimuli to reach threshold CAPs in ganglia from $\alpha 5\beta 4$ KO animals (Fig. 3.4, panel B). Thresholds for eliciting action potentials in nerves depend on the resistivity of the external tissues and a decrease of the thickness of the myelin sheaths (Hayami *et al.*, 2010), which may well be altered in this genotype. Differences in the stimulation threshold are e.g. utilized for distinguishing between different types of afferent fibers to the spinal cord (Chen & Sandkuhler, 2000). Alternatively, a (relatively) larger number of “weak” (sub-threshold) synapses might contribute to the generation of postsynaptic APs. By increasing the number of afferent volleys, some postganglionic neurons would thus reach the threshold for APs by spatial summation of synaptic inputs. Upon supramaximal stimulation, the same number of postganglionic neurons would become activated in $\alpha 5\beta 4$ KO as in the two other genotypes. However, as shown and discussed in Part III of my thesis, I found no evidence in support of the hypothesis that SCG neurons in $\alpha 5\beta 4$ KO have a higher number of “weak” synapses.

Table 3.2: Threshold stimulus strengths necessary to produce CAPs of 15-20 μ V amplitude and maximal CAP amplitudes elicited through 20% supramaximal stimulation in the SCG of different genotypes

	n	Threshold stimulus strength (μ A)	Maximal CAP amplitude (μ V)
WT	30	31.17 $\pm$ 2.92	360.30 $\pm$ 20.54
α5β2KO	33	31.06 $\pm$ 2.90	364.00 $\pm$ 19.38
α5β4KO	36	54.83 $\pm$ 4.86	334.90 $\pm$ 16.74
α5α7β2KO	15	28.53 $\pm$ 3.08	431.50 $\pm$ 45.77
α5α7β4KO	15	59.47 $\pm$ 7.46	408.80 $\pm$ 28.18
α5KO	14	29.57 $\pm$ 1.51	374.90 $\pm$ 17.09
Hetero	14	26.57 $\pm$ 3.92	463.30 $\pm$ 43.42
WT + HMB	10	26.30 $\pm$ 3.20	186.00 $\pm$ 13.56
α5β2KO + HMB	10	37.50 $\pm$ 3.64	183.60 $\pm$ 14.43
α5β4KO + HMB	10	53.40 $\pm$ 3.76	184.40 $\pm$ 21.93
WT + PNU	8	21.88 $\pm$ 1.20	378.90 $\pm$ 10.26
α5β2KO + PNU	8	21.63 $\pm$ 2.54	383.00 $\pm$ 34.53
α5β4KO + PNU	8	58.75 $\pm$ 3.99	375.40 $\pm$ 12.52
WT + NIC	13	28.92 $\pm$ 3.89	180.70 $\pm$ 9.75
α5β2KO + NIC	14	36.07 $\pm$ 5.90	184.90 $\pm$ 11.34
α5β4KO + NIC	15	52.20 $\pm$ 7.98	175.50 $\pm$ 13.61

Values are given as means $\pm$ SEM. HMB = hexamethonium-bromide, NIC = nicotine, PNU = PNU 120596. The concentrations of HMB and NIC are the doses that elicit half-maximal CAP amplitudes, as described in the chapters 3.3 and 3.5. PNU is added at a concentration of 10 μ M to all genotypes, see chapter 3.7.

3.5 The potency of hexamethonium differs between genotypes

Being aware of a previous publication reporting that the so called “ganglion blocker” hexamethonium bromide (HMB) in α 5KO mice strongly inhibited the bradycardia caused by stimulation of the vagal nerve (Wang et al., 2002a), we decided to investigate effects of HMB in a more quantitative manner. The cervical sympathetic trunk was stimulated with supramaximal intensity at 0.033 Hz, and CAPs were recorded in the absence and presence of varying concentrations of HMB (until CAP amplitudes reached a steady-state). As shown in Figure 3.5 and Table 3.4, the IC_{50} for HMB differed significantly between genotypes. α 3 β 2 receptors (in α 5 β 4KO mice) were most potently inhibited (IC_{50} : 22.1 μ M), followed by α 3 β 4 receptors (in α 5 β 2KO mice, IC_{50} : 126.7 μ M) and receptors in WT animals (consisting of α 3 β 4, α 3 β 4 α 5, and α 3 β 4 β 2 receptors: IC_{50} : 389 μ M). The inhibition of receptors in α 5 β 2KO mice was identical to the effects in α 5KO animals (IC_{50} : 119 μ M), suggesting that the presence of (about 25%) β 2-containing

receptors had no effect on the inhibition by HMB. Hence, the main difference in HMB potency between WT and $\alpha 5$ KO (or $\alpha 5\beta 2$ -double KO) is the absence or presence of the $\alpha 5$ subunit. According to my observations, HMB discriminates quite well between receptors with or without the $\alpha 5$ subunit. Hence, HMB at 35 μ M inhibits receptors in $\alpha 5\beta 2$ -double KO animals by 90%, whereas receptors in WT mice are inhibited by just 46%.

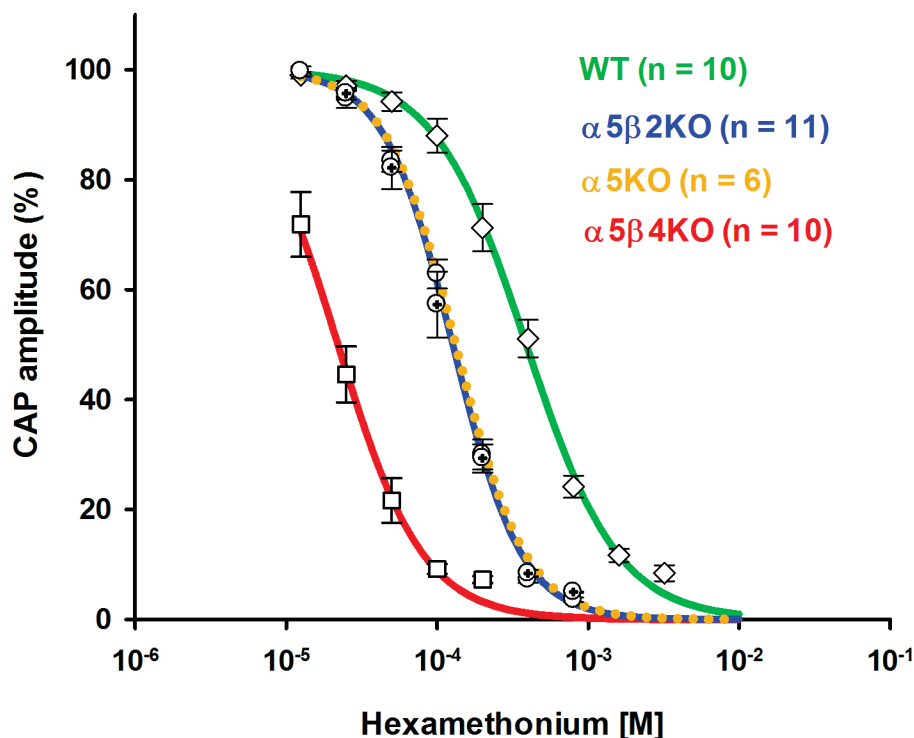


Figure 3.5: Transganglionic inhibition by HMB. Concentration-response curve for HMB in WT, $\alpha 5$ KO, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice. HMB had the highest potency in blocking receptors of $\alpha 5\beta 4$ KO ($IC_{50} = 22.1 \mu$ M), followed by $\alpha 5\beta 2$ KO / $\alpha 5$ KO ($IC_{50} = 126.7 \mu$ M / 119μ M, respectively) and WT ($IC_{50} = 389.0 \mu$ M). Values for Hill slopes were: $\alpha 5\beta 4$ KO = -1.543; $\alpha 5\beta 2$ KO = -1.907; $\alpha 5$ KO = -1.8; WT = -1.432. Statistical analysis is reported in Table 3.3. Data were compared by an extra sum-of-squares F test.

We considered the possibility that $\alpha 5$ -containing receptors might be concentrated at synaptic sites and thus probed effects of HMB in cell cultures of SCG neurons where receptors are likely randomly spread over the cell surface. Here, HMB inhibited $\alpha 3\beta 2$ receptors (in $\alpha 5\beta 4$ KO mice) with an IC_{50} of 0.61 μ M, $\alpha 3\beta 4$ (in $\alpha 5\beta 2$ KO mice) with an IC_{50} of 12.7 μ M, and receptors in WT animals with an IC_{50} of 35.1 μ M (Fig. 3.6). The significantly higher potency of HMB in inhibiting receptors in cultured neurons compared to intact ganglia (about a factor of 10 for receptors in WT and $\alpha 5\beta 2$ KO mice) probably

results from the known use-dependent effect of the inhibitor: While currents in cultured cells were induced by 5-second pulses of ACh, the transmitter is assumed to have a one and only chance to bind to receptors upon release from presynaptic boutons (see Walmsley 1993; Walmsley *et al.* 1998). Interestingly, the potency ratio of HMB to inhibit receptors in WT and $\alpha 5\beta 2$ KO mice is quite similar in intact ganglia (3.0) and cell cultures (2.7).

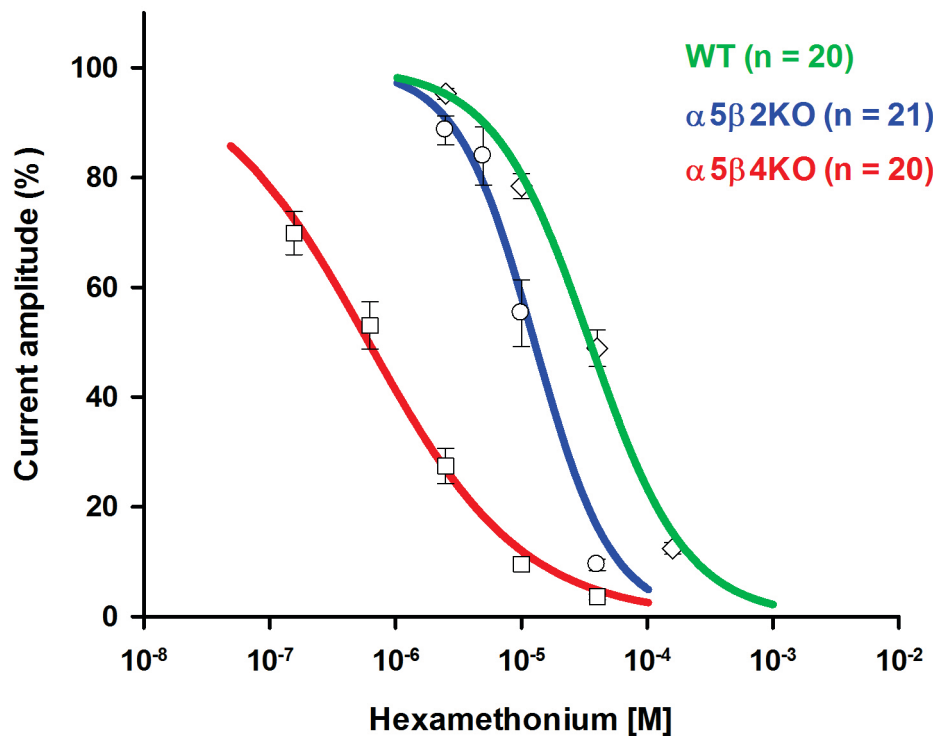


Figure 3.6: Inhibition by HMB in cell culture. Concentration-response curve for HMB in cultured SCGs of WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice. HMB had the highest potency in blocking receptors of $\alpha 5\beta 4$ KO ($IC_{50} = 0.61 \mu M$), followed by $\alpha 5\beta 2$ KO ($IC_{50} = 12.7 \mu M$) and WT ($IC_{50} = 35.1 \mu M$). Values for Hill slopes were: $\alpha 5\beta 4$ KO = -0.71; WT = -1.13; $\alpha 5\beta 2$ KO = -1.42. The number of cells was 23 for $\alpha 5\beta 4$ KO, 13 for $\alpha 5\beta 2$ KO, and 20 for WT. Patch-clamp experiments were performed by Anna Ciuraszkiewicz.

3.5.1. $\alpha 5$ -containing receptors are more concentrated on the cell surface than predicted from IP experiments

According to our previous IP experiments, nAChRs in the WT mouse SCG consist of about 50% $\alpha 3\beta 4$, 25% $\alpha 3\beta 4\alpha 5$, and 25% $\alpha 3\beta 4\beta 2$ receptors (see David et al., 2010; Fig. 1.10). Assuming that HMB inhibits $\alpha 3\beta 4\beta 2$ similar to $\alpha 3\beta 4$ receptors, the inhibition curve of HMB in WT animals reflects a mixture of effects of 75% of receptors with $\alpha 3\beta 4$ properties, and 25% of receptors with $\alpha 3\beta 4\alpha 5$ characteristics. This raises the question of

how the inhibition curve of HMB would look like if all receptors were of the $\alpha 3\beta 4\alpha 5$ type.

We addressed this issue by calculating models of concentration-response curves with higher IC_{50} values and varying Hill coefficients, first for cultured SCG neurons, second for transganglionic transmission. Upon weighing the impact of $\alpha 5$ -containing receptors, we then merged the measured inhibition curve for $\alpha 3\beta 4$ receptors and the model curve for $\alpha 3\beta 4\alpha 5$ receptors. The fused curve should thereafter closely match the results observed in WT animals. Fig. 3.7 shows an example, where inhibition of “pure” $\alpha 3\beta 4\alpha 5$ receptors was modelled by shifting the HMB IC_{50} for $\alpha 3\beta 4$ from 12.7 to 60 μM . The merged curve was thereafter obtained by fusing the $\alpha 3\beta 4$ curve with the model curve at a 1:2 ratio.

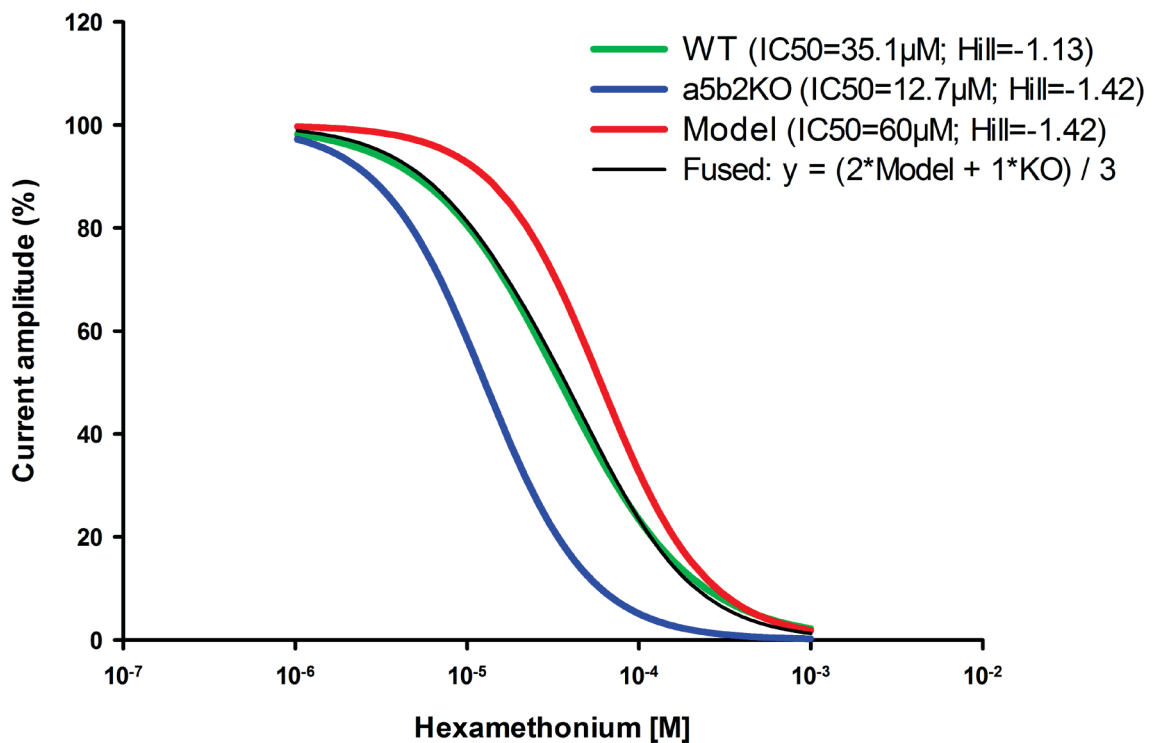


Figure 3.7: Model for the distribution of nAChRs on the cell surface (2:1 ratio). Inhibition curves by HMB. Blue line: curve fitted to experimental data obtained by inhibiting currents induced by 300 μM ACh in cultured SCG neurons from $\alpha 5\beta 2$ KO mice (data taken from Fig. 3.6). Green line: curve fitted to experimental data obtained by inhibiting currents induced by 300 μM ACh in cultured SCG neurons from WT mice (data taken from Fig. 3.6). Red line: Model curve obtained by shifting the HMB IC_{50} for $\alpha 3\beta 4$ receptors (12.7 μM) to 60 μM . Black line: y values of the (experimental) blue curve added to two times the y values of the red model curve, all at the respective HMB concentrations. Results were divided by 3 to construct this fused black curve. Note that this model assumes that $\alpha 3\beta 4\alpha 5$ receptors outnumber $\alpha 3\beta 4$ receptors by a 2:1 ratio.

A quite satisfactory congruency was also obtained by constructing a hypothetical inhibition curve with the parameters $IC_{50} = 90 \mu M$ and Hill coefficient = -1.5 and by merging this curve with the experimental $\alpha 3\beta 4$ inhibition curve at a 1:1 ratio (Fig. 3.8). However, a complete overlay of such a merged curve with my experimental data resulted when shifting the $\alpha 3\beta 4$ inhibition curve with an IC_{50} of $12.7 \mu M$ to an IC_{50} of $45 \mu M$ and by slightly reducing the slope of the hypothetical curve from 1.27 to 1.2. The merged curve was thereafter obtained by fusing the $\alpha 3\beta 4$ curve with the hypothetical $\alpha 3\beta 4\alpha 5$ model curve at a 1:4 ratio (Fig. 3.9). Hence, under this model, $\alpha 3\beta 4\alpha 5$ receptors outnumber $\alpha 3\beta 4$ receptors in the SCG synapse by a factor of 4, reverse to the distribution found in our IP experiments.

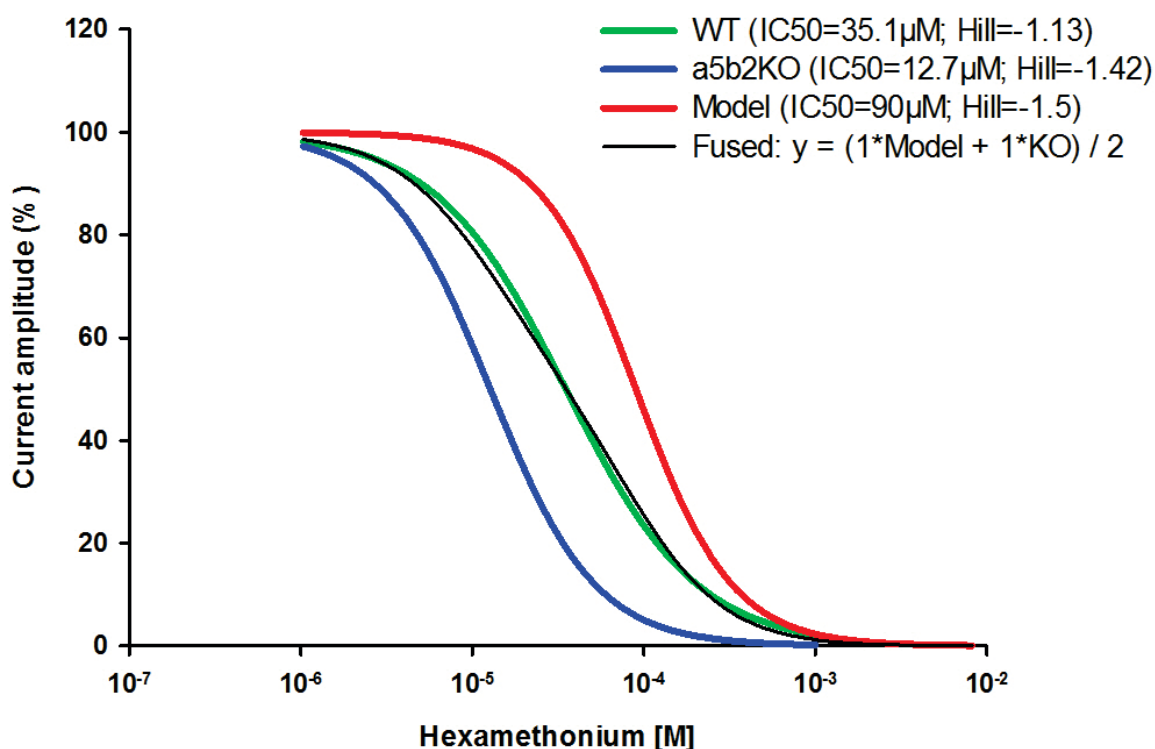


Figure 3.8: Model for the distribution of nAChRs on the cell surface (1:1 ratio). Inhibition curves by HMB. Blue line: curve fitted to experimental data obtained by inhibiting currents induced by $300 \mu M$ ACh in cultured SCG neurons from $\alpha 5\beta 2$ KO mice (data taken from Fig. 3.6). Green line: curve fitted to experimental data obtained by inhibiting currents induced by $300 \mu M$ ACh in cultured SCG neurons from WT mice (data taken from Fig. 3.6). Red line: Model curve obtained by shifting the HMB IC_{50} for $\alpha 3\beta 4$ receptors ($12.7 \mu M$) to $90 \mu M$ and by increasing the Hill coefficient from -1.42 to -1.5. Black line: y values of the (experimental) blue curve added 1:1 to the y values of the red model curve, all at the respective HMB concentrations. Results were divided by 2 to construct this merged curve. Note that this model assumes that $\alpha 3\beta 4\alpha 5$ receptors match $\alpha 3\beta 4$ receptors by a 1:1 ratio.

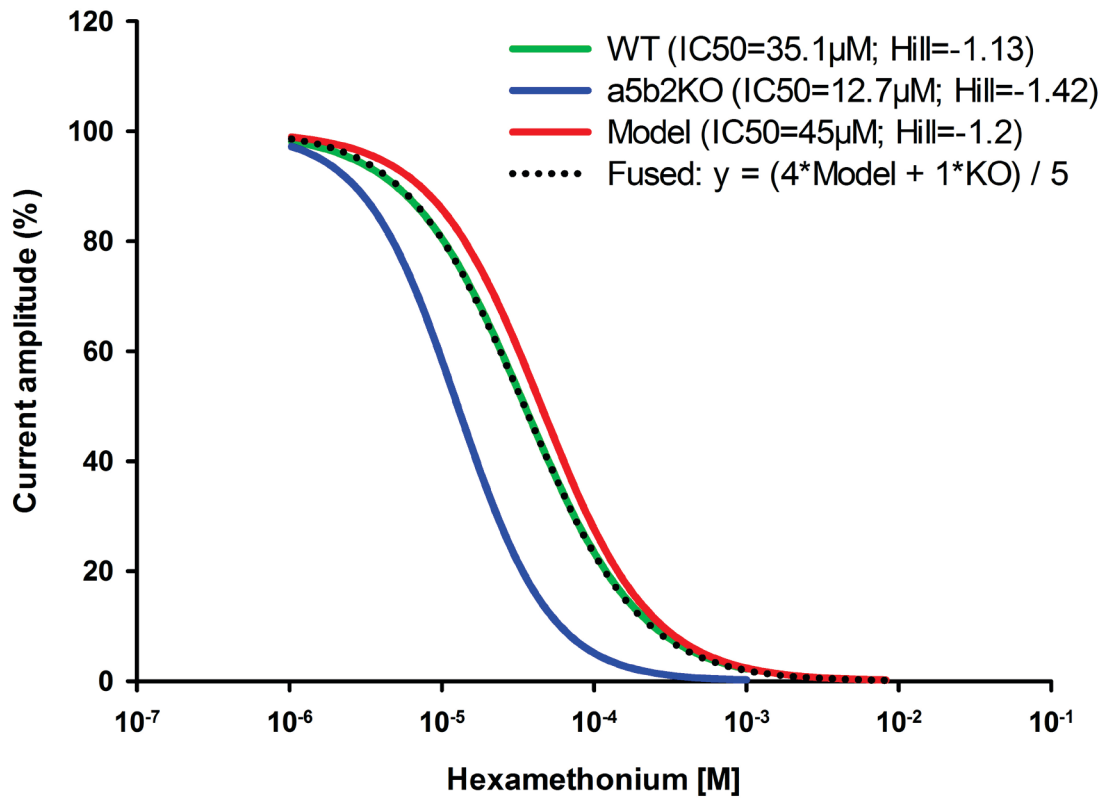


Figure 3.9: Model for the distribution of nAChRs on the cell surface (4:1 ratio). Inhibition curves by HMB. Blue line: curve fitted to experimental data obtained by inhibiting currents induced by 300 μ M ACh in cultured SCG neurons from $\alpha 5\beta 2$ KO mice (data taken from Fig. 3.6). Green line: curve fitted to experimental data obtained by inhibiting currents induced by 300 μ M ACh in cultured SCG neurons from WT mice (data taken from Fig. 3.6). Red line: Model curve obtained by shifting the HMB IC_{50} for $\alpha 3\beta 4$ receptors (12.7 μ M) to 45 μ M and by slightly reducing the Hill coefficient from -1.42 to -1.2. Black dotted curve (complete overlay): y values of the (experimental) blue curve added to four times the y values of the red model curve, all at the respective HMB concentrations. Results were divided by 5 to construct this merged curve. Note that this model assumes that $\alpha 3\beta 4\alpha 5$ receptors outnumber $\alpha 3\beta 4$ receptors by a 4:1 ratio.

In no case we obtained satisfactory congruency of curves when assuming that $\alpha 3\beta 4$ receptors exceed $\alpha 3\beta 4\alpha 5$ receptors by a ratio of 3:1 (75% $\alpha 3\beta 4$ receptors, 25% $\alpha 3\beta 4\alpha 5$ receptors). Fig. 3.10 shows two examples where HMB IC_{50} for $\alpha 3\beta 4$ was shifted from 12.7 μ M to 200 μ M, and with Hill coefficients either increased (to -2) or decreased (to -1). From these model curves we conclude that the percentage of $\alpha 3\beta 4\alpha 5$ receptors on the neuronal cell surface is higher than expected from our IP experiments.

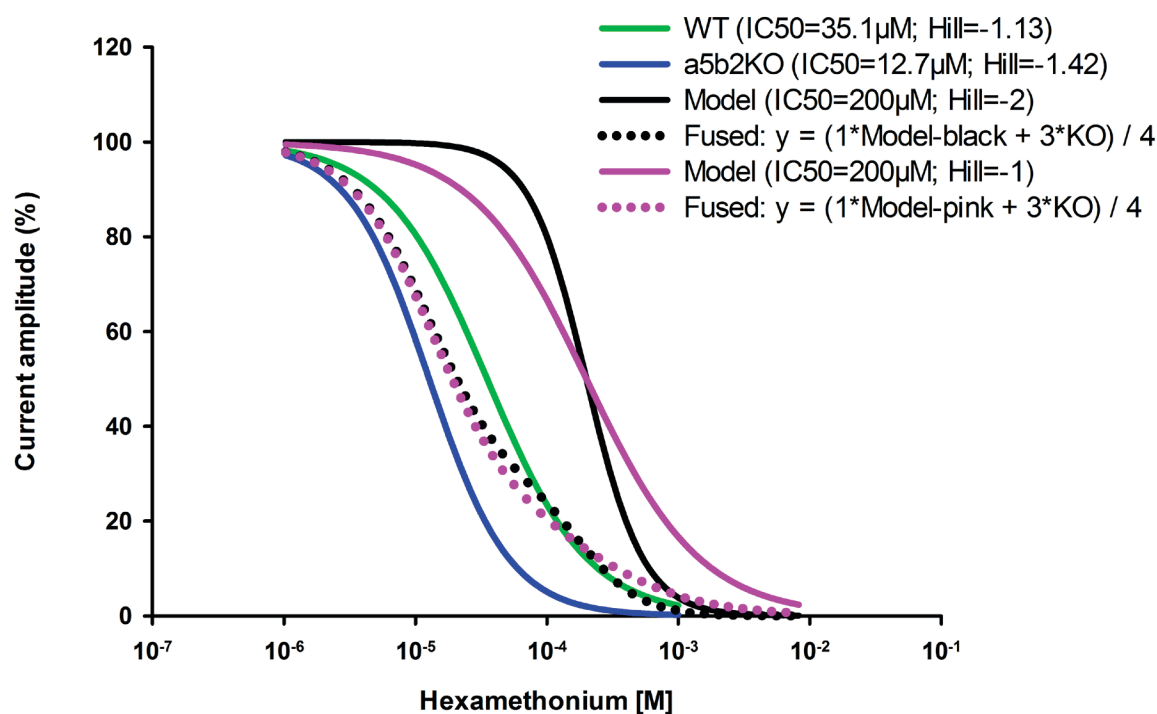


Figure 3.10 Model for the distribution of nAChRs on the cell surface (3:1 ratio). Inhibition curves by HMB. Blue line: curve fitted to experimental data obtained by inhibiting currents induced by 300 μ M ACh in cultured SCG neurons from $\alpha 5\beta 2$ KO mice (data taken from Fig. 3.6). Green line: curve fitted to experimental data obtained by inhibiting currents induced by 300 μ M ACh in cultured SCG neurons from WT mice (data taken from Fig. 3.6). Pink line: Model curve obtained by reducing the Hill coefficient from -1.42 to -1 and by shifting the HMB IC_{50} for $\alpha 3\beta 4$ receptors (12.7 μ M) to 200 μ M. Dotted pink line: Three times the y values of the (experimental) blue curve added to one times the y values of the pink model curve, all at the respective HMB concentrations. Results were divided by 4 to construct this merged curve. Black line: Model curve obtained by increasing the Hill coefficient from -1.42 to -2 and by shifting the HMB IC_{50} for $\alpha 3\beta 4$ receptors (12.7 μ M) to 200 μ M. Dotted black line: Three times the y values of the (experimental) blue curve added to one times the y values of the black model curve, all at the respective HMB concentrations. Results were divided by 4 to construct this merged curve. Note that these models assume that $\alpha 3\beta 4$ receptors outnumber $\alpha 3\beta 4\alpha 5$ receptors by a 3:1 ratio.

3.5.2. $\alpha 5$ -containing receptors are more concentrated in SCG synapses than predicted from IP experiments

We followed up on this issue by constructing models for HMB effects on intact ganglia. Satisfactory results were obtained by the following assumptions: “pure” $\alpha 3\beta 4\alpha 5$ receptors might be inhibited by HMB with IC_{50} of 670.9 μ M (5.3 times the IC_{50} for pure $\alpha 3\beta 4$ receptors) and a Hill coefficient of -2 (was -1.91 for $\alpha 3\beta 4$ receptors). A merged curve was thereafter obtained by fusing the $\alpha 3\beta 4$ curve with the hypothetical $\alpha 3\beta 4\alpha 5$ model curve at a 1:2 ratio (Fig. 3.11).

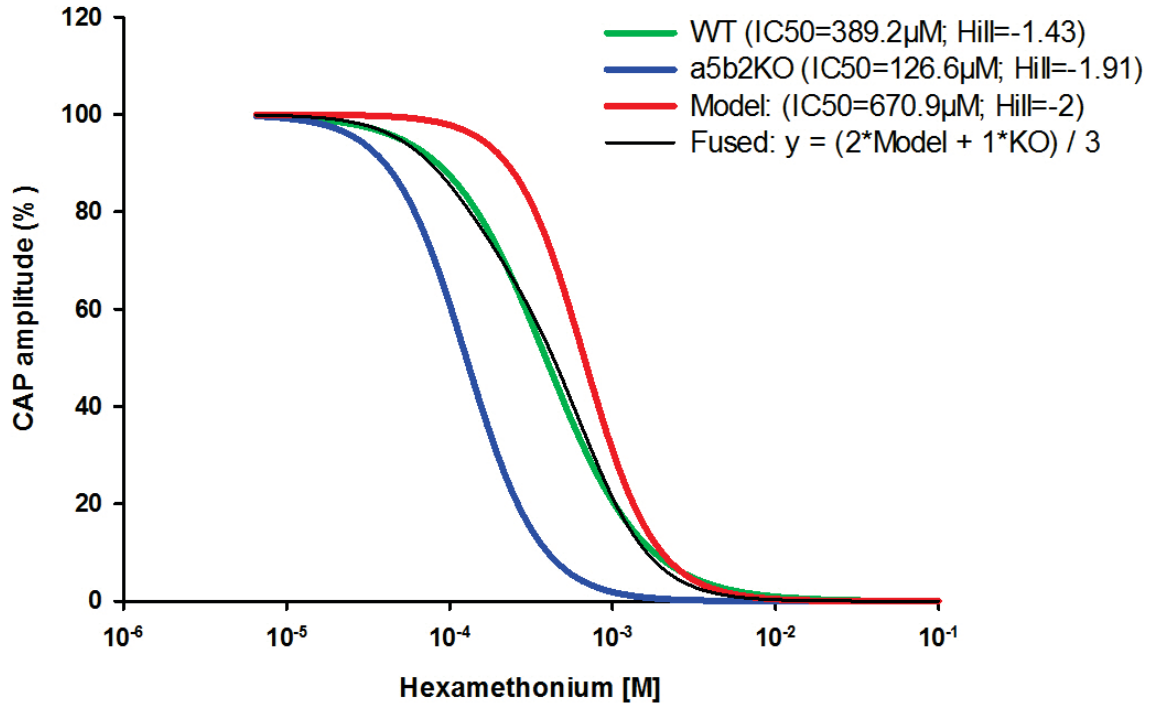


Figure 3.11: Model for the distribution of nAChRs in synapses (2:1 ratio). Inhibition curves by HMB. Blue line: curve fitted to experimental data obtained by inhibiting CAPs in SCGs from $\alpha 5\beta 2$ KO mice. Green line: curve fitted to experimental data obtained by inhibiting CAPs in SCGs from WT mice (data taken from Fig. 3.5). Red line: Model curve obtained by shifting the HMB IC_{50} for $\alpha 3\beta 4$ receptors ($126.6\mu M$) to $670.9\mu M$ and by increasing the Hill coefficient from -1.91 to -2. Black line: y values of the (experimental) blue curve added to two times the y values of the red model curve, all at the respective HMB concentrations. Results were divided by 3 to construct this merged curve. Note that this model assumes that $\alpha 3\beta 4\alpha 5$ receptors outnumber $\alpha 3\beta 4$ receptors by a 2:1 ratio.

However, a complete overlay of such a merged curve with my experimental data resulted when shifting the $\alpha 3\beta 4$ inhibition curve with an IC_{50} of $126.6\mu M$ to an IC_{50} of $600\mu M$ and by reducing the slope of the hypothetical curve to -1.6. The merged curve was thereafter obtained by fusing the $\alpha 3\beta 4$ curve with the hypothetical $\alpha 3\beta 4\alpha 5$ model curve at a 1:4-ratio (Fig. 3.12). Hence, under this model, $\alpha 3\beta 4\alpha 5$ receptors outnumber $\alpha 3\beta 4$ receptors in the SCG synapse by a factor of 4, reverse to the distribution found in our IP experiments.

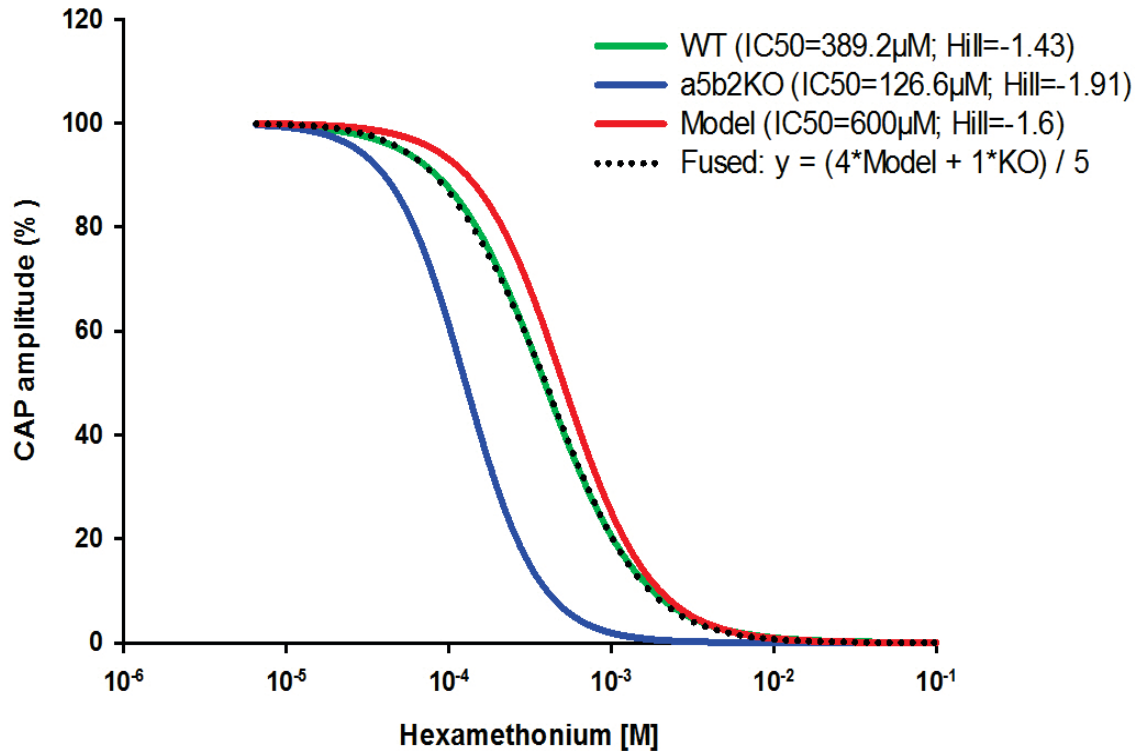


Figure 3.12: Model for the distribution of nAChRs in synapses (4:1 ratio). Inhibition curves by HMB. Blue line: curve fitted to experimental data obtained by inhibiting CAPs in SCGs from $\alpha 5\beta 2$ KO mice. Green line: curve fitted to experimental data obtained by inhibiting CAPs in SCGs from WT mice (data taken from Fig. 3.5). Red line: Model curve obtained by shifting the HMB IC₅₀ for $\alpha 3\beta 4$ receptors (126.6 μM) to 600 μM and by reducing the Hill coefficient from -1.91 to -1.6. Black dotted line (complete overlay): y values of the (experimental) blue curve added to four times the y values of the red model curve, all at the respective HMB concentrations. Results were divided by 5 to construct this merged curve. Note that this model assumes that $\alpha 3\beta 4\alpha 5$ receptors outnumber $\alpha 3\beta 4$ receptors by a 4:1 ratio.

$\alpha 3\beta 2$ receptors (investigated in $\alpha 5\beta 4$ KO mice) were significantly more sensitive to inhibition by HMB than receptors in both WT and $\alpha 5\beta 2$ KO animals (HMB IC₅₀ in cell cultures of the SCG: 0.91 μM; IC₅₀ for inhibiting CAPs: 22.1 μM). $\alpha 3\beta 2$ receptors normally do not occur in WT animals (David et al., 2010) but could play a role in some regions of the CNS (Young *et al.* 2008; see Gotti et al. 2006 for review).

Previous researchers were particularly interested in resolving the mode of action of hexamethonium (and other methonium compounds). In a series of classical experiments, hexamethonium was found to inhibit nAChRs in a use-dependent, non-competitive manner by blocking the receptor channel, e.g. (Ascher *et al.*, 1979; Rang *et al.*, 1982; Gurney & Rang, 1984). Accordingly, hexamethonium primarily reduced channel open times of $\alpha 3\beta 2$, $\alpha 3\beta 4$, and $\alpha 3\beta 4\alpha 5$ receptors expressed in *Xenopus laevis* oocytes (Nelson & Lindstrom, 1999). Given this intricate mechanism, the effects of hexamethonium depend on the frequency at which synapses are activated, on the duration of agonist

application and the membrane potential of the cells that carry the receptors. Hence, we found only few publications for comparison with our results. When expressed in *Xenopus laevis* oocytes (Papke *et al.*, 2010), peak currents induced by ACh at mouse $\alpha 3\beta 4$ receptors were inhibited with an IC_{50} of 15.6 μM , similar to our cell culture experiments (IC_{50} : 12.7 μM). However, contrary to our observations, $\alpha 3\beta 4\alpha 5$ receptors expressed in oocytes were more sensitive to hexamethonium (IC_{50} : 3.5 μM). Likewise, peak current for $\alpha 3\beta 2$ receptors were rather insensitive to hexamethonium (IC_{50} : 221 μM), whereas these receptors were extremely sensitive when probed in our $\alpha 5\beta 4KO$ mice (IC_{50} : 0.61 μM). Inokuchi and McLachlan (1995) reported that hexamethonium EC_{50} values for subthreshold and suprathreshold EPSPs recorded in guinea pig ganglia at 1.25 Hz were 3 μM and 60 μM , respectively. I found IC_{50} values for CAP inhibition of 126 μM (in $\alpha 5\beta 2KO$ mice) and 389 μM (in WT animals), though these data cannot be compared directly.

Interestingly, adding HMB at the respective IC_{50} concentrations for each genotype had no major effect on threshold stimuli (Fig. 3.13, Tab. 3.2). Hence, WT ganglia superfused with 400 μM HMB required similar threshold stimuli (26.30 μA) as non-treated WT (31.17 μA), though thresholds were moderately increased in $\alpha 5\beta 2KO$ ganglia treated with 120 μM HMB (37.50 μA vs 31.06 μA) and $\alpha 5\beta 4KO$ treated with 25 μM HMB (53.40 μA vs 54.83 μA). These observations suggest that synapses made by afferent fibres with the lowest stimulus threshold are also least affected by HMB. As for the IC_{50} values of HMB, $\alpha 5$ single and $\alpha 5\beta 2$ double-KO did not differ significantly regarding the threshold stimulus required for the detection of CAPs (Fig. 3.13).

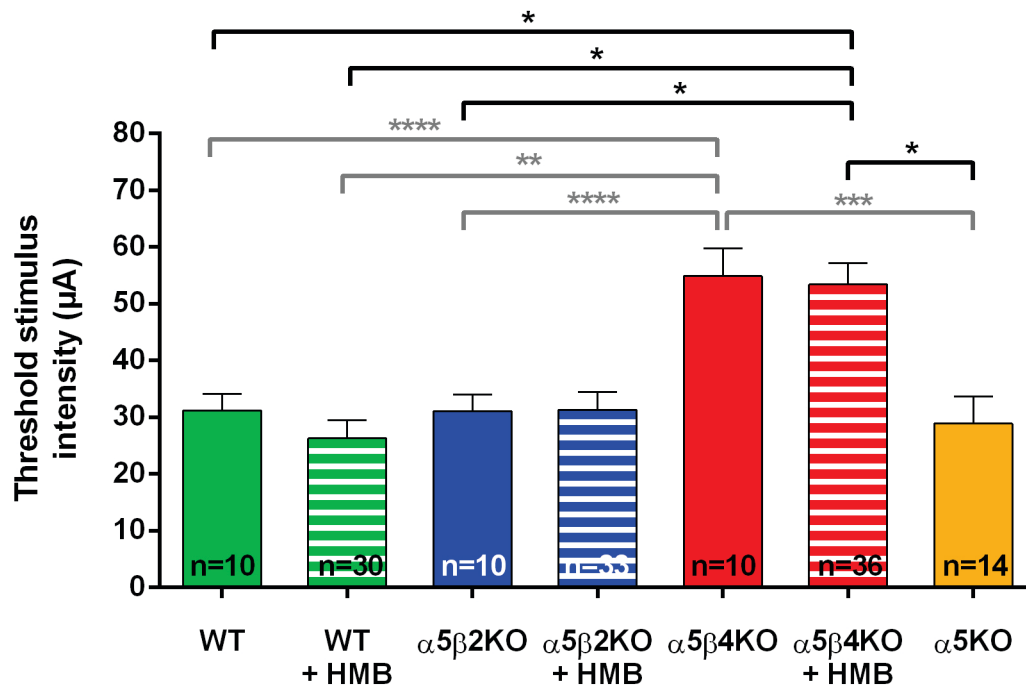


Figure 3.13: Extension of Figure 3.4 B. Threshold stimulus intensities necessary to elicit CAPs of 15-20 μ V amplitude in WT, $\alpha 5\beta 2$ KO, $\alpha 5\beta 4$ KO and single $\alpha 5$ KO mice when hexamethonium bromide at the respective IC_{50} -concentrations (HMB 400 μ M for WT; HMB 120 μ M for $\alpha 5\beta 2$ KO; HMB 25 μ M for $\alpha 5\beta 4$ KO) continuously superfused the SCGs. As for untreated $\alpha 5\beta 4$ KO mice, threshold stimuli of $\alpha 5\beta 4$ KO treated with HMB were significantly higher than in the other genotypes treated or not with HMB. No significant difference was found between HMB-treated $\alpha 5\beta 4$ KO and HMB-treated $\alpha 5\beta 2$ KO. Data were compared using one way ANOVA, $F = 8.243$ ($\alpha 5\beta 4$ KO+HMB vs. WT+HMB: $p < 0.05$; $\alpha 5\beta 4$ KO+HMB vs. $\alpha 5\beta 2$ KO: $p < 0.05$; $\alpha 5\beta 4$ KO+HMB vs. $\alpha 5$ KO: $p < 0.05$), followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM.

3.5.3. No $\alpha 3\beta 2$ receptors in heterozygote mice

We additionally know from our IP experiments that the overall number of receptors analysed in heterozygote mice bred from $\alpha 5\beta 4$ KO and WT mice is significantly larger than in $\alpha 5\beta 4$ KO mice but smaller than in WT animals (Fig. 3.14, panel B; experiments were performed by Florian Grössl). It was thus of interest to see whether any $\alpha 3\beta 2$ receptor might be expressed in these heterozygotes. However, the inhibition of CAPs by HMB was identical between WT and heterozygotes (similar IC_{50} and Hill slopes), indicating that all receptors expressed in this genotype were identical to WT mice (Fig. 3.15, Tab. 3.3). Although in heterozygote mice the lack of $\beta 4$ reduced the number of overall nicotinic receptors, it seemed that no significant numbers of $\alpha 3\beta 2$ receptors reached the synapse. Accordingly, we found no significant difference concerning the threshold stimulus intensity between WT (31.17 μ A) and heterozygote mice (26.57 μ A) (Fig. 3.14, panel A).

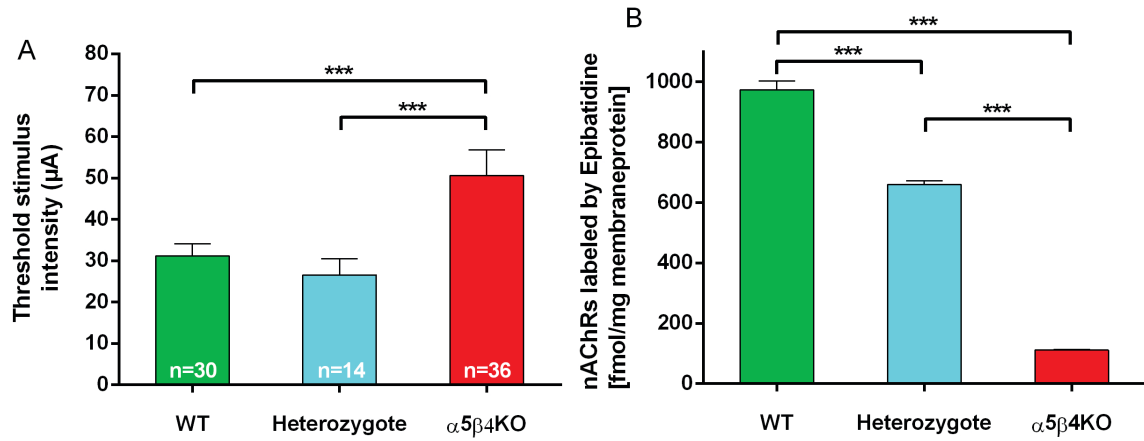


Figure 3.14: Threshold stimuli and binding to nAChRs in WT, heterozygote WT $\alpha 5\beta 4$ KO and $\alpha 5\beta 4$ KO mice. **A)** Threshold stimulus intensities necessary to elicit CAPs of 15-20 μV amplitude were significantly higher in $\alpha 5\beta 4$ KO mice. Data were compared using one way ANOVA, $F = 12.25$ ($\alpha 5\beta 4$ KO vs. WT: $p < 0.001$; $\alpha 5\beta 4$ KO vs. heterozygote: $p < 0.001$), followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM. **B)** Amount of nAChRs labelled by [3H]-epibatidine. Epibatidine binding sites are significantly reduced both in $\alpha 5\beta 4$ KO heterozygote WT $\alpha 5\beta 4$ KO and $\alpha 5\beta 4$ KO mice. Data were compared using one way ANOVA, $F = 574.4$ ($\alpha 5\beta 4$ KO vs. WT: $p < 0.001$; $\alpha 5\beta 4$ KO vs. heterozygote: $p < 0.001$, WT vs. heterozygote: $p < 0.001$), followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM of 6 independent experiments, each performed in triplicate measurements. Epibatidine binding experiments were performed by Florian Grössl.

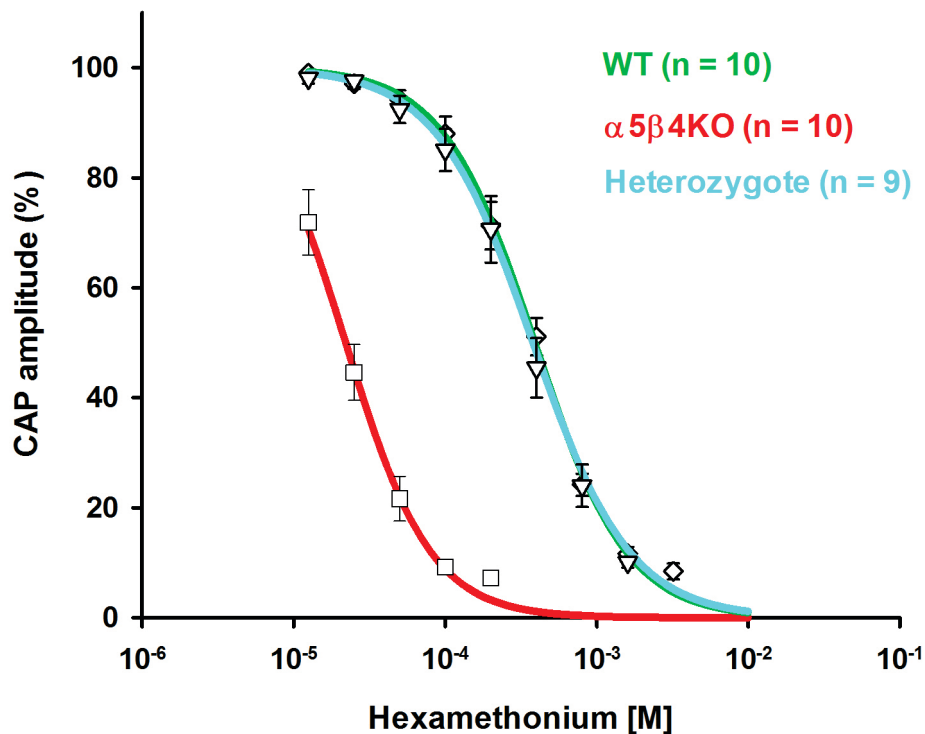


Figure 3.15: Dose-response curve for HMB in WT, $\alpha 5\beta$ KO and heterozygote WT x $\alpha 5\beta 4$ KO mice. The heterozygote genotype ($IC_{50} = 3.54 \times 10^{-4} M$, Hill slope = -1.407), reacted in the same way as the WT ($IC_{50} = 3.89 \times 10^{-4} M$, Hill slope = -1.432) and was significantly different from the homozygote $\alpha 5\beta 4$ KO ($IC_{50} = 2.21 \times 10^{-5} M$, Hill slope = -1.543). Statistical analysis is reported in Table 3.4. Data were compared by an extra sum-of-squares F test.

3.6 Desensitisation potency of nicotine differs between genotypes

Upon activation by an agonist, nicotinic receptors in the continued presence of the agonist may enter a state where channels are not conductive any more (“classical” desensitisation, Giniatullin *et al.* 2005; see Fig. 1.9). The non-conductive state may also be reached without preceding activation if an agonist is present at low concentration for a prolonged period of time (“high affinity” desensitisation, Giniatullin *et al.* 2005). A third state may also exist, which differs from slow desensitisation by a much longer time course for recovery from desensitisation (“deep” desensitisation, Fenster *et al.*, 1999). Nicotinic receptors differ significantly in terms of fast and slow desensitisation, depending on their subunit composition. In general terms, classical desensitisation is more pronounced in $\beta 2$ - than in $\beta 4$ -containing receptors, and $\beta 2$ -containing receptors are more prone to slow desensitisation (Fenster *et al.*, 1997; Giniatullin *et al.*, 2005). Desensitisation is of major interest, as it may contribute to nicotine dependence, along with the activation of nicotinic receptors (Dani *et al.*, 2001).

The stable recording conditions of my preparation facilitate observations on desensitisation by low concentrations of nicotine, as well as recovery from desensitisation upon removal of the substance. Hence, the recording of CAPs is perfectly suited to investigate “high affinity” desensitisation and its impact on nicotinic cholinergic transmission. As for the experiments with HMB, CAP amplitudes were recorded until a steady state effect was achieved (normally within 30 min).

With a nicotine IC_{50} of 1.2 μM , $\alpha 5\beta 4$ KO mice were the genotype most sensitive to nicotine desensitisation (Fig. 3.16). Furthermore, given the rather shallow Hill coefficient of 0.95, CAP amplitudes are already reduced by >20% at a nicotine concentration of 0.1 μM . By comparison, rat $\alpha 3\beta 2$ receptors expressed in *Xenopus laevis* oocytes incubated with low concentrations of nicotine desensitise rapidly ($\tau = \sim 5$ min), with an IC_{50} of 0.33 μM (Fenster *et al.*, 1997).

CAP amplitudes in mice expressing $\alpha 3\beta 4$ (in $\alpha 5\beta 2$ KO animals) were reduced by nicotine with an IC_{50} of ~ 2.9 μM , whereas receptors in WT were desensitised with an IC_{50} of ~ 3.6 μM (Fig. 3.12). Though small, the difference was statistically significant ($p < 0.0001$; Tab. 3.2). Of interest, desensitisation in both genotypes occurred with steep Hill coefficients of 3.76 (WT) and 3.15 ($\alpha 5\beta 2$ KO) (not significantly different, $p = 0.2518$; Tab. 3.2). Hence, synaptic transmission was entirely functional in the presence of about 1 μM but already fully prevented at 10 μM (Fig. 3.16). Data from the literature show that

human $\alpha 3\beta 4$ receptors stably expressed in HEK293 cells and incubated for 30 min with nicotine desensitise with an IC_{50} of 0.7 μM , not significantly different to $\alpha 3\beta 4\alpha 5$ receptors (0.8 μM , Tammimaki *et al.*, 2012). According to a different report, human $\alpha 3\beta 4\alpha 5$ receptors expressed in HEK cells were, on the other hand, somewhat less sensitive to nicotine desensitisation (IC_{50} : 16.7 μM) (Kuryatov *et al.*, 2011). The group of Ken Kellar finally found rat $\alpha 3\beta 4$ receptors stably expressed in HEK293 cells to desensitise with an IC_{50} of 3.2 μM following a 60 min incubation with nicotine (Meyer *et al.*, 2001), a value quite similar to our observations. Desensitisation by nicotine of rat $\alpha 3\beta 4$ receptors expressed in *Xenopus laevis* oocytes occurs with an IC_{50} of 1.15 μM (Fenster *et al.*, 1997).

We concluded from our experiments with HMB that $\alpha 3\beta 4\alpha 5$ outnumber $\alpha 3\beta 4$ receptors on the cell surface (and in synapses) in the SCG of WT mice. Hence, desensitisation should noticeably differ if the two types of receptors varied in their high affinity desensitisation properties. We found an IC_{50} value for the inhibition of CAPs of 2.95 μM for $\alpha 3\beta 4$ receptors (in $\alpha 5\beta 2$ KO mice) and of 3.63 μM in WT animals, suggesting that inclusion of the $\alpha 5$ subunit renders receptors less prone to high affinity desensitisation.

Considering nicotine peak plasma concentrations of up to 620 nM in human brains (see Dani *et al.*, 2001; Rose, 2007), desensitisation of $\alpha 3\beta 2$ receptors may be an issue in brain regions where these receptors occur. However, if the properties of nAChRs in humans are similar to mice, desensitisation of $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ receptors is less likely to take place in smokers.

A factor that has been shown to regulate the slow component of the rate of desensitisation in (muscular) nAChRs is phosphorylation, a common posttranslational modification (Donnelly-Roberts *et al.*, 1996; Eilers *et al.*, 1997; Albuquerque *et al.*, 2009). The common view is that protein phosphorylation modulates allosteric changes between the activated and the desensitised state of the nAChR (Huganir *et al.*, 1986; Eilers *et al.*, 1997; Nisell *et al.*, 1997). Accordingly, phosphorylated receptors are usually activated, while dephosphorylated receptors are desensitised. Instead, for the neuronal nAChR, phosphorylation probably plays a minor role compared to the receptor subunit composition; as described by Fenster and colleagues (1997) in deeply desensitised oocytes, it is the subunit composition of nAChRs to primarily alter the desensitisation properties of the receptor by modifying the receptor conformations.

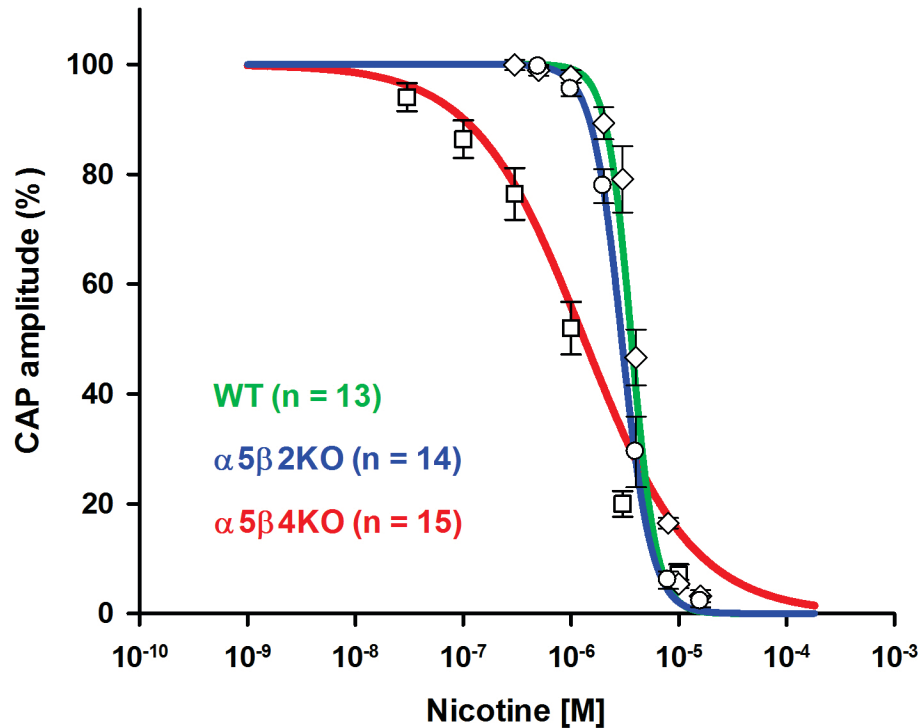


Figure 3.16: Nicotine desensitisation concentration-response-curves for SCGs in WT, $\alpha 5\beta 2$ KO, and $\alpha 5\beta 4$ KO mice. Nicotine has the highest desensitising potency in $\alpha 5\beta 4$ KO ($IC_{50} = 1.19E-6M$), but the desensitisation rate is slower than in the other genotypes (Hill slope = -0.95). Curve shapes of WT and $\alpha 5\beta 2$ KO are similar (Hill slope = -3.760 and -3.157, respectively), whereas their IC_{50} values differ significantly from each other (3.63E-6M and 2.95E-6M, respectively). Statistical analysis is reported in Table 3.2. Data were compared by an extra sum-of-squares F test.

Table 3.3: Shape-defining parameters of concentration-response curves of SCGs desensitised by nicotine and statistical comparison values

		WT	$\alpha 5\beta 2$ KO	$\alpha 5\beta 4$ KO
	N	13	14	15
	Log IC_{50}	-5.440	-5.530	-5.923
	Hill slope	-3.760	-3.157	-0.950
	IC_{50}	3.63E-06	2.95E-06	1.19E-06
WT	p Hill slope	---	$p = 0.2518$	$p < 0.0001$
	p Log IC_{50}	---	$p < 0.0001$	$p < 0.0001$
$\alpha 5\beta 2$ KO	p Hill slope	---	---	$p < 0.0001$
	p Log IC_{50}	---	---	$p < 0.0001$
$\alpha 5\beta 4$ KO	p Hill slope	---	---	---
	p Log IC_{50}	---	---	---

This table summarises n, Hill slope and IC_{50} in WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice continuously superfused with increasing (desensitising) concentrations of nicotine. Data were analysed by an extra sum-of-squares F test. Statistical differences between pairwise compared genotypes are indicated in red.

These results are in line with previous experiments performed in our laboratory, where patch-clamp recordings of cultured SCGs revealed that cells of $\alpha 5\beta 4$ KO mice, pre-treated with nicotine (NIC), desensitised at a ten times lower concentration than SCG cells of $\alpha 5\beta 2$ KO (see chapter 1.5.3, Fig. 1.8).

Threshold stimulus intensities necessary to elicit just discernible CAPs in the three genotypes gave the same results when ganglia were treated or not with nicotine at their nicotine IC_{50} values. As for untreated ganglia, stimulus thresholds were highest in $\alpha 5\beta 4$ KO animals treated with 0.1 μ M nicotine, where mean threshold stimuli equalled 52.2 μ A. Instead, $\alpha 5\beta 2$ KO ganglia (treated with 3.5 μ M nicotine) needed stimuli of ~ 36 μ A, while ~ 29 μ A was already sufficient for WT ganglia (treated with 3.5 μ M nicotine) (Fig. 3.17; Tab. 3.1). Though stimulus thresholds of $\alpha 5\beta 2$ KO+NIC were slightly higher than thresholds of WT+NIC, statistical significances were only found between WT (treated or not with nicotine) and $\alpha 5\beta 4$ KO treated with nicotine ($p < 0.05$) (Fig. 3.17). These findings show that nicotine desensitisation, although reducing the number of available nAChRs for synaptic transmission, has no significant impact on synaptic strength. Thus, regardless whether receptors are desensitised by nicotine or inhibited by HMB, $\alpha 5\beta 4$ KO ganglia always need stimulations around 50 μ A to produce threshold CAPs, whereas WT and $\alpha 5\beta 2$ KO already respond at about 30-36 μ A (see Figs. 3.4B, 3.5). These observations are in line with the hypothesis mentioned above that transmission in the SCG operates with a high safety factor regarding the number of postsynaptic receptors. The threshold for the induction of CAPs thus primarily depends on the excitability of the (afferent) cervical sympathetic nerve.

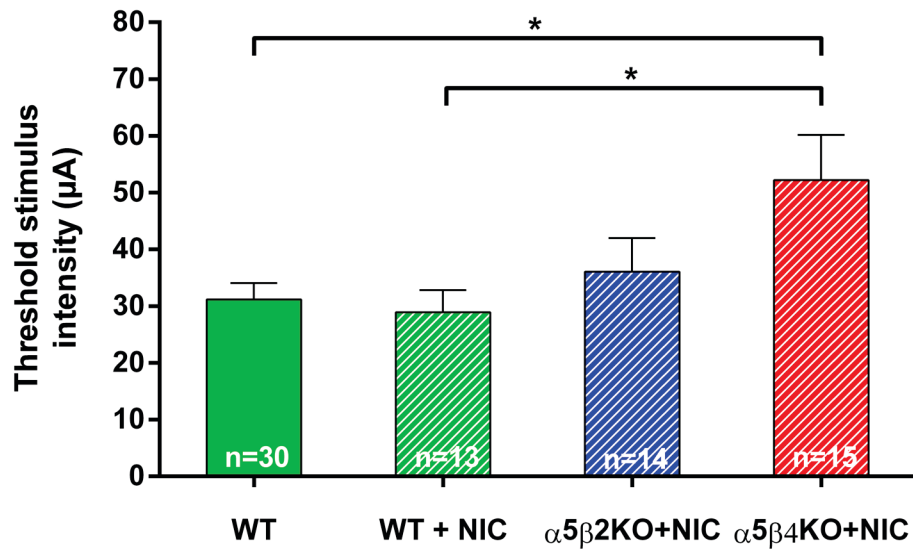


Figure 3.17: CAP thresholds in the presence of nicotine. Threshold stimulus intensities necessary to elicit CAPs of 15-20 μ V amplitude were significantly increased in $\alpha 5\beta 4$ KO when nicotine (nicotine 3.5 μ M for WT and $\alpha 5\beta 2$ KO; nicotine 0.1 μ M for $\alpha 5\beta 4$ KO) was added to the superfusing solution. Data were compared using one way ANOVA, $F = 4.069$ ($\alpha 5\beta 4$ KO+NIC vs. WT: $p < 0.05$; $\alpha 5\beta 4$ KO+NIC vs. WT+NIC: $p < 0.05$), followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM.

I finally merged nicotine and hexamethonium to study their combined effects on the intact ganglion. SCGs of $\alpha 5\beta 4$ KO and $\alpha 5\beta 2$ KO were desensitised by nicotine at their respective nicotine IC_{50} concentrations, and HMB at increasing concentrations was thereafter added to the superfusion. The results in Figure 3.18 show that HMB IC_{50} values were lower in ganglia pre-treated with nicotine, though effects differ between genotypes. Whereas the potency of HMB increased by about a factor of 10 in $\alpha 5\beta 2$ KO ganglia (IC_{50} HMB alone = 120 μ M; IC_{50} after nicotine desensitisation = 23 μ M), the difference was much less for $\alpha 5\beta 4$ KO SCGs (IC_{50} HMB alone = 22 μ M; IC_{50} after nicotine desensitisation = 14 μ M) (see also Fig. 3.4, Tab. 3.3). These observations point at interesting differences between effects of HMB on the two types receptors. It has previously been suggested that (mouse) $\alpha 3\beta 2$ are inhibited by non-competitive mechanisms, whereas inhibition of $\alpha 3\beta 4$ may, in addition, occur through both competitive and non-competitive mechanisms (Papke *et al.*, 2010).

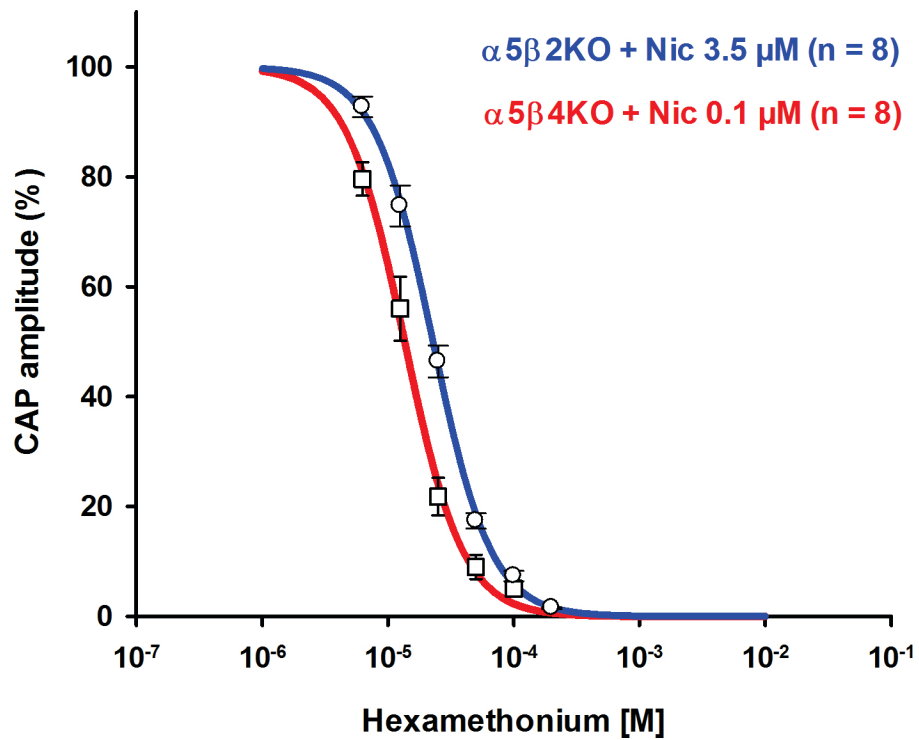


Figure 3.18: The potency of HMB in desensitised ganglia. When SCGs were half-maximally desensitised by nicotine HMB had a higher potency in blocking nAChRs of $\alpha 5\beta 4\text{KO}$ ganglia ($\text{IC}_{50} = 1.36\text{E-}5\text{M}$) desensitised by $0.1 \mu\text{M}$ nicotine than $\alpha 5\beta 2\text{KO}$ ganglia ($\text{IC}_{50} = 2.28\text{E-}5\text{M}$) desensitised by $3.5 \mu\text{M}$ nicotine. Hill slopes (-1.878 for $\alpha 5\beta 2\text{KO}$ and -1.876 $\alpha 5\beta 4\text{KO}$) displayed no difference between genotypes ($p = 0.99$). Statistical analysis is reported in Table 3.3. Data were compared by an extra sum-of-squares F test.

3.7 The role of $\alpha 7$

In addition to the function of $\alpha 3\beta 4$ and $\alpha 3\beta 2$ receptors, we also studied the influence of homo-pentameric $\alpha 7$ receptors on synaptic transmission. In the same way as maximal CAP amplitudes were not changed in the absence of $\alpha 7$ receptors (Fig. 3.19, panel A), stimuli for reaching threshold CAPs did not differ between genotypes containing or not $\alpha 7$ receptors (Fig. 3.19, panel B). Triple KO-mice (lacking $\alpha 7$ homopentamers) required the same stimulus intensity levels as their respective double KO-mice (containing $\alpha 7$ homopentamers). Thus, thresholds for $\alpha 5\alpha 7\beta 2$ KO animals lay at 28.53 μ A and were comparable to the values of $\alpha 5\beta 2$ KO mice, lying at 31.06 μ A. Equally, thresholds for $\alpha 5\alpha 7\beta 4$ KO animals lay at 59.47 μ A and were comparable to the values of $\alpha 5\beta 4$ KO mice, lying at 54.83 μ A. Again, it was the absence of the backbone subunit $\beta 4$, which led to different threshold stimulus intensities. Significant differences were found between $\alpha 5\beta 4$ KO mice and both $\beta 4$ -containing genotypes, as well as between $\alpha 5\alpha 7\beta 4$ KO animals and both $\beta 4$ -containing genotypes (Fig. 3.19, panel B).

One can notice that CAPs of the two triple-KO mice were slightly, but not significantly, higher (60-70 μ V) than CAPs of the other genotypes. This is probably due to a technical improvement of the recording technique (tighter suction of the nerves) occurred with my increasing experience.

Our results show that the presence/absence of the homopentameric nACh receptor $\alpha 7$ seems to have no impact on the correct neurotransmission of the mouse SCG. As described in chapter 1.5.3, the role of $\alpha 7$ is still controversial. Though some studies state that $\alpha 7$ is required for several body functions both in the ANS and CNS, others show the opposite. The relevance of $\alpha 7$ especially for autonomic functions has previously been indicated. Accordingly, Franceschini and colleagues (2000) showed that $\alpha 7$ deficient mice have reduced baroreflex-mediated tachycardia in response to vasodilatations; Chang and Berg (1999) demonstrated that in the chick ciliary ganglion, reliable synaptic transmission is only possible in the presence of $\alpha 7$. On the other hand, the absence of $\alpha 7$ did not alter parasympathetic response of the heart in mice (Deck *et al.*, 2005). Similarly to my results, two other groups could not find a critical contribution of $\alpha 7$ receptors for neurotransmission within the mouse SCG (Brown & Fumagalli, 1977; Chiappinelli & Zigmond, 1978) and in cultured mouse SCG neurons nicotine-induced currents were not modulated by $\alpha 7$ (David *et al.* 2010).

In the CNS, the presence of $\alpha 7$ is crucial for the formation of synaptic plasticity and memory (Dougherty *et al.*, 2003; Puzzo *et al.*, 2008) and has been associated to schizophrenia (Freedman & Goldowitz, 2010) and Alzheimer's disease (Wang *et al.*, 2000), as well as to hippocampal LTP in mice (Sawada *et al.*, 1994; Ji *et al.*, 2001; Mann & Greenfield, 2003; Welsby *et al.*, 2006).

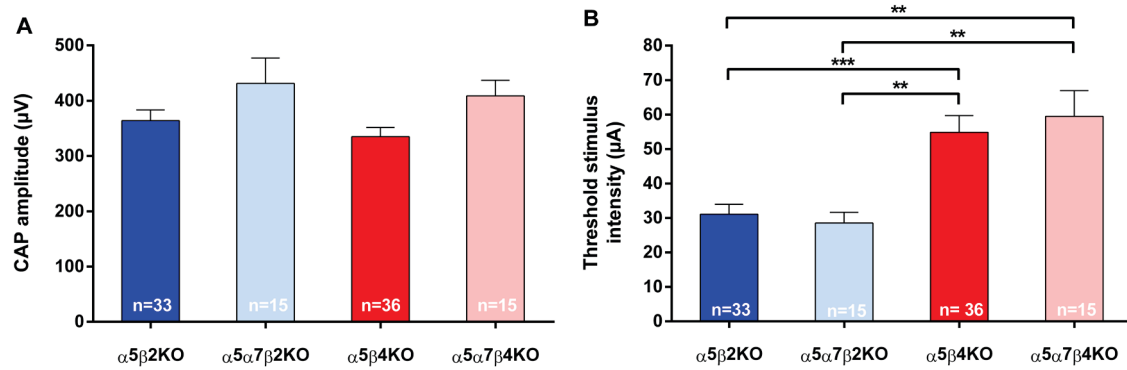


Figure 3.19: CAP amplitudes and threshold stimulus intensities of $\alpha 5\beta 2$ KO, $\alpha 5\alpha 7\beta 2$ KO, $\alpha 5\beta 4$ KO and $\alpha 5\alpha 7\beta 4$ KO mice. **A)** The additional absence of $\alpha 7$ in the triple-KO mice did not alter the CAP amplitude elicited through supramaximal stimulation ($F = 2.913$, $p = 0.038$). **B)** Threshold stimulus intensities necessary to elicit CAPs of 15-20 μV amplitude were significantly higher in $\alpha 5\beta 4$ KO and $\alpha 5\alpha 7\beta 4$ KO mice ($F = 10.25$; $\alpha 5\beta 4$ KO vs. $\alpha 5\beta 2$ KO: $p < 0.001$; $\alpha 5\beta 4$ KO vs. $\alpha 5\alpha 7\beta 2$ KO: $p < 0.01$; $\alpha 5\alpha 7\beta 4$ KO vs. $\alpha 5\beta 2$ KO: $p < 0.01$; $\alpha 5\alpha 7\beta 4$ KO vs. $\alpha 5\alpha 7\beta 2$ KO: $p < 0.01$). Data were compared using one way ANOVA, followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM.

Concentration-response curves with hexamethonium confirm our observation that the presence of $\alpha 7$ nAChRs does not alter synaptic transmission in mouse SCGs (Fig. 3.20). As for the double KO mice, HMB has a higher potency in blocking receptors in $\alpha 5\alpha 7\beta 4$ KO mice ($IC_{50} \approx 25 \mu M$) than in $\alpha 5\alpha 7\beta 2$ KO animals ($IC_{50} \approx 140 \mu M$). Hill slopes and IC_{50} values are similar between $\alpha 5\alpha 7\beta 4$ KO and $\alpha 5\beta 4$ KO, as well as between $\alpha 5\alpha 7\beta 2$ KO and $\alpha 5\beta 2$ KO animals. Significant differences were only found between $\beta 4$ -missing and $\beta 2$ -missing genotypes containing or not $\alpha 7$ (Tab. 3.3). In *Xenopus laevis* oocytes Papke and colleagues (2010) found that pure $\alpha 7$ nAChRs were inhibited by hexamethonium with an IC_{50} of 44 μM (lying in between $\alpha 3\beta 2$ and $\alpha 3\beta 4$ receptors, see chapter 3.5.2). Thus, although $\alpha 7$ receptors can be principally blocked by hexamethonium, in the mouse ganglion $\alpha 3\beta 4$ receptors undertake synaptic neurotransmission on their own.

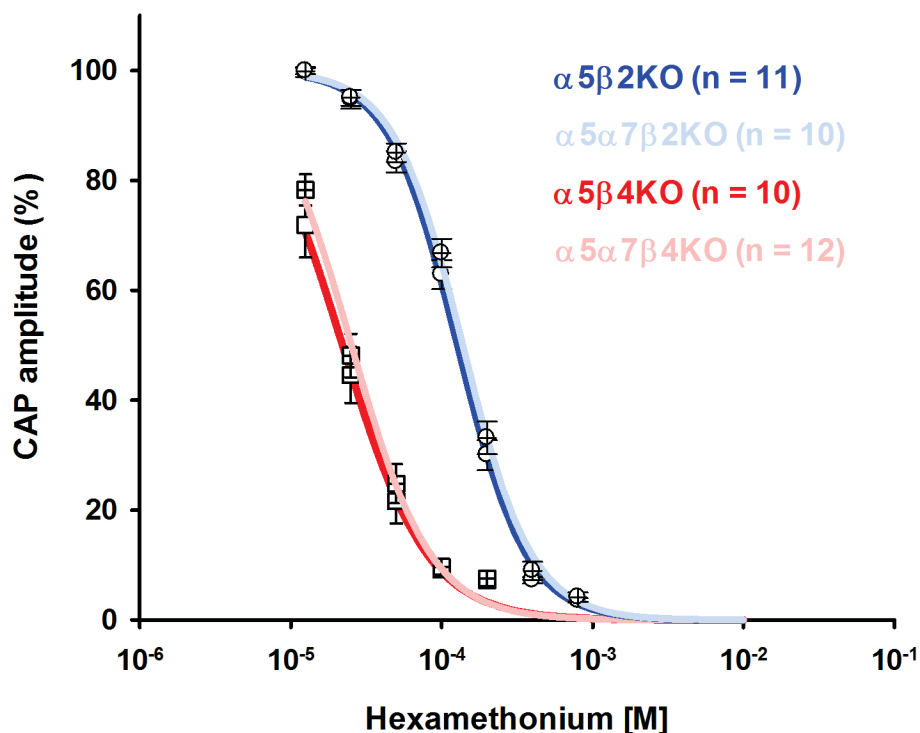


Figure 3.20: HMB dose-response curves in $\alpha 5\beta 2\text{KO}$, $\alpha 5\alpha 7\beta 2\text{KO}$, $\alpha 5\beta 4\text{KO}$ and $\alpha 5\alpha 7\beta 4\text{KO}$ mice. The additional deletion of $\alpha 7$ in the triple knockout mice $\alpha 5\alpha 7\beta 2\text{KO}$ ($\text{IC}_{50} = 1.38\text{E-}4\text{M}$, Hill slope = -1.898) and $\alpha 5\alpha 7\beta 4\text{KO}$ ($\text{IC}_{50} = 2.52\text{E-}5\text{M}$, Hill slope = -1.654) did not alter the responses to HMB, compared to the double knockout mice. Statistical analysis is reported in Table 3.3. Data were compared by an extra sum-of-squares F test.

3.7.1. No modulation by PNU-120596 in heterozygote mice

To further corroborate that in the SCG the homopentameric $\alpha 7$ receptor plays no role for synaptic transmission, we additionally added the $\alpha 7$ -specific (Type II) positive allosteric modulator PNU-120596 to our preparation (see chapter 2.3.7). As expected, PNU did not alter stimulus thresholds (Fig. 3.21), nor did it change supramaximal CAPs in WT, $\alpha 5\beta 4\text{KO}$ and $\alpha 5\beta 2\text{KO}$ mice (Figs. 3.22).

Accordingly, significant differences in stimulus thresholds persisted only within the $\beta 4$ -backbone-lacking $\alpha 5\beta 4\text{KO}$ mice (treated or not with PNU) and the other two $\beta 4$ -backbone-containing genotypes WT and $\alpha 5\beta 2\text{KO}$ (treated or not with PNU) (Fig. 3.21).

In view of that, it is not surprising that HMB blocked all PNU-treated genotypes in the same way as PNU-untreated mice, leading to comparable IC_{50} -values and Hill slopes (Fig. 3.23).

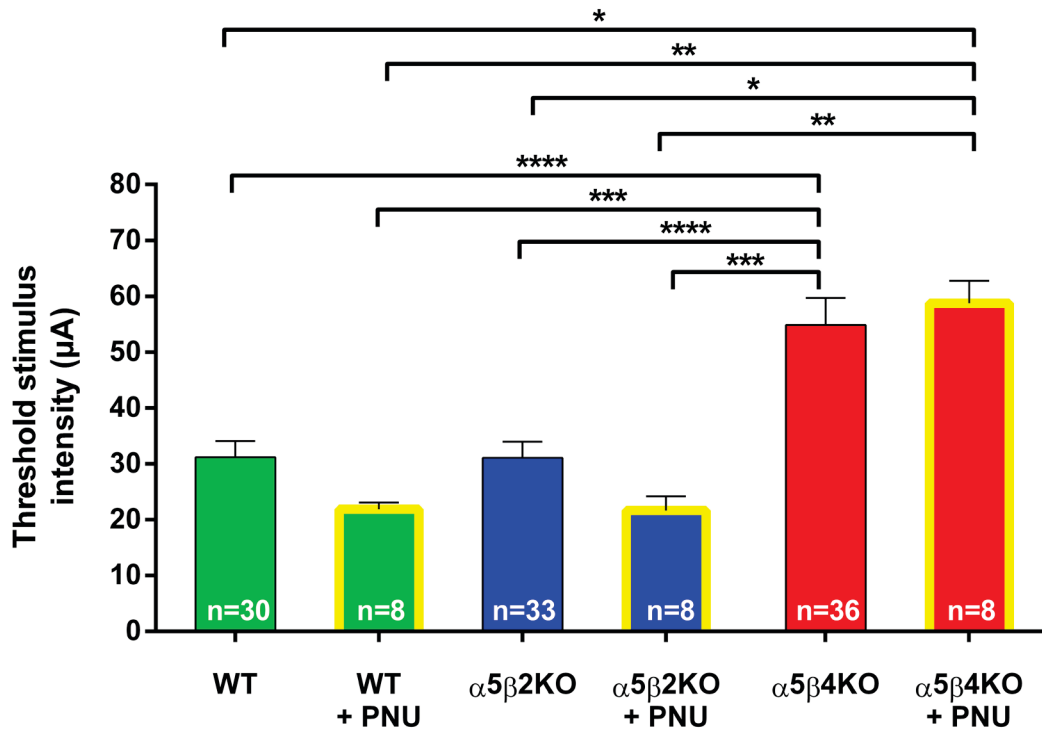


Figure 3.21: Threshold stimulus intensities in ganglia treated with 10 μ M PNU-120596. Significant differences were found between $\alpha 5\beta 4$ KO±PNU with both WT±PNU and $\alpha 5\beta 2$ KO±PNU ($F = 10.29$; $\alpha 5\beta 4$ KO vs. WT: $p < 0.0001$; $\alpha 5\beta 4$ KO vs. WT+PNU: $p < 0.001$; $\alpha 5\beta 4$ KO vs. $\alpha 5\beta 2$ KO: $p < 0.0001$; $\alpha 5\beta 4$ KO vs. $\alpha 5\beta 2$ KO +PNU: $p < 0.001$; $\alpha 5\beta 4$ KO+PNU vs. WT: $p < 0.05$; $\alpha 5\beta 4$ KO+PNU vs. WT+PNU: $p < 0.01$; $\alpha 5\beta 4$ KO+PNU vs. $\alpha 5\beta 2$ KO: $p < 0.05$; $\alpha 5\beta 4$ KO+PNU vs. $\alpha 5\beta 2$ KO +PNU: $p < 0.01$). Data were compared using one way ANOVA, followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM.

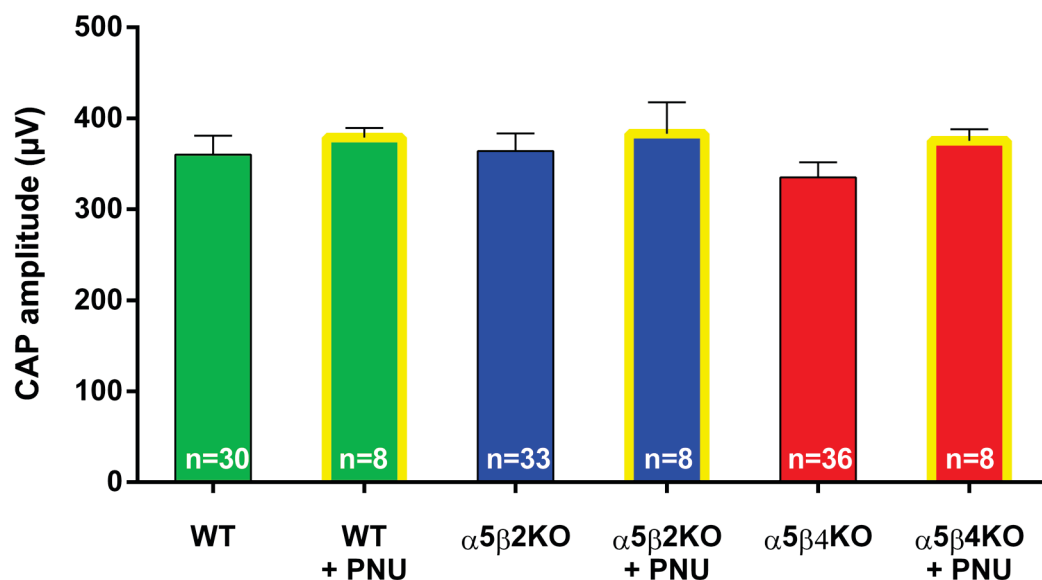


Figure 3.22: CAP amplitudes in ganglia treated with 10 μ M PNU-120596. The treatment with did not alter the CAP amplitudes in WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice. Data were compared using one way ANOVA, $F = 0.6143$, followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM.

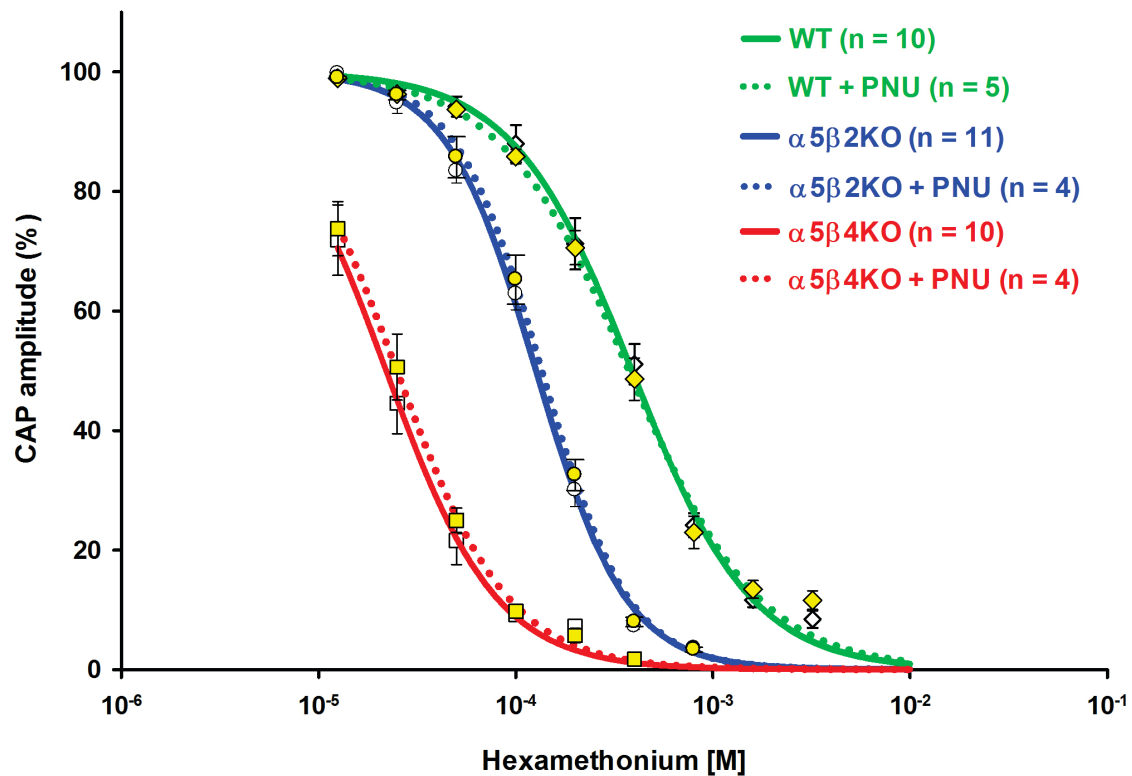


Figure 3.23: HMB dose-response curves in $\alpha 7$ -(not)-containing ganglia treated with PNU-120596. Also this experiment shows that $\alpha 7$ is not necessary for synaptic transmission WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice, since PNU has no modulatory effect on SCGs containing $\alpha 7$ receptors. All values for individual IC_{50} and Hill slopes, as well as statistical differences are reported in Table 3.3. Data were compared by an extra sum-of-squares F test.

Table 3.4: Parameters of concentration–response curves of intact SCGs, blocked by HMB.

	WT	$\alpha\beta 2$ KO	$\alpha\beta 4$ KO	$\alpha 5\alpha 7\beta 2$ KO	$\alpha 5\alpha 7\beta 4$ KO	$\alpha 5$ KO	Hetero	WT+PNU	$\alpha\beta 2$ KO+PNU	$\alpha\beta 4$ KO+PNU	$\alpha\beta 2$ KO+Nic	$\alpha\beta 4$ KO+Nic
n												
Log IC ₅₀	-3.41	-3.897	-4.656	-3.861	-4.598	-3.924	-3.45	-3.424	-3.87	-4.604	-4.643	-4.868
Hill slope	-1.432	-1.907	-1.543	-1.898	-1.654	-1.8	-1.407	-1.32	-1.969	-1.532	-1.878	-1.876
IC ₅₀	3.89E-04	1.27E-04	2.21E-05	1.38E-04	2.52E-05	1.19E-04	3.55E-04	3.76E-04	1.35E-04	2.49E-05	2.28E-05	1.36E-05
WT	---	p = 0.0003 p < 0.0001	p = 0.5268 p < 0.0001	p = 0.0004 p < 0.0001	p = 0.1364 p < 0.0001	p = 0.0119 p < 0.0001	p = 0.8663 p = 0.2175	p = 0.2917 p = 0.5621	p = 0.8213 p < 0.0001	p = 0.5189 p < 0.0001	p = 0.0007 p < 0.0001	p = 0.0131 p < 0.0001
$\alpha\beta 2$ KO	---	---	p = 0.8355 p < 0.0001	p = 0.9261 p = 0.3631	p = 0.7491 p < 0.0001	p = 0.3200 p = 0.2139	p = 0.3108 p < 0.0001	p = 0.1074 p < 0.0001	p = 0.1300 p = 0.2026	p = 0.8096 p < 0.0001	p = 0.1277 p < 0.0001	p = 0.2273 p < 0.0001
$\alpha\beta 4$ KO		---	---	p = 0.0546 p < 0.0001	p = 0.5940 p = 0.0710	p = 0.2243 p < 0.0001	p = 0.5116 p < 0.0001	p = 0.1675 p < 0.0001	p = 0.0665 p < 0.0001	p = 0.9600 p = 0.1929	p = 0.0762 p = 0.6256	p = 0.5940 p = 0.0710
$\alpha 5\alpha 7\beta 2$ KO				---	p = 0.1156 p < 0.0001	p = 0.5274 p = 0.0042	p = 0.0018 p < 0.0001	p < 0.0001 p < 0.0001	p = 0.6789 p = 0.6659	p = 0.0344 p < 0.0001	p = 0.8835 p < 0.0001	p = 0.9024 p < 0.0001
$\alpha 5\alpha 7\beta 4$ KO					---	p = 0.4140 p < 0.0001	p = 0.1619 p < 0.0001	p = 0.0161 p < 0.0001	p = 0.1111 p < 0.0001	p = 0.5360 p = 0.8565	p = 0.1587 p = 0.0501	p = 0.2930 p < 0.0001
$\alpha 5$ KO						---	p = 0.0352 p < 0.0001	p = 0.0002 p < 0.0001	p = 0.3358 p = 0.0264	p = 0.1363 p < 0.0001	p = 0.6052 p < 0.0001	p = 0.7234 p < 0.0001
Hetero							---	p = 0.5520 p = 0.4719	p = 0.0103 p < 0.0001	p = 0.5654 p < 0.0001	p = 0.0053 p < 0.0001	p = 0.0269 p < 0.0001
WT+PNU								---	p < 0.0001 p < 0.0001	p = 0.0756 p < 0.0001	p < 0.0001 p < 0.0001	p = 0.0009 p < 0.0001
$\alpha\beta 2$ KO+PNU									---	p = 0.0060 p < 0.0001	p = 0.5469 p < 0.0001	p = 0.6916 p < 0.0001
$\alpha\beta 4$ KO+PNU										---	p = 0.0270 p = 0.1182	p = 0.1482 p < 0.0001
$\alpha\beta 2$ KO+Nic											---	p = 0.9902 p < 0.0001
$\alpha\beta 4$ KO+Nic												---

This table gives a summarising overview of n, Hill slope and IC₅₀ in all tested genotypes, superfused with increasing doses of the channel blocker hexamethonium bromide (HMB). Data were analysed by an extra sum-of-squares F test. Statistical differences between pairwise compared genotypes are indicated in red.

3.8 Responses to increasing pulse frequencies

Stimulation of the cervical sympathetic trunk will cause EPSPs in SCG neurons and generate APs if the threshold for the activation of voltage-gated Na^+ currents is reached (see Fig. 1.2). At low stimulation frequencies of the afferent nerve (< 1 Hz), the amplitude of the EPSP will remain fairly constant. However, with higher frequencies, amplitudes of the EPSP pass three stages. Stage 1: Initial facilitation (or depression) (McLachlan, 1975a; Birks & Isacoff, 1988); Stage 2: Subsequent depression due to depletion of the (presynaptic) ACh stores (Bennett & McLachlan, 1972a); Stage 3: Steady state maintained by re-synthesis of ACh (Bennett & McLachlan, 1972b). Since re-synthesis depends on the reuptake of choline (see Fig. 1.3), synaptic transmission will cease if reuptake is inhibited by hemicholinium (Bennett & McLachlan, 1972b).

I measured the effect of increasing stimulation frequencies of the cervical sympathetic trunk on CAP amplitudes and observed remarkable differences between WT and KO mice. Hence, CAP amplitudes in response to supramaximal stimulation transiently increased significantly when the frequency was stepped up to 5 Hz (Fig. 3.24, panel A, D). On the contrary, at the same stimulation frequency, no increase in CAP amplitudes occurred in $\alpha 5\beta 2\text{KO}$ (Fig. 3.24, panel B, D), whereas in $\alpha 5\beta 4\text{KO}$ mice the responses significantly decreased (Fig. 3.24, panel C, D).

Ganglia were tested at the following frequencies: 0.5 Hz, 1 Hz, 5 Hz, 10 Hz, 20 Hz and 40 Hz. In WT mice, two-way repeated measures ANOVA using the frequency as the group factor and the pulses as repeated factor showed a highly significant pulse effect ($F_{4, 144} = 59.58$; $p < 0.0001$). The Bonferroni post hoc test indicated that CAPs following 0.5 Hz, 1 Hz and 10 Hz did not significantly change their amplitudes during the train of 30 pulses. At 5 Hz, CAPs significantly increased at all shown pulses (P1 vs. P2: $p < 0.0001$; P1 vs. P3: $p = 0.0001$; P1 vs. P10: $p = 0.0006$; P1 vs. P30: $p = 0.0206$). At 20 Hz and 40 Hz there was a highly significant amplitude decrease at all tested pulses (P1 vs. P2, P3, P10, P30: $p < 0.0001$).

Also in $\alpha 5\beta 2\text{KO}$, two-way repeated measures ANOVA showed a highly significant pulse effect ($F_{4, 144} = 155.1$; $p < 0.0001$). Contrary to WT, no significant increase was observed at 5 Hz, but similarly to WT mice at 20 Hz and 40 Hz CAP amplitudes significantly dropped down (P1 vs. P2, P3, P10, P30: $p < 0.0001$). While in WT at 10 Hz CAPs did not change, in $\alpha 5\beta 2\text{KO}$ CAPs already significantly decreased at this frequency

(P1 vs. P2: $p = 0.022$; P1 vs. P3: $p = 0.016$, P1 vs. P10: $p = 0.001$; P1 vs. P30: $p < 0.0001$). CAP amplitudes remained constant at the frequencies 0.5 Hz, 1 Hz and 5 Hz.

In $\alpha 5\beta 4$ KO animals, two-way repeated measures ANOVA showed as well a highly significant pulse effect ($F_{4, 144} = 203.8$; $p < 0.0001$). Contrary to the other genotypes, CAP amplitudes decreased already when pulses were given at 1 Hz and the drop was highly significant at 5 Hz, 10 Hz, 20 Hz and 40 Hz, as indicated by the Bonferroni post hoc test (P1 vs. P2, P3, P10, P30: $p < 0.0001$).

By comparing the CAPs between genotypes (Fig. 3.24, panel D), two-way repeated measures ANOVA revealed a significant genotype effect at 5 Hz ($F_{2, 18} = 7.602$; $p = 0.0040$) and at 40 Hz ($F_{2, 18} = 4.188$; $p = 0.0321$). Bonferroni's post hoc comparison test indicated that differences of the second pulse were highly significant both at 5 Hz (WT_{5 Hz} vs. $\alpha 5\beta 2$ KO_{5 Hz}: $p = 0.0471$; WT_{5 Hz} vs. $\alpha 5\beta 4$ KO_{5 Hz}: $p < 0.0001$) and at 40 Hz (WT_{40 Hz} vs. $\alpha 5\beta 2$ KO_{40 Hz}: $p = 0.0052$; WT_{40 Hz} vs. $\alpha 5\beta 4$ KO_{40 Hz}: $p < 0.0001$).

A focus on the first two pulses (measured as ratio of the second to the first pulse) also reveals properties of the paired-pulse paradigm which follow, in general, the observations made by continuous stimulation described above. Paired-pulse facilitation and paired-pulse depression are a short time form of synaptic plasticity found in many central and peripheral synapses, see Zucker (1989). I have thus seen paired-pulse facilitation in WT SCGs upon stimulation at 5 Hz (paired-pulse ratio = 1.18; $p < 0.0001$) and paired-pulse depression at 20 Hz (paired-pulse ratio = 0.76; $p < 0.0001$) and 40 Hz (paired-pulse ratio = 0.26; $p < 0.0001$). P2 did not significantly diverge from P1 at 0.5 Hz (paired pulse ratio = 1.00), 1 Hz (paired pulse ratio = 1.05) and 10 Hz (paired pulse ratio = 1.06).

In $\alpha 5\beta 2$ KO mice, paired-pulse ratios were 0.99 at 5 Hz ($p > 0.9999$), 0.91 at 10 Hz ($p = 0.022$), 0.64 at 20 Hz ($p < 0.0001$) and 0.11 at 40 Hz ($p < 0.0001$). Thus, contrary to WT there was no paired-pulse facilitation in this genotype and paired-pulse depression started already at 10 Hz stimulations.

In $\alpha 5\beta 4$ KO mice, paired-pulse ratios equalled 0.98 at 0.5 Hz ($p > 0.9999$), 0.91 at 1 Hz ($p > 0.9999$), 0.82 at 5 Hz ($p < 0.0001$), 0.76 at 10 Hz ($p < 0.0001$), 0.44 at 20 Hz ($p < 0.0001$) and 0.03 at 40 Hz ($p < 0.0001$). Similarly to the other $\alpha 5$ -lacking genotype, also here there was no paired-pulse facilitation, while a clear paired-pulse depression already started at 5 Hz stimulations.

These observations raise some interesting questions: Why do CAP amplitudes increase in WT mice when SCG afferents are stimulated at 5 Hz? After all, stimulation intensity has been supramaximal, meaning that all functional afferents should fire APs. Stimulation of afferents with higher frequency has been attributed to an increase of the quantal content (Birks & Isacoff, 1988), an effect which has been accredited to a build-up in presynaptic calcium (Bennett *et al.*, 1976). One possibility might be that the increase of the quantal content turns some weak synapses into strong synapses, causing excitation of SCG neurons which could not be activated by suprathreshold stimulation. However, it is generally assumed that each presynaptic axon forms at least one strong synapse (Holman & Hirst, 1977). Furthermore, I could not detect weak synapses with intracellular recordings from 5 WT SCG neurons (see PART III). Finally, why did we observe potentiation only in WT but not in KO animals where we might also expect weak synapses?

As an alternative mechanism, postganglionic neurons that have lost their afferent input by the preparation might be excited by indirect mechanisms due to signalling by second messengers or build-up of extracellular potassium; see Galvan *et al.* (1979). Great care has to be taken to not damage the cervical sympathetic trunk in order to be able to see trans-ganglionic transmission. Hence, it is quite likely that some SCG neurons become disconnected from their input during dissection of the SCG. However, why should this explanation only apply to WT but not to the KO SCGs, where I could not observe such potentiation? Whereas accumulation of K^+ already after a single pulse is unlikely to occur, signalling by pertussis toxin-sensitive G-proteins by PACAP or VIP may potentiate nAChRs within 100 ms (Liu *et al.*, 2000; Pugh *et al.*, 2010). However, potentiation of (postsynaptic) nAChRs can only take place if ACh is released upon calcium influx in afferent nerve endings which, in turn, depends on stimulus-induced action potentials. Following the arguments mentioned above, released ACh would thus reach a neighbouring SCG which has lost its afferent connections. An enhanced release with a more widespread diffusion of ACh (due to enhanced quantal size) could also be an attractive explanation for the paired-pulse facilitation observed in WT animals. However, we currently have no evidence that this mechanism takes place. Hence, paired-pulse facilitation (and the transient increase of CAP amplitudes by trains of presynaptic stimuli) in just WT animals but not in mice lacking the $\alpha 5$ subunit remains an unexplained though interesting phenomenon.

The rapid decline of CAP amplitudes at stimulation frequencies of 20 Hz and 40 Hz can likely be explained by depletion of the (presynaptic) ACh stores (Bennett & McLachlan, 1972a). With a stimulation rate of 20 Hz, CAP amplitudes decreased in the order $\alpha 5\beta 4\text{KO} > \alpha 5\beta 2\text{KO} > \text{WT}$ (Fig. 3.24). At 40 Hz, CAP amplitudes did not differ significantly between $\alpha 5\beta 4\text{KO}$ and $\alpha 5\beta 2\text{KO}$ mice. Given that $\alpha 3\beta 2$ receptors differed significantly in their desensitisation properties from $\alpha 3\beta 4$ receptors (see Fig. 3.16; Tab. 3.3), this observation suggests that high affinity desensitisation of receptors at higher stimulation frequencies (possibly leading to the accumulation of released ACh) does not play a major role in the decrease of CAP amplitudes.

As already mentioned above, Wang and colleagues (2002a) observed that WT and $\alpha 5\text{KO}$ mice reacted differently to vagal stimulations, especially at higher frequencies over 40 Hz. While in WT animals, stimulations of 60 Hz caused a complete asystole (i.e. 100% heart rate block), in $\alpha 5\text{KO}$ mice the heart rate was lowered by only 80% and never reached asystole. At low frequencies the heart rates of WT and $\alpha 5\text{KO}$ mice did not significantly differ from each other: at 5 Hz both genotypes lost ~15% of the baseline heart rate and at 10 Hz they both lost ~36%. Differences in response to vagal stimulation started at 40 Hz (~70% block in $\alpha 5\text{KO}$ vs. ~85% in WT) and persisted at 60 Hz and 100 Hz (~85% block in $\alpha 5\text{KO}$ vs. ~100% in WT). Similar experiments were conducted in pure $\beta 2\text{KO}$ (Wang *et al.*, 2005) and $\beta 4\text{KO}$ mice (Wang *et al.*, 2003). There, $\beta 2\text{KO}$ mice behaved nearly like WT mice (no difference at low frequencies and ~92% block in $\beta 2\text{KO}$ at 60 Hz/100 Hz vs. 100% block in WT); in $\beta 4\text{KO}$, differences in responses were already significant at 20 Hz (~45% block in KO vs. ~60% block in WT) and persisted at 40 Hz (~70% block in KO vs. ~80% block in WT), and 60 Hz/100 Hz (~80% block in KO vs. ~100% block in WT). Although my experiments are not directly comparable to Wang's observations, it is worth to point out that in the SCG, effects of stimulation differ from WT already at 5 Hz in $\alpha 5\beta 4\text{KO}$ and at 10 Hz in $\alpha 5\beta 2\text{KO}$ mice, whereas Wang had to stimulate at nearly maximal rates to detect differences. However, both our results show that sympathetic (and parasympathetic) ganglionic transmission induced by nerve stimulation was less effective in knockout animals.

All in all, the discrete disparities in synaptic transmission between my three genotypes point at presynaptic rather than postsynaptic mechanisms. A critical effect of nicotinic receptor function on the expression of the presynaptic choline transporter ChT1 has previously been described in the SCG of mice with a targeted deletion of the $\alpha 3$ nAChR subunit gene (Krishnaswamy & Cooper, 2009). Though deletion of $\alpha 3$ entirely

abolishes synaptic transmission in the ganglion, the removal of the $\alpha 5$ subunit may also have presynaptic effects, albeit more subtle.

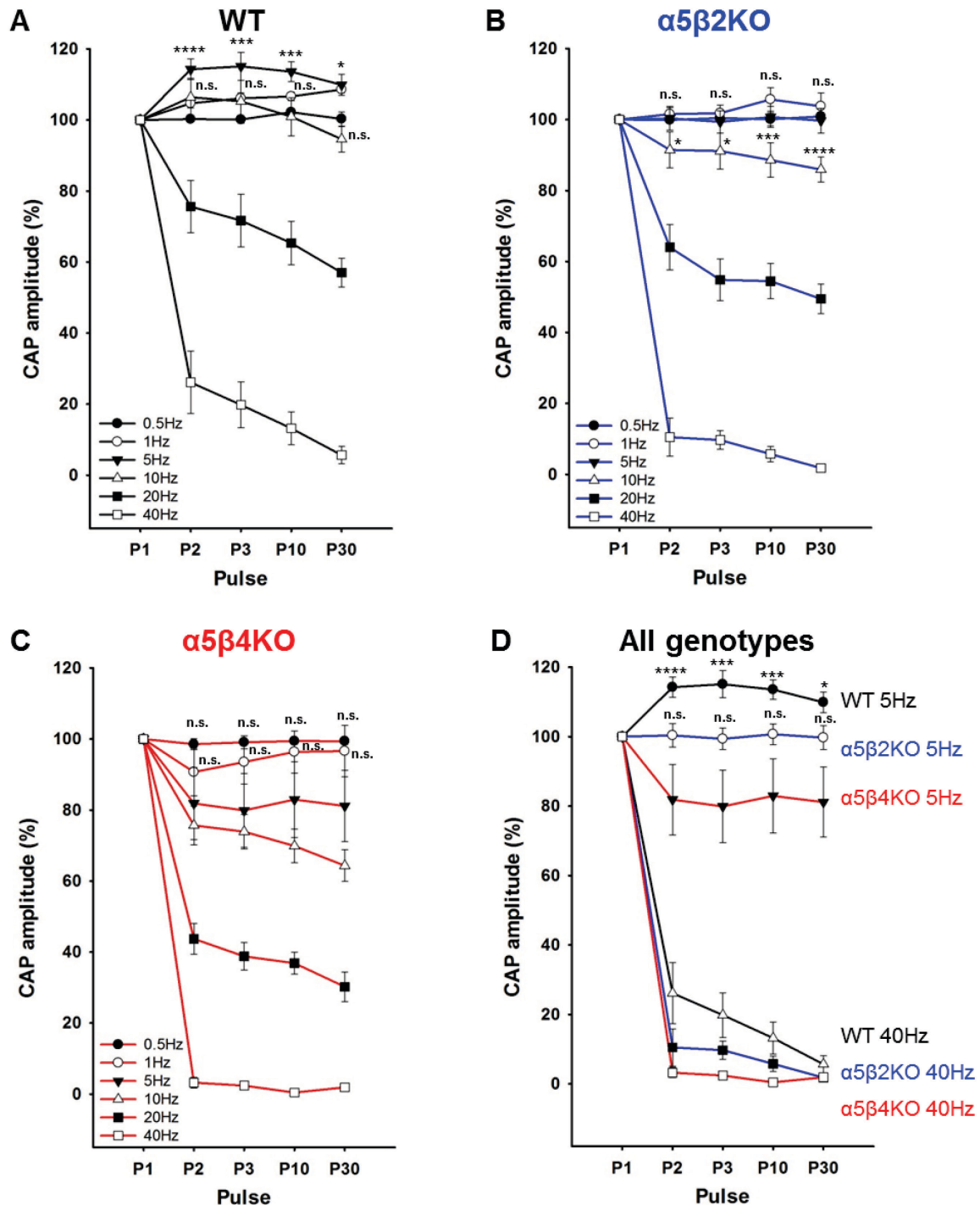


Figure 3.24: CAP responses to a train of 30 pulses given at increasing pulse frequencies. Only the first, second, third, tenth and thirtieth pulse are shown. Inter-stimulus-intervals (ISI) equalled 2 s (0.5 Hz), 1 s (1 Hz), 200 ms (5 Hz), 100 ms (10 Hz), 50 ms (20 Hz) and 25 ms (40 Hz). **A)** WT mice: While pulses given at 0.5 Hz, 1 Hz and 10 Hz did not alter CAP amplitudes, at 5 Hz CAPs significantly increased. High frequency stimulations of 20 Hz and 40 Hz caused strong CAP amplitude drops. **B)** $\alpha 5\beta 2\text{KO}$ mice: contrary to WT, CAPs never increased in amplitude at any tested frequency. No CAP change appeared at 0.5 Hz, 1 Hz and 5 Hz. Significant CAP decrease started already at 10 Hz and was even more pronounced at 20 Hz

and 40 Hz. **C)** $\alpha 5\beta 4$ KO mice: Only at 0.5 Hz CAPs could follow stimulations without incurring amplitude declines. At already 1 Hz there was a visible trend to CAP reduction and at 5 Hz, 10 Hz, 20 Hz and 40 Hz CAPs significantly declined to nearly zero. **D)** Comparison of the responses to 5 Hz trains (upper traces) and 40 Hz trains (lower traces) in all three genotypes. At 5 Hz, the genotypes responded significantly differently from each other ($F_{2,18} = 7.602$; $p = 0.0040$): there was a pulse facilitation to 114% in WT, no change in $\alpha 5\beta 2$ KO mice and a depression to 82% in $\alpha 5\beta 4$ KO mice. At higher frequencies, a pulse depression was visible in all genotypes, the $\alpha 5\beta 4$ KO always being the most susceptible to depression. At the highest tested frequency of 40 Hz, the depressed responses still significantly differed between the genotypes ($F_{2,18} = 4.188$; $p = 0.0321$). The second pulse was significantly reduced to 26% in WT, to 10% in $\alpha 5\beta 2$ KO mice and to only 3% in $\alpha 5\beta 4$ KO mice. Data were compared using two-way ANOVA, followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM. Detailed statistics are reported in the running text.

3.9 Long term potentiation (LTP) in the mouse SCG

Facilitated CAP amplitudes in WT following 5 Hz pulse trains suggested that postsynaptic neurons within the mouse SCG might also be potentiated to produce ganglionic long term potentiation (gLTP) through appropriate stimulation protocols. gLTP is an activity-dependent sustained increase of ganglionic neurotransmission comparable to that described in various regions of the brain (Bliss & Collingridge, 1993).

I looked for the presence of gLTP in intact SCGs taken from WT and $\alpha 5\beta 4$ KO mice. Following the protocol of Alkadhi and colleagues (1996), ganglia were treated with concentrations of HMB necessary to reduce CAPs to half-maximal amplitude (380 μ M in WT and 20 μ M in $\alpha 5\beta 4$ KO). By this way, nicotinic pathways were partially blocked in order to produce submaximal responses. Actually, submaximal postsynaptic responses are necessary to best evaluate enhanced synaptic efficacy (Alkadhi *et al.*, 2005), but submaximal presynaptic nerve stimulations may result in an enhanced recruitment of presynaptic fibres (not present when stimuli are supramaximal, see Briggs *et al.* 1982) and this may lead to an apparent increase of synaptic activity. By using the HMB-method, submaximal responses were achieved through supramaximal stimuli (Briggs *et al.*, 1985; Alkadhi *et al.*, 2005).

My results showed that in WT mice, a high frequency tetanic stimulation (train of pulses at 20 Hz for 20 s) resulted in a post-tetanic CAP potentiation (PTP) up to $\sim 180\%$, which exponentially decreased and restored to the normal CAP amplitude within 20-25 min (Fig. 3.25. panel A). Statistical differences as calculated by two-way ANOVA ($F_{45,180} = 24.11$; $p < 0.0001$) were found only until minute 6 after the control pulse (last pulse before tetanus) in WT, and until minute 30 in $\alpha 5\beta 4$ KO mice. In $\alpha 5\beta 4$ KO mice, the PTP reached $\sim 240\%$ and CAPs required about 60 min to resume their initial values

(Fig. 3.25. panel B). Thus, in both untreated genotypes only a short time post-tetanic potentiation appeared, but no LTP.

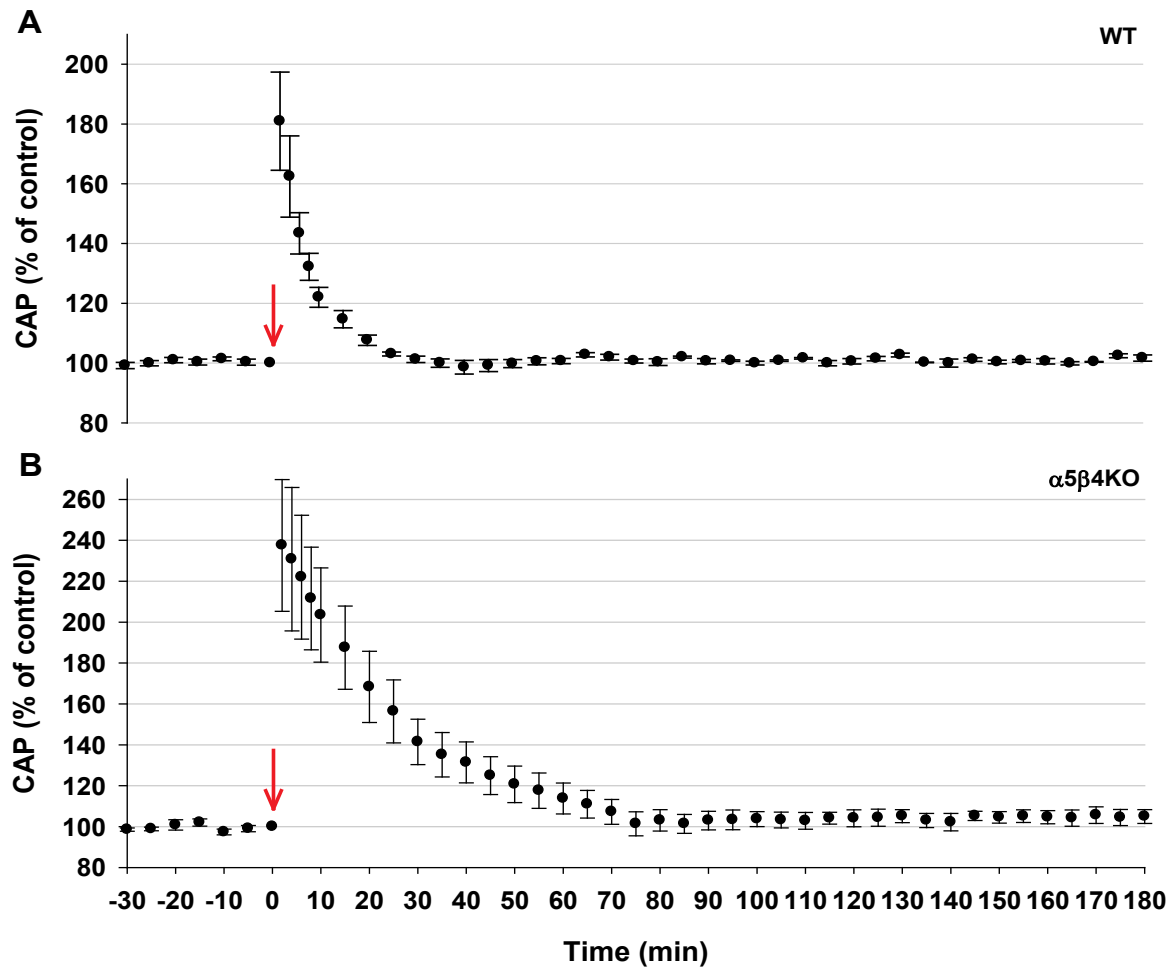


Figure 3.25: Lack of ganglionic long term potentiation (gLTP) in the isolated SCG. A) WT treated with 380 μ M HMB to get halfmaximal responses. **B)** $\alpha 5\beta 4$ KO mice treated with 20 μ M HMB to get halfmaximal responses. A train of pulses (20 Hz for 20 s; arrowhead) induces a post-tetanic potentiation. No gLTP is induced in any genotype. The response is expressed as percentage of a control CAP recorded immediately before the tetanus. CAPs were evoked by supramaximal pulses to the cervical sympathetic trunk. For both genotypes, data show the mean of 3 ganglia $\pm$ SEM.

In 1996, Alkadhi and colleagues showed that in the rat SCG, the magnitude of gLTP could be enhanced by pre-treating ganglia with 10 μ M 5HT (Alkadhi *et al.*, 1996). In this study, untreated SCGs produced a tetanus-induced gLTP with a CAP increase to 120-130% lasting more than 3h. Instead, SCGs pre-treated with 5HT produced a gLTP that reached even 140-150% of the control CAP and also lasted over three hours. LTP was similarly enhanced in the presence of the 5HT-reuptake inhibitor fluoxetine and completely blocked by the 5HT-receptor antagonist MDL 72222 (Alkadhi *et al.*, 1996). The authors concluded that gLTP in the rat SCG requires activation of 5HT₃-receptors

both for induction and maintenance of LTP. For this reason, I added 10 μ M 5HT to my preparation, expecting to gain a gLTP. 5HT was pre-applied for 20 min before tetanus and continuously superfused over the tissue. Actually, in the presence of 5HT, the PTP lasted longer: 30 min in WT ganglia (Fig. 3.26, panel A) and 80 min in α 5 β 4KO (Fig. 3.26, panel B). Maximal CAP amplitudes that were reached during post tetanic potentiation were insignificantly minor ($\sim$ 160% in WT and $\sim$ 220% in α 5 β 4KO). Two-way ANOVA revealed a time effect corresponding to a classical PTP in both genotypes ($F_{45,180} = 152.4$; $p < 0.0001$). Hence, in WT+5HT CAPs were significantly increased until minute 25, and in α 5 β 4KO+5HT CAPs were significantly increased until minute 75.

The little increase of $\sim$ 10% that seemed to persist for two more hours in ganglia pre-treated with 5HT was not significant. Nevertheless it shows a trend that would verify the role of 5HT₃-receptors for gLTP as described by Alkadhi *et al.* 1996 in rats.

The exact function of 5HT₃-receptors for gLTP is not known, but it is presumed that 5HT₃-receptors (which are highly permeable to calcium) enhance presynaptic intracellular Ca²⁺-levels (Nichols & Mollard, 1996; Ronde & Nichols, 1998), which lead to a larger release of ACh that is responsible for gLTP (Brown & McAfee, 1982). In the marine gastropod *Aplysia*, 5HT was shown to modulate ACh release indirectly by increasing Ca²⁺ influx through N-type channels (Baxter & Byrne, 1989; Hochner & Kandel, 1992). In the mammalian SCG, serotonin is presumably released from small intensely fluorescent (SIF) cells that are thought to act presynaptically to modulate the quantity of acetylcholine (North *et al.*, 1980; Dun & Karczmar, 1981; Hadjiconstantinou *et al.*, 1982; Alkadhi *et al.*, 1996). In rat SCGs, SIF cells occupy 1% of all ganglion cells, while in mouse SCGs only 0.3% of the cells are SIF cells (Madariaga-Domich & Taxi, 1986). This could explain why I was not able to measure gLTP in untreated mice and why the treatment with 5HT enhanced the potentiation to only 110% instead of 140% as recorded in rats.

In the past, the expression of gLTP in sympathetic ganglia has been found both *in situ* (Bachoo *et al.*, 1992; Morales *et al.*, 1994; Alzoubi *et al.*, 2010) and *in vitro* (Brown & McAfee, 1982; Briggs *et al.*, 1985; Minota *et al.*, 1991; Alkadhi *et al.*, 1996) in different animal models including the rat SCG (Brown & McAfee, 1982; Briggs & McAfee, 1988; Alkadhi *et al.*, 1996; Heppner & Fiekers, 2003), the cat superior cervical, stellate and lumbar ganglia (Bachoo & Polosa, 1991; Morales *et al.*, 1994), the guinea pig SCG (Weinreich *et al.*, 1995; Albuquerque *et al.*, 1997), the chick parasympathetic ciliary

ganglion (Scott & Bennett, 1993a) and the bullfrog sympathetic ganglia (Kuba & Kumamoto, 1986; Kumamoto & Kuba, 1986; Minota *et al.*, 1991). Different methods including CAP recordings, EPSP recordings and measurement of released ACh have been applied to study gLTP.

To my knowledge, gLTP has never been found or tested in mice. Unlike for other animal models, my data indicate that mouse SCGs are not able to produce gLTP *per se* in response to tetanic stimulations, possibly because of their reduced content of SIF cells. Only after a pre-treatment with 5HT, CAPs increased their amplitudes by ~10%, this enhancement being very small and not significant compared to rat SCGs. Nevertheless this trend lasted over three hours, a prerequisite for LTP.

Interestingly, this little (not significant) enhancement of ~10% could be similarly induced both in WT and in $\alpha 5\beta 4$ KO mice when they were treated with 5HT. Assuming that WT contain 85% more nAChRs than $\alpha 5\beta 4$ KO mice, this would indicate that our “reduced form of LTP” is not dependent on the number of postsynaptic receptors. Although it is still unclear whether in general LTP is due to pre- or post-synaptic mechanisms, postsynaptic mechanisms for gLTP that have been previously proposed include the number or postsynaptic receptor (and therefore increased nicotinic receptor sensitivity) caused by the titanic stimulation (for review see Cifuentes *et al.* 2013). If, on the other hand, the overall number of receptors cannot be directly related to a specific genotype, my results support the idea that presynaptic mechanisms as the SIF-cell-induced release of ACh prevail in generating gLTP in mouse SCGs and suggest that the amount of SIF cells is similar in WT and KO mice.

Even though initially proposed, I did not proceed in investigating LTP mechanisms (in terms of changing extracellular Ca^{2+} levels, checking for the role of the $\alpha 7$ receptor) as I was unable to see a robust LTP in mice SCGs as described for other mammals.

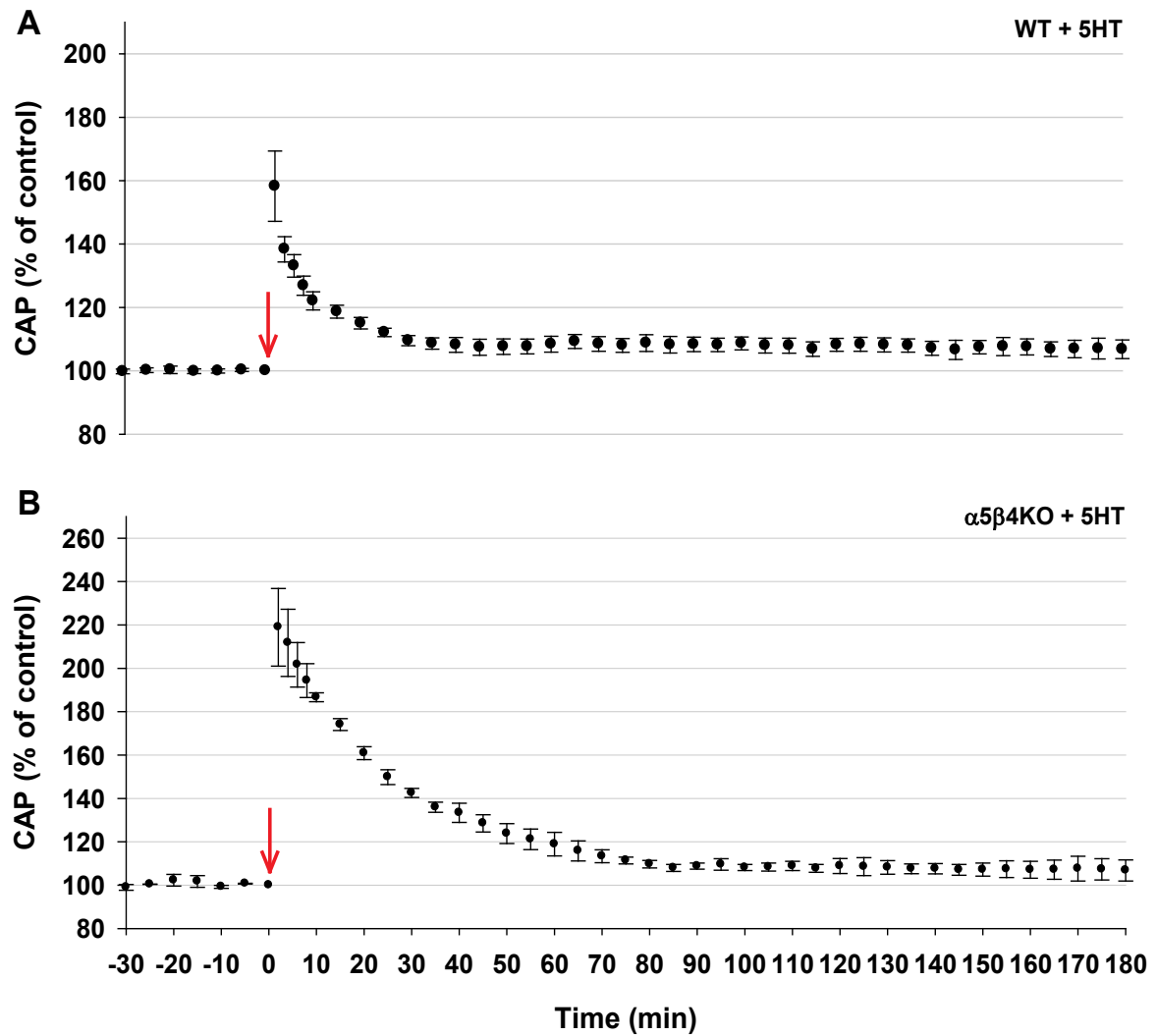


Figure 3.26: Trend to gLTP in the isolated SCG treated with serotonin (5HT). **A)** WT treated with 380 μ M HMB to get halfmaximal responses. **B)** $\alpha 5\beta 4$ KO mice treated with 20 μ M HMB to get halfmaximal responses. Both genotypes were pre-treated for 20min with 5HT 10 μ M. A train of pulses (20 Hz for 20 s; arrowhead) induces a brief post-tetanic potentiation, followed by a subtle potentiation (of 10%) lasting over 3 hours. This increase is not significant. The response is expressed as percentage of a control CAP recorded immediately before the tetanus. CAPs were evoked by supramaximal pulses to the cervical sympathetic trunk. For both genotypes, data show the mean of 3 ganglia $\pm$ SEM.

PART III:

INTRACELLULAR RECORDINGS OF THE EPSP

3.10 Excitatory postsynaptic potentials (EPSPs)

CAP recordings showed that neurotransmission in untreated mouse SCGs was in general similar in all genotypes, except of higher stimulus thresholds needed for $\alpha 5\beta 4$ KO mice. As the stimulus thresholds are a property of the presynaptic neurons, we further wanted to examine the impact of distinct receptors at the postsynaptic site. Thus, the second part of the study consisted in intracellular recordings of excitatory postsynaptic potentials (EPSPs) generated by postsynaptic neurons within the SCG.

Figure 3.27 shows a typical waveform of 20 superimposed EPSPs from a WT mouse.

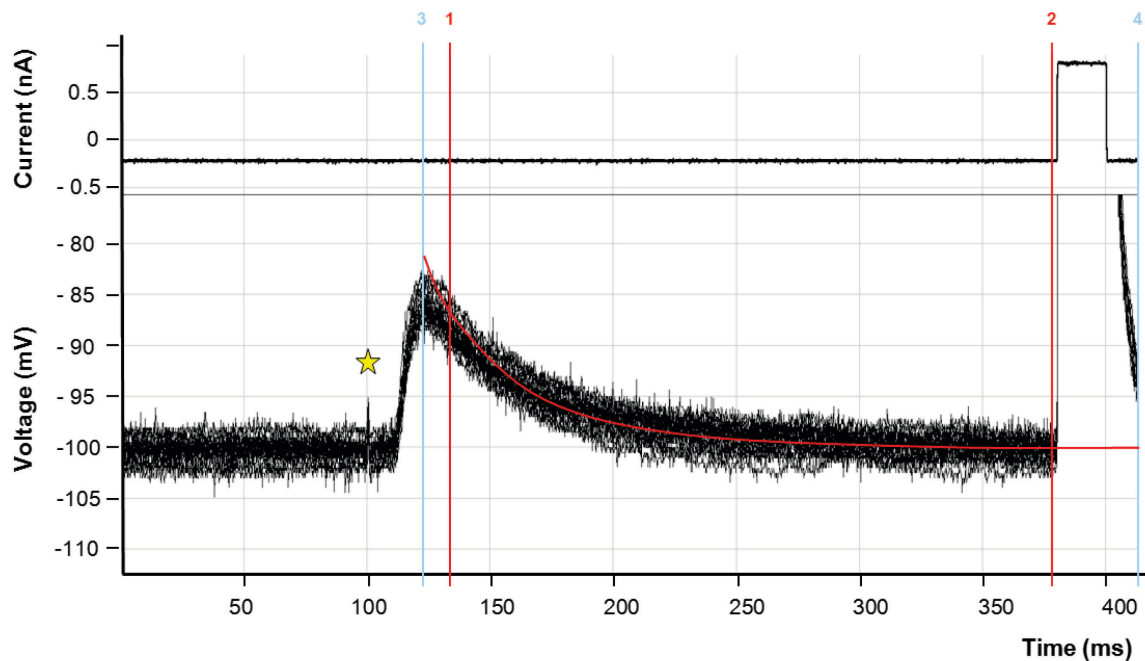


Figure 3.27: Typical recording of an excitatory postsynaptic potential (EPSP). EPSP (20 superimposed traces, lower part) were recorded from the SCG of a 6 week old WT-mouse. The membrane potential was adjusted to -100 mV via current injection of -0.2 - -0.4 nA (upper part) to avoid the generation of APs. The traces were averaged, fitted by a double exponential decay between cursors 1 and 2 and fits were extrapolated to cursors 3 and 4. Electrical stimuli were given at supramaximal intensity at 0.33 Hz. The yellow star indicates stimulus artefact. A depolarising pulse (20 ms) given at the end of the sweep directly through the recording electrode helped monitoring the steadiness of the membrane resistance (depolarization cut, y-axis has been adjusted to better see the EPSP).

3.11 Membrane properties: EPSP amplitudes and time constants

Table 3.5 summarises the passive membrane properties and physiological characteristics of the tested neurons. Minor, not significant, differences were found between genotypes. Neurons in all ganglia had resting membrane potentials of around -50 mV, a value that equals findings by Jobling and Gibbins 1999 (-49.7 ± 0.8). Compared to this group, the amplitudes of the action potentials that I measured (~ 63 -66 mV) were about 25mV lower (89.5 ± 1.4 mV), and input resistances (~ 170 -215 M Ω) were consistently higher (109.7 ± 5.7 M Ω). These differences may be related to the mouse age, Jobling and Gibbins possibly using older adult mice than I did.

Table 3.5: Electrophysiological properties of intracellular recordings in mouse SCGs

	WT (n = 5)	$\alpha 5\beta 2$ KO (n = 5)	$\alpha 5\beta 4$ KO (n = 5)
RMP (mV)	-49.3 ± 2.3	-48.5 ± 2.3	-53.3 ± 2.9
R_{in} (MΩ)	213.2 ± 20.5	206.8 ± 28.3	175.6 ± 18.5
AP amplitude (mV)	64.5 ± 4.3	65.8 ± 3.2	63.7 ± 3.3
Threshold stimulus (μA)	20.6 ± 8.5	15.6 ± 2.7	18.6 ± 8.2
Holding C (nA)	-0.3 ± 0	-0.4 ± 0.1	-0.2 ± 0
EPSP amplitude (mV)	8.8 ± 1.3	9.3 ± 1.9	11.8 ± 1
Extrp EPSP amplitude (mV)	9.8 ± 1.3	11 ± 2.2	13 ± 0.9
EPSP τ_1 (ms)	32.4 ± 4.3	35.3 ± 2.9	77.2 ± 6.3
EPSP τ_2 (ms)	9.7 ± 0.3	10.7 ± 2.5	23.9 ± 5.2

Values are given as means $\pm$ SEM. AP = action potential, C = holding current required to maintain RMP at -100 mV, Extrp = extrapolated, R_{in} = input resistance, RMP = resting membrane potential, τ_1 = time constant slow component, τ_2 = time constant fast component. Statistical differences as determined by a one-way ANOVA were found only between the time constants (see Figure 3.30.)

The lowest stimulations (50 μ s duration) that produced a postsynaptic response required current intensities ranging between 15 μ A and 20 μ A. This is about half as much needed for CAPs (~ 30 μ A in WT and $\alpha 5\beta 2$ KO, ~ 70 μ A in $\alpha 5\beta 4$ KO). Contrary to CAP recordings, no difference in thresholds was found between genotypes.

At RMP, I could not detect any weak input since no neuron responded with subthreshold EPSPs: Against our expectations, all tested cells produced one AP (phasic discharge) as a first response to the threshold stimulus, indicating that either all inputs triggered by the minimal stimulus were strong or that multiple EPSPs were activated simultaneously (Wang *et al.*, 2010). This finding not only diverges from the hypothesis to find relatively more weak inputs in $\alpha 5\beta 4$ KO mice, but it also contrasts the majority of the SCG studies made in rodents (mostly rats), stating that SCG neurons receive at least one

strong input and *additionally* several weak inputs (see chapter 1.4.3; Jänig 1995; McLachlan and Meckler 1989; Wang *et al.* 2010). On the other hand, Jobling and Gibbins also had difficulties in discerning between weak and strong inputs, as voltage threshold to stimulate one or the other input class was too similar. Quite differently, Vernino's group found several stepwise increasing EPSPs in the mouse SCG by gradually increasing the stimulation intensity. Only 20% of the neurons generated action potentials as the first synaptic response (Wang *et al.*, 2010).

Hence, in order to see the shape of the EPSP underlying the AP, cells had to be hyperpolarized down to -100 mV so that the threshold for the generation of APs was not reached. In most cases, this high hyperpolarisation was necessary because neurons still fired APs at -80/-90 mV. About 10% of the neurons still showed an AP at -100 mV that could not be blocked even injecting more hyperpolarising current (up to -180mV). Along these lines, Jobling and Gibbins also reported that in the mouse SCG the AP following orthodromic nerve stimulation was difficult to prevent by hyperpolarising currents (Fig. 4 C and D in Jobling and Gibbins, 1999). EPSP amplitudes of hyperpolarised cells did not statistically differ between WT (8.8 mV), $\alpha 5\beta 2$ KO (9.3 mV) and $\alpha 5\beta 4$ KO (11.8 mV). Similarly, no difference could be found between extrapolated amplitudes, which were slightly increased respect to the measured amplitudes (Fig. 3.28, Tab. 3.5). Vernino's group who could record (weak input) EPSPs at resting membrane potential, reports EPSP amplitudes of 5.2 ± 0.8 mV (n=24).

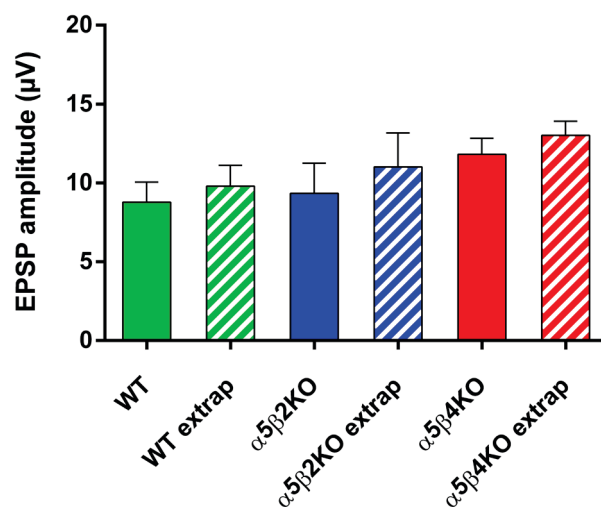


Figure 3.28: EPSP amplitudes. Amplitudes of measured and extrapolated EPSPs of WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice. No significant differences could be found between EPSP amplitudes of the tested genotypes. Columns were compared using one way ANOVA, $F = 1.247$, $p = 0.3222$, followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM. $n = 5$ for every genotype.

The shape of an EPSP is determined not only by its amplitude, but also by the time course of the rising and the falling phase. Trying to fit the EPSPs, I found that a double exponential decay best described their falling phase. The time constants τ_1 (slow component) and τ_2 (fast component) differed between genotypes. While WT and $\alpha 5\beta 2$ KO mice displayed similarly shaped curves and comparable time constants, EPSPs in $\alpha 5\beta 4$ KO mice declined with significantly shallower slopes (Figs. 3.29, 3.30). The slow component τ_1 lasted ~ 32 - 35 ms in WT and $\alpha 5\beta 2$ KO mice and was approximately doubled in $\alpha 5\beta 4$ KO mice (~ 77 ms). Similarly, the fast component τ_2 lasted ~ 10 - 11 ms in WT and $\alpha 5\beta 2$ KO mice and was more than doubled in $\alpha 5\beta 4$ KO mice (~ 24 ms) (Fig. 3.29).

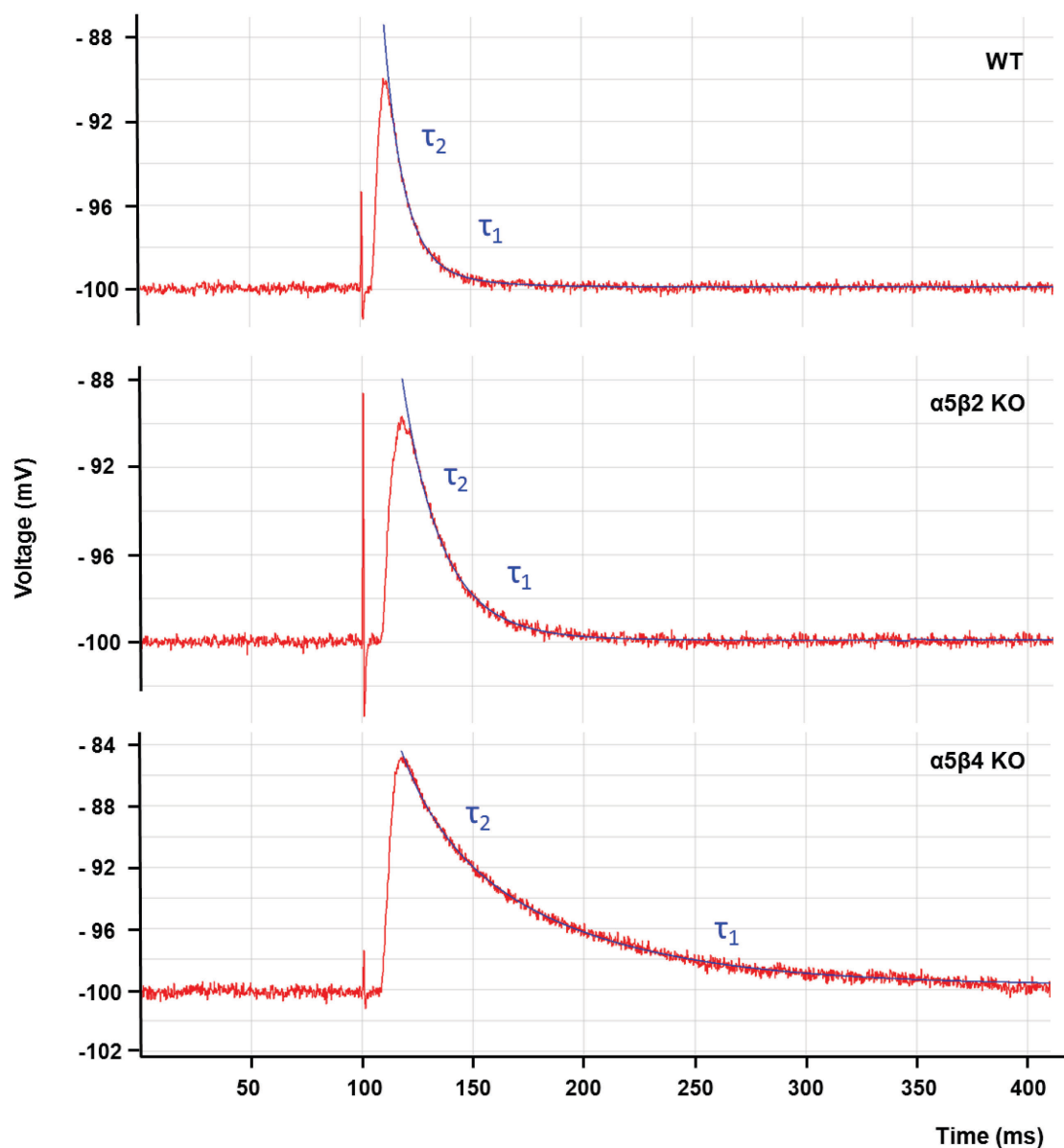


Figure 3.29: EPSP shapes. Examples of typical EPSPs (20 averaged traces, red line) of WT (upper trace), $\alpha 5\beta 2$ KO (middle trace) and $\alpha 5\beta 4$ KO (lower trace) mice. EPSPs were fitted (blue line) to a double exponential curve. Both the slow component (τ_1) and the fast component (τ_2) are slower in $\alpha 5\beta 4$ KO than in WT or $\alpha 5\beta 2$ KO mice.

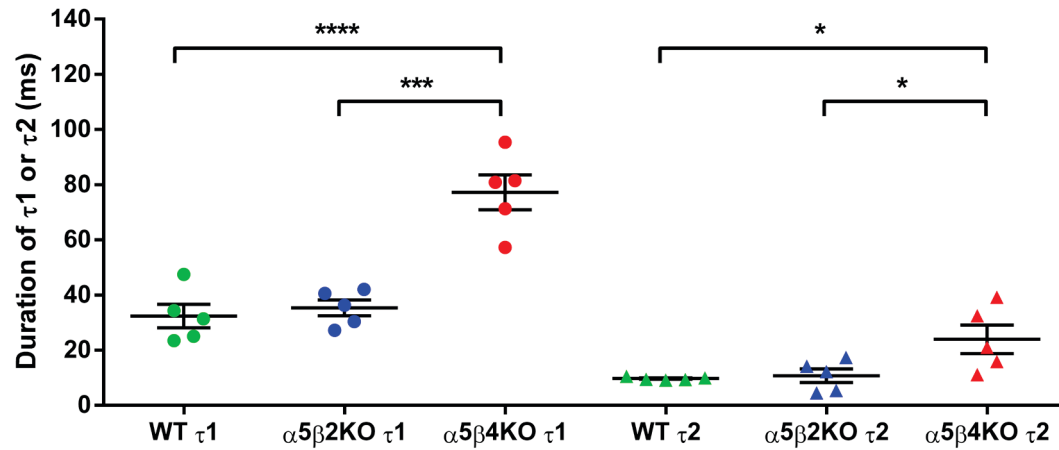


Figure 3.30: Time constants of EPSPs. Duration of the slow and fast component (τ_1 and τ_2 , respectively) of the double exponential EPSP decay in WT, $\alpha 5\beta 2$ KO and $\alpha 5\beta 4$ KO mice. Both τ_1 and τ_2 differed significantly between $\alpha 5\beta 4$ KO mice and the other two genotypes. Data were compared using one way ANOVA ($F_{\tau_1} = 28.55$; WT τ_1 vs. $\alpha 5\beta 4$ KO τ_1 : $p < 0.0001$; $\alpha 5\beta 2$ KO τ_1 vs. $\alpha 5\beta 4$ KO τ_1 : $p < 0.001$; $F_{\tau_2} = 5.665$, WT τ_2 vs. $\alpha 5\beta 4$ KO τ_2 : $p < 0.05$; $\alpha 5\beta 2$ KO τ_2 vs. $\alpha 5\beta 4$ KO τ_2 : $p < 0.05$), followed by Bonferroni's post-hoc multiple comparisons test. Data are mean $\pm$ SEM. $n = 5$ for every genotype.

Desensitisation of $\alpha 3\beta 2$ receptors occurs at a much faster rate than desensitisation of $\alpha 3\beta 4$ receptors (David et al., 2010; Papke & Heinemann, 1994; Fenster et al., 1997; Gerzanich et al., 1998). Hence, if the shape of the EPSP depended on the desensitisation properties of nAChRs, a significantly faster decay of the EPSP had to be expected in the $\alpha 5\beta 4$ KO mice. My findings thus support the view that the time course of EPSPs primarily depends on the gating properties of postsynaptic receptors. Due to the highly efficient breakdown of ACh by the enzyme ACh-esterases (AChE), transmitter released into the synaptic cleft has a one and only chance to bind to its receptor. Channel properties are then defined by the burst properties, resolved at the single channel level. Several studies have shown a correlation between the time constant(s) of macroscopic current/potential decays (EPSC/EPSP) and the time constant(s) of burst durations recorded in single channels (Colquhoun *et al.*, 1997; Wyllie *et al.*, 1998; Grosman & Auerbach, 2001). Accordingly, for nAChR – with their typical kinetic transition scheme between open states, short-lived shut states and long-lived shut states – the slowest time constant of the synaptic response (τ_1 in our case) coincides with the time constant of the slowest burst duration component. Applied to our recordings and the measured values for the slow EPSP decay component τ_1 , this would mean that receptors in WT and $\alpha 5\beta 2$ KO mice, when opening, produce bursts lasting 32-35 ms, while nAChRs in $\alpha 5\beta 4$ KO ganglia produce bursts of about 78 ms (see Table 3.5). These results fit to the single channel recordings obtained by our colleague Anna Ciuraszkiewicz (Ciuraszkiewicz *et al.*, 2013).

She showed that burst duration time constants of $\alpha 3\beta 4$ -receptors (recorded in WT and $\alpha 5\beta 2$ KO) ranged between 27 ms and 38 ms, thus corresponding to the values that I recorded ($\tau_1 = 32$ -35 ms). However, unlike her observation that bursts of $\alpha 5$ -containing receptors (in WT and $\beta 2$ KO mice) lasted significantly longer (115 ms-150 ms) than bursts of $\alpha 3\beta 4$ -receptors (in $\alpha 5\beta 2$ KO mice), I could not detect significant differences in the decline of EPSPs between these genotypes. If the above mentioned hypothesis - that the shape of an EPSP/EPSC depends on the burst duration - holds true, then bursts recorded from $\alpha 3\beta 2$ -receptors should last significantly longer. Since we have not performed single channel recordings in $\alpha 5\beta 4$ KO animals, these data are missing. However, burst times of human $\alpha 3\beta 2$ expressed in *Xenopus laevis* oocytes were shorter (0.82 ± 0.12 and 5.3 ± 0.7 ms) than burst times of $\alpha 3\beta 4$ receptors (1.7 ± 0.1 and 16 ± 1 ms) (Nelson & Lindstrom, 1999).

3.12 Summary and outlook

Taken together, my results can be summarised as follows:

1) Different morphology:

$\alpha 5\beta 4$ KO mice gain weight more slowly than other genotypes; their SCGs are sometimes malformed, seem to need longer time to mature, and are less tightly ensheathed by connective tissue.

2) Normal neurotransmission:

When stimulated supramaximally, SCGs produce CAPs that do not differ between genotypes and are not reduced in $\alpha 5\beta 4$ KO mice, although they have just 15% of overall nAChRs found in WT mice.

3) High stimulus thresholds in $\alpha 5\beta 4$ KO:

Compared to the other genotypes, the stimulus necessary to produce threshold CAP responses requires nearly two times the currents in mice lacking the $\beta 4$ subunit.

4) $\alpha 5$ affects pharmacology and affinity of nAChRs:

The potency of HMB differs significantly between $\alpha 3\beta 4$, $\alpha 3\beta 45$, and $\alpha 3\beta 2$ nAChRs.

5) More $\alpha 5$ on the cell surface and in SCG synapses than predicted by IP:

Our modelling based on the effects of HMB suggests that functional $\alpha 3\beta 4\alpha 5$ receptors present in WT ganglia and in cell cultures outnumber $\alpha 3\beta 4$ receptors. Quite the opposite, previous IP experiments have shown that $\alpha 3\beta 4$ outnumber $\alpha 3\beta 4\alpha 5$ with a 3:1 ratio.

6) $\alpha 7$ plays no role in the SCG:

$\alpha 7$ does not contribute to neurotransmission in the mouse SCG.

7) Paired pulse facilitation in WT:

WT mice exhibit a paired-pulse facilitation of CAPs when stimulation is done with a time gap of 200 ms (5 Hz). In contrast, the response to a second pulse is never facilitated in $\alpha 5\beta 2$ KO or $\alpha 5\beta 4$ KO mice.

8) No gLTP in the mouse SCG

Ganglionic long term potentiation cannot be induced in mice. When pre-treated with serotonin, both WT and $\alpha 5\beta 4$ KO SCGs show a long lasting facilitation of the synaptic response by ~10%.

9) Only strong inputs in the mouse SCG:

When intracellularly recorded, no difference in terms of synaptic strength exists between genotypes. First responses at resting membrane potential are always APs and the underlying EPSPs at -100 mV have similar amplitudes.

10) Prolonged EPSP decay in $\alpha 5\beta 4$ KO:

EPSPs in $\alpha 5\beta 4$ KO mice decay more than two times slower ($\tau 1 \sim 80$ ms) than EPSPs in WT or $\alpha 5\beta 2$ KO mice ($\tau 1 \sim 30$ ms).

It is most widely accepted that synaptic transmission is regulated by the amount of released neurotransmitter, and that postsynaptic receptors are present in surplus (see Walmsley et al., 1998). My observations based on the use of $\alpha 5\beta 4$ KO mice are in general agreement with this hypothesis. However, in the mouse SCG, postsynaptic nAChRs have been shown to send retrograde signals to the presynaptic site which induces the expression of the high affinity choline transporter in the afferent neurons. Hence, re-uptake of choline ceases when postsynaptic nAChRs (in $\alpha 3$ KO mice) are missing (Krishnaswamy & Cooper, 2009). Given that CAP amplitudes in $\alpha 5\beta 4$ KO mice decline at lower frequencies of stimulation compared to the other genotypes, and since the stimulation threshold for eliciting CAPs is also increased, it is possible that such a mechanism also takes place in these mice.

Contrary to our IP experiments, which showed that the number of $\alpha 3\beta 4$ exceeds the number of $\alpha 3\beta 4\alpha 5$, our recordings imply that more $\alpha 3\beta 4\alpha 5$ receptors than $\alpha 3\beta 4$ receptors are expressed on the neuronal cell surface and at the postsynaptic site. These receptors may play an important role in short term synaptic plasticity.

In conclusion, my data demonstrate that $\beta 4$, the classical β -backbone subunit of nAChRs in the autonomic nervous system, is dispensable and can be efficiently replaced by $\beta 2$, the classical β -backbone subunit of nAChRs in the central nervous system. Furthermore, neurotransmission was unimpaired in $\alpha 5\beta 4$ KO mice when the afferent nerve was stimulated with maximal intensity, even though $\alpha 5\beta 4$ KO contain only 15% of nAChRs of WT and $\alpha 5\beta 2$ KO mice. These observations allow for two explanations: $\alpha 3\beta 2$ receptors in $\alpha 5\beta 4$ KO mice are either targeted more efficiently to postsynaptic sites, or synaptic transmission in the SCG functions with a high safety factor. Future experiments based on immunocytochemistry with subunit-specific nAChR antibodies may help to resolve this question.

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09/10	Consultant for a “cost-of-living-survey” at the International Atomic Energy Agency (IAEA), Div. of Human Resources, UNO, Vienna, Austria
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e) Associations

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- David, R., Ciuraszkiewicz, A., Simeone, X., Orr-Urtreger, A., Papke, R. L., McIntosh, J. M., Huck, S. and Scholze, P. (2010) Biochemical and functional properties of distinct nicotinic acetylcholine receptors in the superior cervical ganglion of mice with targeted deletions of nAChR subunit genes. *The European journal of neuroscience*, 31, 978-993.

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Poster: Simeone X., Draskovits M., Siebert D., Schnürch M., Mihovilovic M., Willeit M., Ernst M (2015).
Novel GABA_A receptor ligands
- 04/13 Neuroscience Vienna Network Meeting, IST Austria, Klosterneuburg, Austria.
Poster: Simeone X., Burgstaller M., Tichy H. (2013): ON and OFF dichotomy in the cockroach olfactory system
- 05/11 Nicotinic Acetylcholine Receptors 2011, Wellcome Trust Scientific Conference, Hinxton, Cambridge, UK.
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- 07/10 VII FENS-meeting (Forum of European Neuroscience) in Amsterdam, Netherlands.
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- 09/09 XI ANA-meeting (Austrian Neuroscience Association) in Salzburg, Austria.
Poster: Simeone X., Scholze P., Huck S. (2009). Trans-ganglionic neurotransmission in the sympathetic nervous system of mice with deletions of distinct nicotinic acetylcholine-receptor subunit genes
- 09/07 IX ESITO congress (European Symposium for Insect Taste and Olfaction) in Roscoff, France.
Poster: Barrozo R., Simeone X., Gadenne C., Anton S. (2007). Olfactory plasticity in insects: the role of biogenic amines on pheromone sensitivity

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