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The role of the Cholinergic System on Plasticity in the Basolateral Nucleus of the Amygdala

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Abstract

The amygdala and the cholinergic system play important roles in learning and memory. The amygdala receives substantial cholinergic innervation and in itself expresses differences in this innervation. $p75^{NTR}$ is one of the primary receptors of cholinergic neurons and transgenic mice that are missing exon IV of the p75 neurotrophin receptor locus, display a change in cholinergic innervation. The loss of $p75^{NTR}$ can induce changes in learning and memory so it was hypothesized $p75^{EXIV}$ animals would display an enhancement in cholinergic induced plasticity in the amygdala, due to increased cholinergic innervation in these mice, and a difference between the BL and La nuclei would be seen. As expected, ChAT immunohistochemistry showed a stark difference in cholinergic innervation between the BL and La nuclei of the amygdala. Field potential recordings, as well as PPF and LTP, did not show any differences between BL and La in wt animals therefore sharp microelectrode recording and a standard plateau firing paradigm were used to determine intrinsic differences in projection neurons for the BL and La nuclei. Sharp recordings revealed a difference in plateau firing induction for BL neurons as well as a hyperpolarizing membrane deflection. As in wt animals, there were no differences seen between the BL and La for field recordings in ko mice; although, paired pulse facilitation and LTP elucidated a reduction in transmitter release as well as disrupted postsynaptic maintenance in $p75^{EXIV}$ animals as compared to wt. Field potential recordings with cholinergic challenge also indicated a difference in cholinergic signaling in $p75^{EXIV}$ animals. In contrast with the original hypothesis, $p75^{EXIV}$ animals did not display an enhancement of cholinergic induced plasticity in the amygdala but rather impairment.

Zusammenfassung

Die Amygdala und das cholinerge System spielen eine wichtige Rolle bei Lern- und Gedächtnisprozessen. Die Amygdala wird sehr reichhaltig von cholinergen Fasern innerviert und zeigt dabei in sich selbst ausgeprägte Unterschiede bezüglich dieser

Innervation. P75NTR ist einer der primären Rezeptoren cholinerger Neurone und transgene Mäuse, welchen der Gen-Lokus Exon IV des p75-Neurotrophinrezeptors fehlt, haben eine phänotypisch deutlich veränderte cholinerge Innervation. Der Verlust des P75-Neurotrophinrezeptors kann Veränderungen bezüglich von Lern- und Gedächtnisprozessen induzieren. Bei entsprechenden transgenen P75ExIV-Mäusen wurde eine durch das cholinerge System induzierte erhöhte Plastizität in der Amygdala aufgrund der hier generell reichhaltigeren cholinergen Innervation und diesbezüglich auch Unterschiede zwischen BL- und La-Nukleus postuliert. ChAT-immunohistochemische Untersuchungen zeigten dabei erwartungsgemäß, dass sich die entsprechende Innervation bei beiden Kernen erheblich unterscheidet. Durch extrazelluläre Feldpotentialableitungen (fEPSPs), sowohl PPF-Messungen und auch Registrierung der LTP, konnten bei Wildtyp-Mäusen keine Unterschiede zwischen BL- und La-Kern gezeigt werden. Daher wurden intrazelluläre Ableitungen mit der Glasmikroelektrode und standartisierte *plateau-firing* Paradigmen angewandt, um etwaige Unterschiede bezüglich der intrinsischen Eigenschaften der jeweiligen Projektionsneurone beider Kerne zu untersuchen. So konnte gezeigt werden, das die Induktion des *plateau firing* bei BL-Neuronen und La-Neuronen verschieden ist und dass BL-Neuronen eine Hyperpolarisationsablenkung des Membranpotentials zeigen. Bei Knockoutmäusen konnten durch fEPSP-Messungen ebenso keinerlei Unterschiede zwischen BL- und La-Kern festgestellt werden, wenn auch PPF- und LTP-Messungen bei transgenen Tieren im Vergleich zu Wildtypmäusen eine etwas verringerte Transmitterausschüttung sowie einen veränderten Zeitverlauf der LTP aufzeigten. Wurden fEPSPs unter cholinerger Anregung gemessen, war bei den transgenen P75ExIV-Tieren das cholinerge Signaling verändert. Im Gegensatz zur ursprünglichen Hypothese war die durch das cholinerge System induzierte Plastizität in der Amygdala bei P75ExIV-Mäusen nicht erhöht, sondern sogar reduziert.

Abbreviations

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$
µl	micro liter
µs	micro second
2-APB	Diphenylboric acid 2-aminoethyl ester
AcCoA	acetyl-Coenzyme A
ACh	acetylcholine
AChE	acetylcholinesterase
AChRs	acetylcholine receptor(s)
aCSF	artificial cerebral spinal fluid
AD	Alzheimer's Dieseas
AHP	after hyperpolerization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AP	action potential
BDNF	brain derived neurotrophic factor
BFCNs	basal forebrain cholinergic neurons
BL	basolateral nucleus
BLA	basolateral amygdala
BM	basomedial nucleus
BV	basoventral
CaMKII	calcium/calmodulin-dependent protein kinase II
CAN	calcium activated nonspecific cation current
Ce	central nucleus
CED	cambridge electronic design
ChAT	choline acetyltransferase
CHT	sodium dependant choline transporter

CNS	central nervous system
CS	conditioned stimulus
DAG	diacyglycerol
DAPI	2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride
DG	Dentate Gyrus
DNA	dxeoxyribonucleic acid
EC	external capsule
EEG	electroencephalogram
E-LTP	early phase long term potentiation
fEPSPs	field excitatroy synaptic potential(s)
fp	field potential
FST	fine science tools
GDP	guanosine diphospate
GEF	guanine nucleotide exchange factor
GPCRs	G protein coupled receptors
GTP	guanosine triphospate
HDB	horizontal limb of the diagonal band of Broca
HFS	high frequecncy stimulation
HS	horse serum
Hz	Hertz
I_{AHP}	afterhyperpolerization current
IP_3	inositol 1,4,5-triphosphate
ISI	interstimulus interval
K_{Ca}	calcium activated potassium current
ko	knock out animal
La	lateral nucleus
LGIC	ligand gated ion channel
LTD	long term depression
L-LTP	late phase long term potentiation

LTP	long term potentiation
mAChRs	muscarinic acetylcholine receptor(s)
Me	medial nucleus
mGluR	metabotropic glutamate receptors
ml	mililiter
ms	milisecond
MS	medial septum
MΩ	mega ohm
nA	nano ampre
nAChRs	nicotinic acetylcholine receptor(s)
NB	nucleus basalis
NbM	nucleus basalis of meynert
NGF	nerve growth factor
NMDAR	N-Methyl-D-Aspartate Receptor
NT-4	neurotrophin
NTRs	neurotrophin receptor(s)
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFC	prefrontal cortex
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
PPF	paired pulse facilitation
PTP	post tetanic potentiation
PTSD	post traumatic stress disorder
REM	rapid eye movement

RT	room temperature
s	second
sem	standard error of the mean
SI	substantia innominata
SK	small conductance calcium-activated K potassium channels
TBE	tris-borate-EDTA
TNFR	necrosis factor receptor
TrkA	tropomyosin receptor kinase A
UR	unconditioned response
US	unconditioned stimulus
VAChT	vesicular ACh transporter
VDB	vertical band of Broca
VGCCs	voltage gated calcium channel
V_M	membrane potential
wt	wild type animal

1 Introduction

Classically seen as the region responsible for emotions and emotion based learning, the amygdala is now being implicated in other forms of learning as well as cognitive performance. It displays a key feature in the consolidation of memory as greater amygdala activation determines which sensory inputs will be stored long term rather than transiently. Experiences that are able to provoke a strong amygdala response generally give rise to late phase long term potentiation (L-LTP) resulting in lasting and sustainable plasticity and therefore memory retention; whereas weakly evoked amygdala responses usually result in the deterioration over time of resulting memories or their complete lack of retention [1].

As part of the overall limbic system, the amygdala shares connections with the hippocampus, the entorhinal cortex, dentate gyrus as well as other cortices [2]. It receives substantial innervations from the Nucleus Basalis of Meynert (N. Meynert) linking it with basal forebrain cholinergic neurons (BFCNs). The primary cholinergic ligand is Acetylcholine, which binds to either nicotinic acetylcholine receptors (nAChRs) or muscarinic acetylcholine receptors (mAChRs) [3]. Survival of BFCNs is highly dependent upon the neurotrophin Nerve Growth Factor (NGF) and its high affinity receptor tropomyosin receptor kinase A (TrkA). NGF has also been shown to facilitate neurotransmission between the N. Meynert and that of the amygdala [4].

Degeneration of Basal Forebrain Cholinergic Neurons has been shown to be one of the principal contributing factors to Alzheimer's disease (AD). The loss of BFCNs gives rise to lower innervation of cortical regions resulting in memory lost, the inability to retain new information, as well as attention deficits [5]. One of the primary causes for this loss of neurons is the lack of binding of NGF to TrkA receptors. Mice that are lacking TrkA receptors show a substantial decrease in amygdala cholinergic innervation as well as decimation in the size of cholinergic neurons [6], whereas mice that

are lacking the low affinity neurotrophin receptor $p75^{NTR}$ have an increase in the amount of afferent amygdala cholinergic fibers as well as larger neuronal size [7].

As already stated, the amygdala is now being seen as playing a pivotal role in memory consolidation. The ability of the amygdala to modulate other members of the limbic system has been demonstrated in great detail of late. Electrical stimulation of the amygdala can reinforce LTP in the Dentate Gyrus (DG) as well as transform early phase LTP (E-LTP) into L-LTP [8]. The ability of the amygdala to produce persistent firing due to cholinergic modulation has also been demonstrated [9]. It is thought that persistent firing gives a single neuron the ability to retain transient information until such information can be consolidate.

The cholinergic system is integral to the central and peripheral nervous systems and to the neuromuscular junction of skeletal and smooth muscles. Due to its wide distribution, the cholinergic system has an extremely broad physiological relevance both in normal and pathological states [10]. In the brain, the cholinergic system plays a central role in analgesia, cognition, memory and neuroprotection, whereas in the skeletal muscle the system is responsible for contraction. Impairment in cholinergic transmission results in selective loss of cholinergic neurons with subsequent neuropathological conditions.

2 Literature Review

2.1 Cholinergic System

The broad scope of the cholinergic system is far too vast for the purposes of this dissertation. It is involved in regulating the CNS [11], PNS [12], as well as other areas where the cholinergic system is considerably overlooked; and plays a role in matters such as cell proliferation and differentiation [13]. Highly specialized systems, such as that of the immune system, have their own integrated cholinergic system [10, 14]. Therefore, the brief literature review here will focus on the cholinergic system of the basal forebrain.

2.1.1 Discovery of Acetylcholine

The existence of neurotransmitters is a process that took 35 years and much controversy to fully establish. It was tempestuously contested whether transmission across synapses was electrical or chemical and the early trailblazers were forced to work with the peripheral nervous system (PNS) prolonging the debate for another 30 years in the central nervous system (CNS) [15].

Henry Hallett Dale is contributed as being the first to discover acetylcholine. While working with the fungus ergot, Dale extracted multiple compounds which mimicked either sympathetic or parasympathetic nerve stimulation including an amine, with similar properties to adrenaline, histamine, and ergotoxine, with properties similar to acetylcholine, and finally acetylcholine [16, 17]. Acetylcholine is not a normally occurring substance in ergot but as chance favors the prepared, the ergot happened to be infected with the bacterium *Bacillus acetylcholini* and since cholinesterase is also not naturally present in ergot, there was an abundance of stable acetylcholine [15]. Dale also made a substantial contribution to the understanding of synaptic transmission as he observed that acetylcholine and muscarine exhibited the same effects at some sites but not others. However, low doses of nicotine displayed the same effect as acetylcholine at sites where muscarine did not. Thus Dale foreshadowed that the effects must be due to “receptor substances” and he labeled the differences as muscarinic and nicotinic [18]. Dale also observed that the effect of acetylcholine was “intense”, “immediate” and “extraordinarily evanescent” and thus concluded that an esterase must also exists which rapidly breaks down acetylcholine into acetic acid and an inactive choline [19].

Otto Loewi has been distinguished as the first to demonstrate that synaptic transmission was indeed chemical although it was not conclusively accepted for a conspicuously length of time. It is best to describe the work of Loewi through his own words:

The night before Easter Sunday of that year I awoke, turned on the light, and jotted down a few notes on a tiny slip of thin paper. Then I fell asleep again. It occurred to me at six o’clock in the

morning that during the night I had written down something most important, but I was unable to decipher the scrawl. The next night, at three o'clock, the idea returned. It was the design of an experiment to determine whether or not the hypothesis of chemical transmission that I had uttered seventeen years ago was correct. I got up immediately, went to the laboratory, and performed a simple experiment on a frog heart according to the nocturnal design.

The hearts of two frogs were isolated, the first with its nerves, the second without. Both hearts were attached to Straub cannulas filled with a little Ringer solution. The vagus nerve of the first heart was stimulated for a few minutes. Then the Ringer solution that had been in the first heart during the stimulation of the vagus was transferred to the second heart. It slowed and its beats diminished just as if its vagus had been stimulated. Similarly, when the accelerator nerve was stimulated and the Ringer from this period transferred, the second heart speeded up and its beats increased. [20]

Loewi named the first substance secreted via stimulation of the vagus nerve *Vagusstoff* and the other *Acceleransstoff*. His initial publication "Über humorale Übertragbarkeit der Herznervenwirkung" [21] started what would be a fire storm debate as to whether or not synapses did indeed release chemicals for the next 30 years. For their revolutionary work, both Dale and Loewi were awarded The Nobel Prize in Physiology or Medicine in 1936. A better appreciation of the two men can be obtained from reading Feldberg 1970 [22] and Dale 1962 [23].

2.1.2 Acetylcholine Synthesis, Release, and Metabolism

Acetylcholine (ACh) synthesis is facilitated by the enzyme choline acetyltransferase (ChAT) in nerve terminals. The enzyme catalyzes the acetylation of choline by acetyl-Coenzyme A (AcCoA) which is derived from glucose [24]. Following synthesis, ACh is then transferred into synaptic vesicles via a vesicular ACh transporter (VAChT) where it is stored until release [25].

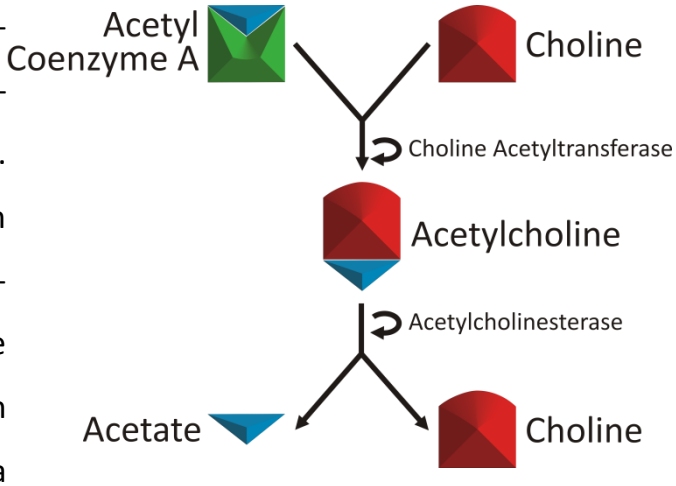


Figure 2.1.1 | ACh Synthesis and Hydrolysis ACh is acetylated with choline by AcCoA. It is hydrolyzed into acetate and choline via AChE

Release occurs when cholinergic neurons are sufficiently depolarized creating an action potential (AP) allowing synaptic vesicles to bind to the presynaptic terminal and releasing ACh into the synaptic cleft via exocytosis. Upon release, ACh is quickly hydrolyzed by the enzyme acetylcholinesterase (AChE) into choline and acetate. AChE is extremely efficient as one molecule of AChE has the ability to hydrolyze 5000 molecules of ACh per second [26, 27]. Choline is then returned to the presynaptic terminal via a high affinity, sodium dependent choline transporter (CHT) located at the plasma membrane of cholinergic neurons [28, 29]. Since ChAT and VAChT are both necessary for the creation and storage of ACh, markers for ChAT and VAChT are acceptable means for elucidating cholinergic neurons [30, 31].

2.1.3 Acetylcholine Transmission

The mode of transmission of acetylcholine is under great scrutiny at the moment due to its brazen contradictory effects. It is suggested that there exist underlying basal levels of ACh that incur fluctuations and thus accounts for the actions of ACh; implying a form of volume transmission. Volume transmission being that, neurotransmitter release moves into the extra synaptic space and produces effects by binding to receptors outside the synapse [32]. On the other side of the coin, is the more classical form of neurotransmitter release known as wired transmission. This being the

case that ACh is released into the synaptic cleft and its actions are solely contrived in the synapse; bringing forth yet two more arguments of phasic or tonic action [33].

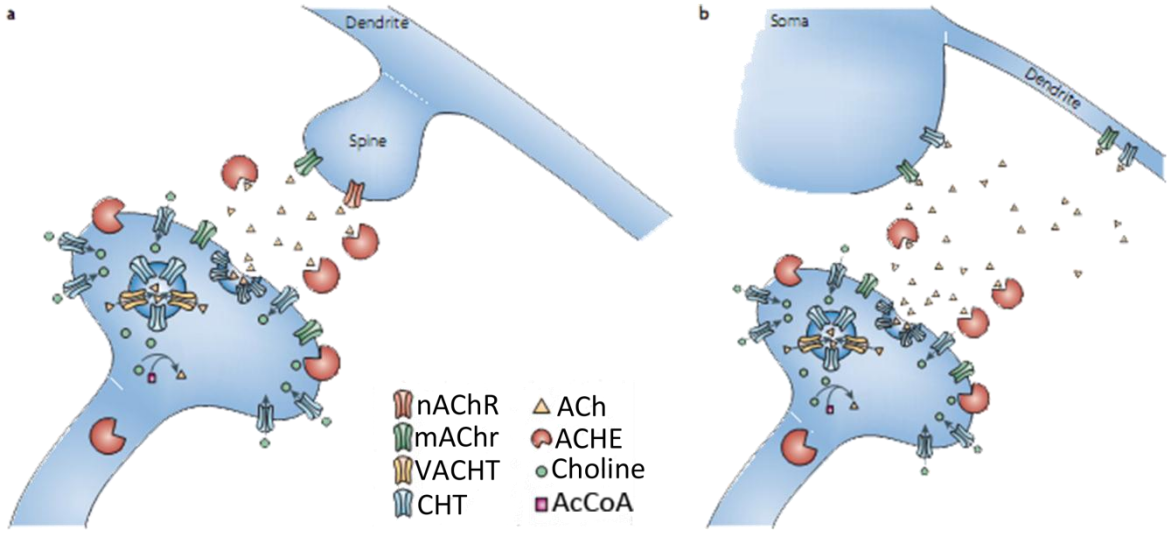


Figure 2.1.2 | The main characteristics of wired and volume transmission In the wired model of cholinergic neurotransmission (a), the presence and high catalytic activity of AChE restricts the neurotransmission to classic synapses or junctional complexes. By contrast, in the volume model of cholinergic neurotransmission (b), most presynaptic cholinergic terminals in the cortex do not form junctional complexes and so neurotransmission is mediated by ACh that escapes hydrolysis because of insufficient or regulated availability and/or activity of AChE. This ACh reaches the extracellular space and can stimulate non-junctional nAChRs and mAChRs. Adapted from Sater et al 2009 [34]

2.1.4 Cholinergic Receptors

Cholinergic receptors, also known as acetylcholine receptors (AChRs) are classified either as muscarinic acetylcholine receptors (mAChRs) or as nicotinic acetylcholine receptors (nAChRs). Both receptor types respond to their endogenous ligand ACh, however, as their name implies, only muscarinic receptors respond to muscarine while nicotinic receptors respond to nicotine.

2.1.5 Muscarinic Cholinergic Receptors

Muscarine was first discovered when the psychopharmacological actions of the mushroom *Amanita muscaria* were being investigated in 1869 [35]. There are believed to be possibly 9 different subdivisions of muscarinic receptors [36] the current consensus is that there exist 4 pharmacologically identified subdivisions designated with upper case letters as M1-M4 and 5 immunologically/genetically identified receptor subclasses identified with lower case letters as m1-m5 [37, 38]. Muscarinic

receptors consist of single unit proteins folded into 7 transmembrane domains and a large cytoplasmic domain between regions 5 and 6 [39]. mAChRs belong to the super class of G protein coupled receptors (GPCRs) and thus induce signal transduction via catalyzing the exchange of guanosine diphospate (GDP) for guanosine triphosphate (GTP) when activated, acting as a guanine nucleotide exchange factor (GEF) [40, 41]. As with all GPCRs, muscarinic receptors can further be classified by the type of the G protein they are associated with; primarily due to the alpha subunit which determines which downstream effectors are activated upon the disassociation of the G protein complex. When mAChRs are activated, the G protein disassociates into α and βγ subunits, which in turn, activate corresponding downstream effectors [42, 43]. Classically, most attention has been giving to the alpha subunit which is further divided into four subtypes determining its activation capacity. However, the capacity of the beta-gamma complex to primarily activate ion channels is becoming ever more lucid [43]. Table one illustrates the mAChRs subtypes along with their associated G proteins, including their known second messenger cascades.

Table 2.1.1 | mAChRs: signal transduction

	mAChR subtype				
	M_1	M_2	M_3	M_4	M_5
Preferred signal transduction	$G_{q/11}$ $PLC/IP_3/DAG/CA^{2+}$ PKC	$G_{i/o}$ AC (inhibition)	$G_{q/11}$ $PLC/IP_3/DAG/CA^{2+}$ PKC	$G_{i/o}$ AC (inhibition)	$G_{q/11}$ $PLC/IP_3/DAG/CA^{2+}$ PKC

PLC, phospholipase-C; IP_3, inositol 1,4,5-triphosphate; DAG, diacyglycerol; PKC, protein kinase C; AC, adenylate cyclase Adapted from Langmead et al 2008 [44]

2.1.6 Nicotinic Cholinergic Receptors

Tobacco and indirectly nicotine has been used for thousands of years. The genus *Nicotiana* received its name from the French ambassador Jean Nicot who introduced tobacco to France and indirectly the whole of Europe. Wilhelm Heinrich Posselt and Karl Ludwig Reimann coined the term alkaloid and gave nicotine its name in their thesis submission to the University of Heidelberg in 1828 as they were the first to successfully isolate nicotine in its pure form [45]. Ironically, the pioneering research in nicotine was conducted by the R. J. Reynolds Tobacco Co. which is now the second

largest tobacco company in the U.S.A and continues to lead research in the fields of nicotine addiction and Alzheimer's Disease therapeutics [46].

Unlike mAChRs, nAChRs are ligand gated ion channel receptors (LGIC) that consist of five subunits (α,β, γ, δ,ε) surrounding a cation permeable core. Each subunit has four transmembrane segments, a long N-terminal domain, an intracellular loop between transmembrane segments 3 and 4, and finally a short C-terminal domain [47]. Neuronal nicotinic receptors are composed of heteromer combinations of α (2-6) and β (2-4) subunits as well as homomers of α7 and α9 and finally heteromers of α9 and α10 subunits [48]. The combination of nAChRs subunits determines the channel kinetics for the given receptor as well as its physiopharmacological properties. Currently the best understood neuronal receptor types are the α4β2 heteromer and the α7 homomer , however, the diverse possible subunit combinations gives rise to a near infinite possibility of nAChR receptor types [49]. Nicotinic receptors exist primarily in three principal states: activated, inactivated, and desensitized. In the desensitized state, the channel is inactivated and incapable of ligand binding. Upon ligand binding (usually 2 ligands), the channel becomes activated and permeable to K^+, Na^+, and Ca^{2+} ions. The ratio between which ions are permeable or favored is directly related to the subunits constituting the nAChR. Presynaptically located, nAChRs can directly induce neurotransmitter release via the influx of Ca^{+2} or indirectly through activating voltage gated calcium channels (VGCCs). Postsynaptically, nAChRs can activate intracellular signaling cascades and gene transcription [50].

2.2 Cholinergic Projections

In 1967, Lewis and Shute made the first substantial contributions to the understanding of cholinergic neurons and their projections [51-53]. Then sixteen years thereafter, Mesulam introduced a *Ch* classification system to distinguish cholinergic groups based upon their topographical location as well as their cortical and subcortical projections [54].

2.2.1 Basal Forebrain

Cholinergic neurons of the basal forebrain innervate the entire cerebral cortex and contain the cholinergic areas Ch1 to Ch4 based upon Mesulam nomenclature [54]. Ch1 neurons refer to cholinergic neurons of the medial septum (MS) which project primarily to the hippocampus but as well to the cingulated cortex, olfactory bulb and hypothalamus [55]. Cholinergic neurons of the vertical limb of the diagonal band of Broca (VDB) are classified as Ch2 and project also primarily to the hippocampus along with the fore mentioned projections of Ch1 [55]. Ch3 neurons comprise those of the horizontal limb of the diagonal band (HDB) and constitute the major cholinergic pathway of the olfactory bulb [56]. The final cholinergic constituent of the basal forebrain is area Ch4 which refers to the densest set of cholinergic neurons located in the nucleus basalis of Meynert (NbM) also commonly referenced as nucleus basalis (NB) or substantia innominata (SI). The NB projects primarily to the amygdala as well as to the rest of the cerebral cortex that does not receiving other cholinergic projections [57].

2.2.2 Brain Stem

The brainstem cholinergic system consists of four cholinergic cell groups Ch5 to Ch8. Ch5 designates cholinergic cells in the pedunculopontine nucleus, and Ch6 cells in the laterodorsal tegmental nucleus and rostral brainstem. The Ch5 and Ch6 cells project to the thalamus. Ch7 cells in the medial habenula innervate the interpeduncular nucleus, and Ch8 cells in the parabigeminal nucleus project to the superior colliculus [54, 58].

2.2.3 Identification of Cholinergic Neurons

For the identification of cholinergic neurons, there are numerous methods and markers available. The most widely used methods consist of immunohistochemistry, immunocytochemistry, *in situ* hybridization, as well as polymerase chain reaction (PCR) and its derivatives [59]. Commonly used markers for cholinergic neurons include choline acetyltransferase, vascular acetylcholine transporter, acetylcholinesterase, and finally the choline high affinity transporter [60-62]. It is generally accepted that identification with either ChAT or VChAT is conclusive enough for cholinergic neurons. AChE, as a cholinergic marker, is considered to be unspecific and therefore normally only used in conjunction with another marker. CHT, although considered a highly specific conclusive cholinergic marker, is limited to nerve terminals and therefore not as widely used [63].

2.2.4 Characteristics of Cholinergic Neurons

The prime characteristic of basal forebrain cholinergic neurons (BFCNs) is their expression of the tropomyosin receptor kinase A (TrkA) and the binding of the endogenous ligand nerve growth factor (NGF) gives the BFCNs their respected phenotype [64]. Most BFCNs also express all neurotrophin receptors in varying ratios but it is the loss of TrkA expression which leads to the onset of mild cognitive impairment and later Alzheimer's disease [65]. While a vast majority of BFCNs also express the low affinity $p75^{NTR}$ receptor, more than 90 percent in the MS and diagonal band of Borca [66], there exist a subpopulation of cholinergic neurons in the nucleus basalis which do not. Cholinergic lesions with 192 IgG-saporin directed against $p75^{NTR}$ abolishes BFCNs in all areas except for that of the NB and cholinergic neurons in this area show high ChAT reactivity but very low $p75^{NTR}$ expression following 192 IgG-saporin [67, 68].

2.3 Acetylcholine Neuromodulation

The cholinergic system plays a pivotal role in modulating learning and memory, arousal, sleep, and cognition [69]. It achieves these effects through the modification of intrinsic functions on the neuronal level as well as setting the oscillatory tone on the network level [33].

2.3.1 Intrinsic Modulation

Intrinsically, ACh modifies action potential adaptation as well as membrane potential and resistance. It is believed that one of the primary purposes of ACh modulation is to limit intrinsic neuronal excitability and thus increase the fidelity of extrinsic excitation [70]. The increased intrinsic inhibition also functions as a neuroprotective mechanism against over excitation [71]. Intrinsic inhibition and excitation from afferent projections facilitates network contiguity during ACh release [33]. Both nAChRs and mAChRs have the ability to activate slow conductance calcium activated potassium channels (SK) [70, 72]. SK channels are heteromeric complexes of SK-α subunits and calmodulin and are gated by changes in intracellular Ca^{2+} in a voltage-independent manner [73, 74]. These channels are activated by Ca^{2+} binding to calmodulin that induces conformational changes resulting in channel opening [75]. SK channel activation results in K^+ ions entering the cell and driving cell hyperpolarization forming the medium part of which is commonly referred to as the after hyperpolarization current (I_{AHP}) [76]. The after hyperpolarization current is typically initiated by the firing of action potentials and its primary action is to limit AP firing causing adaptation and decreasing firing frequency [77]. Paradoxically, the I_{AHP} is both enhanced and abolished by ACh activation of mAChRs in pyramidal neurons located in the basolateral nucleus of the amygdala [70].

2.3.2 Network Modulation

Acetylcholine plays an essential role in the network integration of neurons of various cortical regions allowing for the processing of information via oscillatory synchronization occurring primarily in the beta and gamma ranges (12 to 80 Hz) [78]. Cholinergic

activity is closely associated with cortical arousal [79] and is also strongly linked with enhanced sensory processing in the cortex [80] including heightened top-down interactions, from higher associative cortices to lower sensory cortices [81]. Activation of nAChRs facilitates afferent inputs to the cortex, whereas the activation of mAChRs is important for enhancing intra laminar connections [82]. Muscarinic receptor activation is associated with gamma frequency oscillations and arousal [83]. In contrast, enhanced cortical activation by nicotine is associated with increased visual attention and working memory [84] including an increase in beta rhythm generation [85]. Acetylcholine induces hippocampal oscillations in the 40 Hz range via the activation of mAChRs; the oscillation is dependent upon rhythmic inhibition through γ-aminobutyric acid A receptors ($GABA_A$) [86]. As in the hippocampus, nAChR induced beta rhythms in the auditory cortex are also dependent upon the activation of $GABA_A$Rs [78].

2.3.3 Learning and Memory

Acetylcholine agonists enhance memory and learning performance when applied to brain regions associated with such learning and memory task at hand, whereas ACh antagonists disrupt performance [87, 88]. The underlying cellular mechanisms for the effect of ACh on learning and memory include the enhancement of afferent input relative to excitatory feedback, regulation of inhibition and oscillations, increased persistent spiking for active maintenance, and augmentation of synaptic modification (LTP) [69]. High levels of acetylcholine enhances the fidelity of sensory inputs via the activation of nAChRs allowing information encoding while inhibiting the retrieval of already stored information by de-

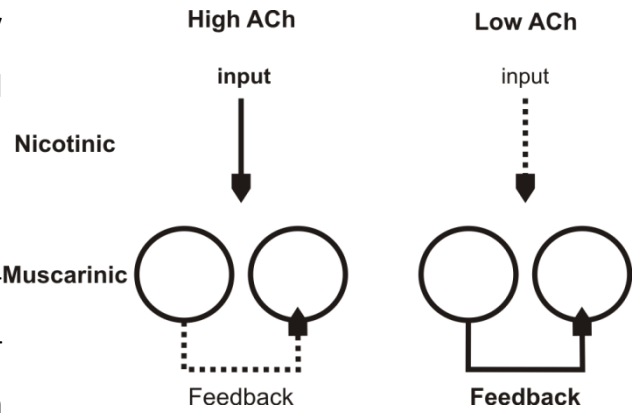


Figure 2.3.1 | ACh on cortical dynamics High ACh levels enhances afferent input through action of nAChRs and suppresses feedback excitation via presynaptic inhibition of glutamate release through mAChRs. Low ACh levels results in weaker afferent input and a strengthening of excitatory feedback. Adapted from Hasselmo 2006 [69]

creasing excitatory feedback through presynaptic inhibition elicited by the activation of mAChRs. Low levels of ACh reduce information from afferent sensory inputs and increases excitatory feedback by the removal of presynaptic inhibition and causing greater spiking activity and allowing the consolidation of newly learned information [33, 89].

2.4 Field Recordings

2.4.1 Long-term Potentiation

LTP is the prolonged strengthening of synapses between neurons following brief but intense activation. It is believed to be one of the primary processes underlying the neuronal correlation to learning and memory.

2.4.2 Hebbian Synapse

In 1949, Donald Hebb revolutionized the way neuronal plasticity was viewed. Hebb predicted that plasticity was driven by the temporal juxtaposition of pre- and postsynaptic activity. Arguably Hebb's most quoted statement, "When an axon of cell A is near enough to excite B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" [90] led to what is commonly referred to has Hebb's Rule and is popularly summarized as "Neurons that fire together wire together". Hebb also argued that neurons functioned in cell assemblies and postulated that a specific function of the Hebbian synapse was the conversion of short term memory into that of long term [91].

2.4.3 Finding LTP

Hunting for synaptic analogues to learning, many techniques were deployed following the postulations of Hebb. One of the building blocks was that of facilitation shown by Feng in 1941, which consists of the ability of an excitatory synapse to release more neurotransmitters following a single synaptic activation [92]. Following up on facilitation, in 1955 Cragg and Hamlyn demonstrated augmentation in the hippocampus [93]. Augmentation occurs when a synapse is repeatedly activated and

there is an enhancement of synaptic response during and briefly following the repeated activations. Edging ever closer to a working demonstration of the Hebbian synapse, many groups reported on the ability of post tetanic potentiation (PTP) to induce fleeting synaptic augmentation following the initial activation. PTP is the ability of low frequency stimulation, generally below that of 50 Hz, or a short, high frequency stimulation to briefly induce an enhancement of synaptic excitability. Yamamoto and McIlwain came extremely close in 1966 when they elicited what was termed PTP, due to the short potentiation duration, using a 10 second 100 Hz stimulation paradigm [94]. Finally, in 1973 Bliss and Loemo induced what they, at the time, called long lasting potentiation [95] which was later coined long-term potentiation in 1975 by Douglas [96]. What was believed to be the long sought mechanism underlying learning and synaptic plasticity was found and Hebb's Rule became firmly grounded [97, 98].

2.4.4 Induction of LTP

Classical induction of LTP is in agreement with the Hebbian synapse in that presynaptic activity induces substantial postsynaptic activation resulting in the potentiation of the synapse. This form of LTP induction is N-Methyl-D-Aspartate Receptor (NMDAR) dependent. Presynaptic activity depolarizes the postsynaptic neuron through the activation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPA) [99-101]. Upon sufficient depolarization, the magnesium block of NMDARs is alleviated in the postsynaptic neuron [102]. Upon removal of the voltage dependent Mg^{2+} ion blockade, NMDARs then become permeable to potassium, sodium and calcium ions [103]. The increased concentration of Ca^{2+} in the postsynaptic neuron through NMDA receptors is the principle initiator of LTP [104]. Ca^{2+} build up in the postsynaptic neuron triggers an increase in the amount of AMPA receptors present on the neuron's membrane, which thus further depolarizes the postsynaptic neuron activating more NMDARs and further increasing the levels of Ca^{2+} [105]. When Ca^{2+} concentrations reach a sufficient level, then calcium/calmodulin-dependent protein kinase II (CaMKII) is activated [106, 107]. CaMKII autophosphorylation leads to the induction

of LTP by phosphorylating AMAPRs which increases their conductance to Na^{+} ions and thus synaptic potentiation [108].

Most LTP studies have been conducted in the hippocampus and are the classical NMDAR dependent form of LTP [109]. However, other brain regions express other forms of LTP and the hippocampus also expresses non NMADAR dependent LTP. Generally LTP induction, which is not dependent upon the NMDA receptor, is termed non-Hebbian; as presynaptic activation is not a requisite for its induction. This non NMDAR dependent form of LTP requires the activation of G protein coupled receptors and the release of internal Ca^{+2} stores via activation of the inositol 1,4,5-triphosphate pathway [110, 111]. In hippocampus, non-Hebbian LTP is mediate by the activation of metabotropic glutamate receptors (mGluR) while in the amygdala, this form of LTP is dependent upon the activation of mAChRs [110, 112, 113]. Although the receptor types are different in both brain areas, the primary mechanisms underlying LTP induction are virtually the same. Activation of GPCRs activates phospholipase C (PLC) which induces IP_3 and diacylglycerol (DAG) production from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) [111, 114]. IP_3 causes the release of intracellular Ca^{+2} from endoplasmic reticulum stores initiating LTP induction by triggering the buildup of AMPARs on the cell membrane while DAG activates protein kinase C (PKC) [112]. In turn, PKC phosphorylates AMPA receptors increasing their conductance to sodium ions resulting in the potentiation of the synapse [115].

2.4.5 Paired Pulse Facilitation

Paired pulse facilitation is a short term enhancement of a synapse on a time scale of hundreds of milliseconds. It is primarily considered to be a presynaptic phenomenon in which a second postsynaptic potential is enhanced relative to the first if the second potential quickly follows the first. The enhancement is considered to be the result of a transient increase in calcium levels in the presynaptic terminal resulting from the first pulse [116, 117]. The ratio of the second pulse compared to that of the first is used to determine if facilitation or depression took place, which both are con-

sidered short term forms of synaptic plasticity. A PPF ratio greater than that of 1 indicates that the synapse has under gone facilitation whereas all ratios blow that of 1 are an indication of synaptic depression. The rational of PPF is twofold: first the activity of the presynapse determines the extent of transmitter release which in turn determines the amount of activation at the postsypase (first pulse); second, the residual calcium level in the presynaptic cell determines the magnitude of the second paired pulse in relation to the first pulse (second pulse). This gives rise to the necessity to determine the intensity of the first pulse as well as the time frame between the first and second pulses [117]. When PPF is induced following LTP, it is controversially considered to be indicative of whither LTP occurred pre- or postsynaptically as reports are conflicting [118, 119].

2.5 Plateau Firing

Plateau firing, also referred to as plateau potential, persistent activity, or persistent firing, is a phenomenon in which single cells repeatedly fire action potentials for a sustained period following initial induction. It is believed to be a cellular mechanism for maintaining working memory as well as performing cognitive processes such as reward based decisions [120, 121]. Currently there are two concepts for how persistent firing occurs, either intrinsically through a calcium activated nonspecific cation current or by reverberations in neuronal networks providing reciprocal positive feedback [122, 123]. Neurons which display intrinsic firing have been shown to demonstrate graded firing levels in direct response to depolarizing and hyperpolarizing pulses which can either increase or decrease firing frequency respectively [9, 124]. It is currently believed that the mechanism underlying graded firing exists of two opposing calcium dependent currents with differing calcium concentration thresholds. In normal conditions, the calcium activated potassium current (K_{Ca}) exceeds that of the calcium activated nonspecific cation current (CAN) so that persistent firing does not occur. When the initial stimulus is strong enough, calcium influx can surpass a necessary threshold for CAN activation and persistent firing will take place. Spiking can be further increased by increasing CAN conductance with a second

stimulus and greater calcium influx. Firing can be degraded or ceased by membrane repolarization and reducing CAN conductance and replacing the K_{Ca} current above the CAN current. It is believed that biochemical processes such as phosphorylation and dephosphorylation set calcium threshold levels. Entry of calcium through voltage gated channels during action potential firing is essential for maintaining the CAN current but insufficient to increase CAN conductance providing stable levels of firing [121, 122].

2.6 The Amygdala

The amygdala is a structurally and functionally heterogeneous collection of nuclei that is classically viewed to be the "emotional center" of the brain. First being identified and named by Friedrich Burdach [125], it is still under some controversy over its actual existence as a structure [126]. The amygdala is composed of two primary complexes, the centralmedial; containing the central (Ce) and medial (Me) nuclei and the Basolateral complex (BLA); which contains the lateral (La), basolateral (BL), basomedial (BM), and basoventral (BV) nuclei [127, 128]. Growing from the groundbreaking work of Pavlov [129], the amygdala has been considerably connected to conditioning, primarily that of fear, and emotional behavior [130-134]. As of late, more understanding is emerging in the relationship of the amygdala and memory, in particular consolidation [1, 33, 135, 136], and that of cognition [137-141].

2.6.1 Connectivity

Vast sensory input arrives at the amygdala from advanced levels of visual, auditory, and somatosensory cortices. The olfactory system and polysensory brain regions, such as the parahippocampal gyrus and the perirhinal cortex, also project profusely to the amygdala [142, 143]. The primary target of sensory input is the La while processing of this information emerges almost exclusively in the BL via intrinsic connections between the two nuclei [144, 145]. Projections from the amygdala target a wide sphere of structures including the prefrontal cortex (PFC), striatum, sensory cortices, hippocampus, perirhinal cortex, entorhinal cortex, as well as the basal fore-

brain. The amygdala also targets subcortical structures that are related to physiological responses such as autonomic and hormonal [146]. Subcortical projections originate in the Ce while projections to cortex and striatum arise from the BL [147].

2.6.2 Learning and Memory

Learning of new information and the ability to recall this information later is crucial for survival. A single acute traumatic experience can induce an intense life long memory which is dependent upon BLA activation [148]. Stressful and emotional events are well remembered and it is believed that hormones and neurotransmitters released during such events are responsible for the enhanced memory formation [1]. The infusion of epinephrine, norepinephrine or corticosterone enhances memory while lesions of the BLA block this enhancement [149]. Intra amygdala infusions of cholinergic agonist and antagonist enhance and inhibit, respectively, memory induced via many forms of behavioral animal training [150]. Stress hormone induced amygdala memory enhancement is also dependent upon cholinergic activation as muscarinic antagonist blocks such effects [151]. It has been shown that lesions of the BLA or its efferents severely impair learning and memory consolidation but do not extinguish such learning, thus indicating that the amygdala modulates other brain regions and is not the primary memory locus [152, 153]. In this regard, amphetamine infused into the caudate nucleus enhanced memory of visual cued training while amphetamine infused into the hippocampus enhanced spatial memory, yet amphetamine infused into the amygdala was able to enhance both types of memory. Inactivation of either the hippocampus or the caudate nucleus with lidocaine blocked spatial or visual memory respectfully, but amygdala inactivation had no effect on either memory type [153]. BLA electrical stimulation also modulates hippocampus LTP [154] and enhances induction of LTP in the dentate gyrus [155] while BLA lesions block the induction of LTP in the dentate gyrus [156]. Not limited to inducing strong amygdala activation, acute stress, as well as chronic stress, induces plasticity changes within the amygdala and thereby making the amygdala oversensitive to future stressful stimuli [157-159]. This enhanced amygdala response due to plasticity changes leads

to increases in anxiety, mood related disorders, as well as severe emotional disorders such as post-traumatic stress [160].

2.7 p75 Neurotrophin Receptor

The $p75^{NTR}$ is the first member of the tumor necrosis factor receptor (TNFR) super-family to be characterized and is structurally characterized as having four, tandemly arranged, cysteine-rich, extracellular domains consisting of a sequence of 40 amino acids and an intracellular death domain [161, 162]. $p75^{NTR}$ has the ability to bind all neurotrophins with low affinity but only NGF, BDNF and NT-4 have been shown to induce signaling [163-165]. A characteristic of $p75^{NTR}$ which distinguishes it from other TNFRs is the existence of a disulfide linked dimer formed via cysteinyl residues within the transmembrane domains. These transmembrane domains are necessary for $p75^{NTR}$ interaction with other neurotrophin receptors (NTRs) [166, 167].

2.7.1 $p75^{NTR}$ signaling

Paradoxically, activation of the p75 neurotrophin receptor has the ability to promote neuronal survival as well as induce apoptosis. For general purposes, it is accepted that lone activation of $p75^{NTR}$ induces apoptosis while the synergetic activation of $p75^{NTR}$ with another NTR promotes survival [168]. Upon binding NGF, $p75^{NTR}$ forms a asymmetric complex which allows interaction with only one tyrosine kinase receptor such as TrkA and thus, improving the affinity of TrkA to NGF [169]. $p75^{NTR}$ is cleaved by α-secretase freeing the extracellular domain preventing the further binding of neurotrophic factors, however the transmenbrane domain is still able to interact with tyrosine kynase receptors. Following α-secretase cleavage, $p75^{NTR}$ is further cleaved by γ-secretase freeing the intracellular domain which is then translocated to the cell nucleus. Surprisingly, NGF binding to $p75^{NTR}$ inhibits γ-secretase cleavage [170]. Interestingly it has been discovered that the precursors to mature neurotrophic factors also bind to $p75^{NTR}$ inducing apoptosis, so far this has been shown to be the case for proNGF as well as proBDNF [171]. However, the most striking $p75^{NTR}$ signaling is that the cleaved intracellular tail has been shown to induce apop-

tosis autonomously [172]. Aside from inducing apoptosis and promoting cell survival, $p75^{NTR}$ is also capable of regulating neurite growth, reduction, and repair [173]. Signaling induced by $p75^{NTR}$ can best be surmised as being dependent on the activating ligand as well as $p75^{NTR}$'s promiscuity as a coreceptor.

2.7.2 $p75^{NTR}$ transgenic mice

There currently exist two forms of transgenic mice with regard to $p75^{NTR}$. The first mouse was produced by deleting 3 of the 4 cysteine-rich extracellular domains by targeting exon III of the locus. This prevented the ability of $p75^{NTR}$ to bind any neurotrophic factors; however the deletion left the transmembrane and intracellular regions. This shorten formed of the receptor was termed s-$p75^{NTR}$ and was revealed to be coexpressed with the full length receptor at lesser quantities during embryonic development. Therefore, exon IV of the $p75^{NTR}$ locus was deleted to give a full ablation of the receptor. Transgenic mice expressing the truncated s-$p75^{NTR}$ receptor are referred to as $p75^{EXIII}$ while mice with a complete ablation of the receptor are called $p75^{EXIV}$ [174]. Unfortunately it has been shown that the exon IV transgenic mouse also displays a truncated version of the receptor which is capable of signaling [175].

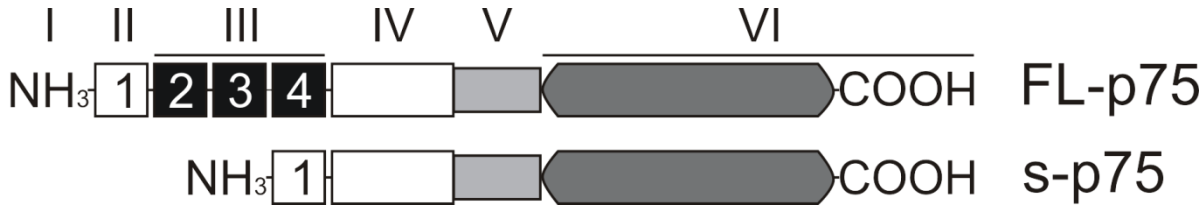


Figure 2.7.1 | p75NTR Locus The full length (upper) and short (lower) forms of the $p75^{NTR}$ locus. Above the loci are the corresponding exon domain numbers. In the truncated locus, domain III is deleted leaving behind the first 2 extracellular domains, the transmembrane and intracellular domains. The short locus is left behind in $p75^{EXIII}$ mice whereas $p75^{EXIV}$ mice are thought to be lacking both forms

2.7.3 Known p75 deficits

Mice lacking the exon III or exon IV region of the locus show specific phenotypes and deficits. Lesioning of neurons expressing the p75 receptor by various methods such as 192-IgG injections also leads to specific deficits related to the loss of the low affinity nerve growth factor receptor. Exon IV animals show mobility problems due to hind limb ataxia [174], which also hampers the ability for nullizygous male mice to breed

(own observations) as well as a thinning of blood vessels resulting in fewer births of ko animals [174]. Both transgenic mouse lines show an increase in target innervation by cholinergic afferents as well as an increase in cholinergic neuronal size; interestingly the number of cholinergic neurons is reduced [7, 174]. Aside from deficits in neuronal development, a loss of the receptor or the lesioning of neurons which express it greatly interferes with varying forms of learning and memory [176-179]. Most studies concerning the loss of the p75 receptor and changes in plasticity have been performed in hippocampus. $p75^{EXIV}$ animals have demonstrated an impairment in LTD maintenance but no difference in LTP when induced by Schaffer collateral stimulation and recorded in region CA1. The mice also showed alterations in AMPA receptor subunits GluR2 and GluR3 [180]. It was further reported that signaling of proBDNF via $p75^{NTR}$ enhanced LTD in hippocampus and p75 ko animals showed reduced expression of NMDA receptor subunit NR2B which is uniquely involved in LTD [181].

Table 2.7.1 | Phenotype of p75NTR-deficient mice

Cell population	Phenotype
Dorsal root ganglia	Reduced neuronal survival due to impaired trkA signaling Failure of Schwann cells to migrate and related effects on axonal growth
Basal forebrain	Increased cholinergic neuron size, neurotransmitter expression and axonal branching in the adult, Increased/decreased cholinergic neuron number (controversial)
Superior cervical ganglia	Increased neuron number during period of developmental cell death
Retinal ganglia neurons	Increased neuron number during period of developmental cell death
Trigeminal neurons	Increased neuron number during period of developmental cell death
Spinal motor neurons	Increased neuron number during period of developmental cell death
Facial motor neurons	Increased survival following axotomy
Hippocampal neurons	Increased survival after seizure, Increased dendritic branching and spine density, Altered LTD
Spiral ganglion neurons	Progressive hearing loss
Subventricular zone	Reduced adult neurogenesis
Vasculature	Thinner blood vessels
Hepatic stellate cells	Inhibited hepatocyte proliferation, and liver regeneration
Alternative splice forms	Low levels of an extracellularly truncated death-inducing protein are expressed on certain congenic backgrounds in each of the two strains

Summary of the phenotype displayed by transgenic mice for the p75 neurotrophin receptor adapted from Underwood et al 2008 [182]

3 Objectives

The amygdala plays a substantial role in learning and memory and is a key regulator of memory consolidation. It also displays one of the highest cholinergic densities in the brain; as such, it was desired to elucidate differences in plasticity based upon cholinergic innervation of the BL and La nuclei of the amygdala. Since the vast majority of cholinergic projection neurons in the substantia innominata also display the p75 neurotrophin receptor, and it has been shown that ko animals for $p75^{NTR}$ show significant augmentation of cholinergic innervation, plasticity differences in $p75^{EXIV}$ mice were also studied.

ChAT immunohistochemistry was used to determine cholinergic innervation differences between the BL and La as well as a determination of stimulation and recording sites for electrophysiological experiments.

Field potential recordings and sharp microelectrode recordings were used to determine if differences in cholinergic density plays a role in plasticity in the La and BL amygdala nuclei.

LTP was used to determine if $p75^{EXIV}$ animals had an enhancement of LTP related to increased cholinergic innervation.

Field potential recordings were conducted to study differences in the amygdala between $p75^{EXIV}$ ko and wt animals and if the variation in cholinergic fiber density plays a role.

Paired Pulse Facilitation was used to elucidate any change in presynaptic transmitter release as well as possible changes in cholinergic mediated presynaptic inhibition between $p75^{EXIV}$ mice and wt animals.

Biocytin staining was used to complement electrophysiological neuronal characterization as a further determination of projection neurons based upon their morphology.

4 Materials

4.1 Electrophysiology

4.1.1 Equipment

A.M.P.I. Master 8 programmable stimulator ISO-flex stimulus isolator	**A.M.P.I.**
Hameg HM507 50 MHz CombiScope oscilloscope	**HAMEG Instruments GmbH**
Axon Instruments AxoClamp 2B amplifier with remote buzzer two HS-2A-x0.1LU head stages	**Molecular Devices**
CED Micro 1401 mk 2 data acquisition unit Spike2 and Signal software	**Cambridge Electronic Design Limited**
Gilson Miniplus3 Peristaltic Pump with 2 channel head	**Gilson, Inc.**
NI EMPS-07 modular housing two DPA-2FS filters	**npi electronic GmbH**
FST Interface Recording Chamber TR-200 temperature regulator	**Fine Science Tools GmbH**
Schott KL 1500 LCD cold halogen lamp	**SCHOTT AG**
Askania SMT4 stereomicroscope with stand	**Mikroskop Technik Rathenow GmbH**
Siskiyou manual micromanipulator SD-MX110R SD-MGB 3.0 base SD-MX630R hydraulic drive	**Siskiyou Corporation**
Märzhäuser MM33 manual micromanipulators with base	**Märzhäuser Wetzlar GmbH & Co. KG**
Active Air Suspension Isolation Table including working plate granite with stainless steel top Steel Faraday Cage Borosilicate glass microelectrodes with filament	**Science Products GmbH**

Sutter Instrument P-97 Flaming/Brown Micropipette Puller	**Sutter Instrument Company**
Exadrop precision gravity infusion	**B. Braun Melsungen AG**
Bipolar stainless steel electrode	**Rhodes Medical Instruments Inc.**
Flexible silicone rubber heater	**Minco Worldwide Headquarters**
Nanoliter 2000 Injector	**World Precision Instruments**
Slice Holding Chamber	**University of Heidelberg**

4.1.2 Slice Preparation

Non-Sterile Scalpel Blades #20 Non-Sterile Scalpel Blades #10 Standard Pattern Scissors- Large Loops sharp/blunt 14.5cm Scalpel Handle #4- 13cm Extra Thin Iris Scissors- 10.5cm Scalpel Handle #3- 12cm Spring Scissors- 10mm Blades Angled Side Dumont #7b Medical Forceps- Inox Standard Tip Halsted-Mosquito Hemostats- 12.5cm Dumont #7 Forceps- Titanium Biologie Dumont #2AP Forceps- Inox Epoxy Coated Dumont #5 Mirror Finish Forceps- Inox Biologie	**Fine Science Tools GmbH**
Pattex Blitz Pinsel	**Henkel KGaA**
Multifix razor blades	**Apollo Herkenrath GmbH & Co. KG**
Vibroslice MA752	**Campden Instruments**

4.1.3 Gas Assembly

Carbogen (95% O_2 5%CO_2) Compressed air	**Air Liquide**
PUN 6x1BL pneumatic tubing	**Festo**
Tygon tubing	**LabMarket GmbH**
Couplings and adapters	**neoLab Migge Laborbedarf-Vertriebs GmbH**
Messer 3-O-200 BG88 and 3-D-200 Pressure regulators	**Messer Group GmbH**

4.1.4 Chemicals

Apamin UCL 1648 2-APB APV (AP-5) Atropine Kynurenic Acid Eserine (Physostigmine) CNQX Picrotoxin (Cocculin) Biocytin Carbachol Glucose	**Sigma-Aldrich**
NaCl	**J.T. Baker Inc.**
$NaHCO_3$ KCl $CaCl_2$ $MgSO_4$	**Carl Roth GmbH + Co. KG**
NaH_2PO_4	**Grüssing GmbH**

4.2 Genotyping

4.2.1 Equipment

Mastercycler PCR cycler Thermomixer comfort	**Eppendorf AG**
Alphaimager mini gel documentation system	**Alpha Innotech**
PowerPac 300	**Bio Rad Laboratories**
EasyPhor gel chamber	**Biozym Scientific GmbH**
Thermo printer P93DW	**Mitsubishi**
Vortex Genie 2	**Scientific Industries**

4.2.2 Primers

Mp75Ex4-S3 5'-TGT-TGG-AGG-ATG-AAT-TTA-GGG-3'	**Metabion International AG**
2590 5'-GAT-GGA-TCA-CAA-GGT-CTA-CGC-3'	
Pgk-2 5'-AAG-GGG-CCA-CCA-AAG-AAC-GG-3'	

4.2.3 PCR

QuickExtract DNA Extraction Solution	**EPICENTRE Biotechnologies**
Platinum Taq DNA Polymerase 10X PCR buffer 50 mM MgCl 100 mM dNTP set TrackIt 50 bp DNA Ladder TrackIt Cyan/Orange Loading Buffer	**Invitrogen**

4.3 Immunohistochemistry

4.3.1 Equipment

Leica VT100 S vibrating blade microtome	**Leica Microsystems**
Super Frost Plus slides	**R. Langenbrinck Labor und Medizintechnik**
Axioplan2 Imaging	**Zeiss**
Axiocam HRc	**Zeiss**
Stretching Table OTS 40	**Medite GmbH**

4.3.2 Markers

DAPI	**Boehringer**
Alexa Fluor	**Molecular Probes**
ChAT	**Chemicon**
Biotinylated IgG	**Vector Laboratories**
Streptavidin Cy3	**Jackson ImmunoResearch**

4.4 Solutions

50x TAE buffer

242 g Tris

57.1 ml acetic acid

100 ml 0,5 M EDTA (pH 8)

1x PBS
8 g NaCl
0.2 g KCl
1.15g Na_2HPO_4
0.2 g KH_2PO_4
to 1 L distilled water

0.1 M PB
2.62 g NaH_2PO_4
14.41 g Na_2HPO_4 x 2 H_2O
to 1 L distilled water

4% PFA
40 g PFA
to 1 L 0.1 M PB

Mowiol
2.4 g Mowiol
6 g Glycerol
6 ml Distilled water
12 ml 0.2M Tris (pH 8.5)

3% HS 0.3% Triton in PBS
6 ml HS
0.6 ml Triton
to 200 ml with PBS

Ringer

$NaHCO_3$	26 mM
KCl	3 mM
$CaCl_2$	1.6 mM
$MgSO_4$	1.8 mM
Glucose	10 mM
NaCl	124 mM
NaH_2PO_4	1.25 mM

5 Methods

5.1 Electrophysiology

5.1.1 Slice Preparation

Acute horizontal brain slices were prepared from adult mice following decapitation under diethyl-ether anesthesia. The brain was then rapidly removed and placed into ice cold artificial cerebral spinal fluid (aCSF) saturated with carbogen gas. After an incubation time of 20 minutes, the brain was then hemisected in the midsagittal plane and the cerebellum was removed. It was then cut dorsally 30 degrees from rostral to caudal and fixed along this edge with a cyanoacrylate adhesive (Pattex Blitz Pinsel) to the cutting block so that the brain was sitting oblique to the block surface with the ventral side upward and the hemisections facing another caudally. The cutting block was then submerged in ice cold aCSF saturated with carbogen and 400µm thick horizontal sections, containing the hippocampus, amygdala, entorhinal and perirhinal cortices and substantia innominata, were then cut using a Campden vibroslicer from lateral to medial. Horizontal slices were then placed into a holding chamber (built in house) and incubated for at least one hour, after which slices were transferred to an interface recording chamber (FST) and held at 33° C ±1 degree for at least another hour.

5.1.2 Electrophysiological Recording

Horizontal slices were placed in an interface recording chamber (FST) and allowed to accumulate at least for one hour to continuous profusion of aCSF at a rate of 2 ml min^{-1} and a temperature of 33° C ±1 degree. Extracellular field potential recordings were then recorded using an Axoclamp-2B amplifier (Molecular Devices) in conjunction with a DPA-2FS extracellular amplifier (npi Electronics). Signals were filtered online at 1 kHz and digitized at 5–10 kHz by an analog to digital computer interface (CED) and then were stored using Spike2 software (CED). Field potential (fp) recordings were obtained with aCSF filled borosilicate glass electrodes with a tip resistance

of 1 MΩ placed in either the La, BL or both of the amygdala which was identified with a dissecting microscope using trans-illumination. An extracellular bipolar electrode (Rhodes Medical Instruments) was used to induce synaptic fp responses by local stimulation of the external capsule. Pulses (0.1 ms, 4–25 V) were delivered with an Iso-Flex stimulus isolator (AMPI) triggered by a Master-8 pulse generator.

For LTP induction, an input/output (I/O) response curve was constructed by varying the intensity of single-pulse stimulation and then averaging 6 responses at each intensity. The stimulus intensity that evoked a mean fp equal to 50% of the maximum amplitude was then used for all subsequent stimulations (duration: 0.1 ms frequency: 0.1 Hz). A stable baseline was obtained by presenting a single stimulus (100 µs duration) every 10 seconds. Once a stable baseline of responses was obtained for at least 20 min, HFS was delivered as 2 trains at a frequency of 100 Hz (duration: 1 s, 30 s apart). Drug-induced changes in baseline activity were considered. Subsequent responses to single stimuli were recorded for at least 60 min, and their amplitude was quantified as percent change with respect to baseline.

Paired Pulse Facilitation (PPF) was performed by delivering 2 pulses (duration: 0.1 ms frequency: 0.1 Hz) with an inter-stimulus interval (ISI) of 10 to 100 ms and a stimulation intensity which evoked a potential equal to 50% of the maximum amplitude following an I/O curve and baseline. The amplitude of each pulse was quantified as percent change to baseline or as a ratio between the second and first pulse. Pulses were delivered in PPF and LTP paradigms as already stated in the extracellular methods as well as placement of the stimulator and recording electrodes see Fig. 6.2.1 in Results section and Fig. 11.1.2 appendix.

Intracellular recordings were obtained using sharp microelectrodes pulled on a Brown-Flaming puller P-97 (Sutter Instruments) from 1.5-mm borosilicate glass with filament and backfilled with 2 molar potassium-acetate resulting in a tip resistance of 60– 120 MΩ. Signals were amplified using an Axoclamp-2B amplifier and were digitally stored using Spike 2 laboratory software (CED). Intracellular potentials were rec-

orded in bridge mode and the bridge balance was monitored and compensated throughout the experiments. Resting membrane potential was determined by subtraction of the tip potential following withdrawal from the cell. Input resistant was calculated by current injections (intensity: 0.2 to 0.4 nA duration: 200 ms) through the recording electrode and measuring the resultant voltage deflections. Membrane potential was manually adjusted by intracellular injection of DC current through the recording electrode and holding recorded cells to near firing threshold when eliciting persistent activity. An extracellular bipolar electrode was used to induce synaptic responses by stimulation of the substantia innominata (SI) or within the BLA. Trains of pulses (0.1 ms, 5–20 V) were delivered at 10–100 Hz for 0.1–10 s with an Iso-Flex stimulus isolator (AMPI) triggered by a Master-8 pulse generator.

Plateau firing was induced using a standard paradigm [124]. In the presence of 10 µM carbachol, cells were held near AP threshold and a suprathreshold 4 s 0.3 to 0.4 nA pulse was given to induce plateau firing. Alternatively, 1 µM eserine was bath applied and an extracellular bipolar electrode was used to stimulate cholinergic SI afferents. The cell was either held near AP threshold and SI stimulation (10 Hz) was used to induce plateau firing or while the cell was at V_M rest, repeated SI stimulation was given once every 30 s at 10 to 50 Hz to induce plateau firing. SI stimulation strength was always subthreshold and was given between 8 and 14 mV with 10 mV and 10 Hz being the most prevalent.

5.1.3 Biocytin Neuronal Labeling

During intracellular recordings some neurons were labeled with biocytin in order to elucidate cell morphology. Sharp microelectrodes were prepared as previously stated with the exception that the tips were first filled with biocytin and then the electrodes were back filled with 2 molar potassium acetate. Biocytin was prepared by dissolving in Milli-Q water. Following experimental recordings, biocytin was injected using pulses of positive and negative current at 0.4 nA with a varying duration of 10 to 200 ms. Slices were left in the interface chamber, after current injections, for a minimum of

20 minutes to allow biocytin to diffuse throughout the cell arborization. Slices were then removed from the interface chamber and placed into 4% PFA in PB overnight at 4° C.

5.1.4 Data Analysis

Extracellular data were collected and averaged with Signal 2 software (CED). Field potential amplitude was defined as the absolute DC voltage of a vertical line running from the minimal point of the fp to its intersection with a line running tangent to the points of fp onset and offset. It is assumed that the recorded negative wave reflects a summation of both excitatory post-synaptic potentials and synchronized action potentials (population spike component) [183, 184] Watanabe et al. [184] have carried out intracellular recordings of evoked potentials and confirmed that the latency of peak negative fps (5–6 ms) corresponds well with that of intracellular recorded action potentials, indicating that the extracellular recorded sharp negativity is a population spike. Therefore, the amplitude of fps was analyzed. In addition, the slope measure in the lateral amygdala is more sensitive to variability and signal noise, making it difficult to analyze [183]. To calculate the significance of differences between groups, the Mann-Whitney test was primarily used (GraphPad Prism). $P < 0.05$ was considered significant. Where the Mann-Whitney test (2 groups, one factor) was not applicable then either Kruskal-Wallis (3 or more groups, one factor) or Two-way ANOVA (3 or more groups and 2 or more factors) were performed with their appropriate posttest. To express and compare changes of fp amplitudes between groups in LTP paradigms, the averaged responses from either the 20 to 30 or 50 to 60 min period after HFS were used. PPF responses were analyzed by a ratio comparison of the averaged signals of pulse 2 to pulse 1 $[(P_2 - P_1 \times 100\%)/P_1]$ and plotted as a ratio of pulse 2 to pulse 1. All data are shown as mean ± sem unless otherwise stated.

Intracellular data were analyzed offline using Spike 2 software (CED). The amplitude of the SI afferent stimulus induced deflection was measured in relation of the mem-

brane potential and presented as mean ± sem. Peristimulus histograms were performed using Spike 2 as needed.

5.2 Immunohistochemistry

5.2.1 Biocytin Development

Slices were stored overnight in 4% PFA in PB then transferred to a 24 well plate. Slices were washed using 1% PBS, repeated 3 times for 5 minutes each wash. Incubation for 2 hours at room temperature was then performed using Streptavidin-Cy3 in PBS (concentration: 1:1500-1:2000, volume: 250-500 µl/well). Slices were then rewashed with PBS, as stated, followed immediately by a counter stain with DAPI (concentration 1:100000) for 2 minutes at room temperature. Two washes, in PBS as previously mentioned, were then conducted followed by a quick wash in Milli-Q water for 30 seconds. Slices were then allowed to dry and were mounted and cover-slipped using Mowiol on a glass slide.

5.2.2 ChAT Staining

Animals were transcardially perfused with PBS followed by 4% PFA. The brain was then removed and stored in 4% PFA at 4° C. Horizontal slices with a thickness of 25 to 40 µm were cut on an automated, vibrating, blade microtome (Leica Microsystems) and collected in PB solution. Slices were then washed 3 times in PBS for a period of 5 minutes each wash followed by membrane permeabilization with 0.4% Triton-X 100 in PBS for 30 minutes. Slices were re-washed, as stated above, and a blocking solution consisting of 3% horse serum and 0.3% Triton-X in PBS was applied for the duration of 1 hour. A repeated wash cycle as stated was performed followed by incubation with ChAT antibody (concentration: 1:100 in 1% HS|0.1% Triton-X|PBS) for a 2 day period at 4° C. Again a wash cycle was executed on the slices and incubation with the secondary antibody at a concentration of 1:1000 was carried out either over night at 4° C or at room temperature for 5 hours. Following secondary antibody incubation, slices were re-washed and counterstained with DAPI (1:100000) for 2 minutes. A final wash (3 X PBS and 1 X Milli-Q water) was administered and slices

were then dried and mounted on glass slides with Mowiol. Alternatively, in order to strengthen fidelity in small fiber tracts, in place of the normal secondary antibody a biotinylated antibody was used (concentration: 1:2000, incubation: 2 hours RT). Slices were then washed as previously stated proceeded by incubated with Streptavidin-Cy3 (1:2000) for 2 hours at room temperature. The regular protocol was then followed until mounting. Staining was carried out using a free floating technique and a volume of 500 µl for all steps.

5.2.3 ChAT Analysis

Slices were observed and photographed using an Axoplan 2 microscope coupled to an Axiocam HRc digital camera (Zeiss). RGB images were then analysed using ImageJ software (NIH). Areas corresponding to the BL and La nuclei were manually selected according to brain atlases [185, 186] and RGB pixels were converted into intensity values based upon the formula V=(R+G+B)/3 using standard weighing (R=0.299, G=0.587, B=0.114). The Mann-Whitney test was used to determine statistical difference and data are reported as mean ± sem. Photos were merged, when applicable, and adjusted for contrast and brightness using Adobe Photoshop CS4 for presentation. All analyses were performed on unedited images and images were obtained using a calibrated standard conducted on multiple slices.

5.3 Mice

$p75^{EXIV}$ mice, which were originally created in a 129/SvJ strain and backed crossed into a C57/BL6 strain, were bred in house. Homozygous, heterozygous and wild type animals were maintained. Wild type C57/BL6 animals were ordered via normal suppliers. $p75^{EXIV}$ animals were originally provided by Prof. Dr Yves-Alain Barde (University of Basel, Switzerland).

5.4 Genotyping

5.4.1 DNA Extraction

Tail tips obtained from experimental animals were used for DNA extraction. The tails were placed into 1.5 ml microcentrifuge tubes and 200 µl of QuickExtract DNA Extraction Solution 1.0 was added to each tube. The tubes were then vortexed for 15 seconds and placed into a thermomixer pre-heated to 65° C and incubated for 6 minutes. Following initial incubation, the tubes were again vortexed for 15 seconds and a final incubation was carried out at 98° C for 2 minutes. Genomic DNA was then stored at -20° C or immediately used for PCR.

5.4.2 PCR

Due to the incompatibility of the primers for genotyping, two PCR reactions were created for each DNA sample. A master mix was created with the following components according to manufacturer's protocol: Taq DNA Polymerase, $MgCl_2$, dNTPs, PCR reaction buffer, primers (mix A: 2590/Mp75Ex4-S3, mix B: 2590/Pgk-2), and sample DNA. The reactions were then filled with the necessary amount of pure water for either 20 µl or 50 µl reactions. PCR was then carried out using the following program:

CNTRL TUBE

1. HOLD 95° C ENTER
2. T=95° C 0:03:00
3. T=95° C 0:00:30
4. T=58° C 0:00:30
5. T=72° C 0:01:00
6. GOTO 3 REP 39
7. T=72° C 0:10:00
8. HOLD 6° C ENTER

END

5.4.3 Gel Electrophoresis

A 1.5% agarose solution was created by adding 3 grams of agarose to 200 ml of 1% TBE buffer and heating in a microwave. The solution was allowed to cool for approximately 10 minutes allowing the addition of ethidium bromide. The solution was then poured into a gel electrophoresis chamber and allowed to solidify. 10 to 20 µl of amplified product was then pipetted in to the corresponding well for each product and the gel was then run for 1.5 to 2 hours. Visualization was then carried out under UV light and the gel was photographed for analysis.

6 Results

6.1 Immunohistochemistry

6.1.1 ChAT Staining

Based upon observations in the literature as well as brain atlases [185, 186], it was decided that a horizontal slice preparation would be the best method for electrophysiological studies. 25 to 40 µm horizontal slices from adult mice were incubated with a specific antibody for choline acetyltransferase and counterstained with DAPI to determine cholinergic cell and fiber locations. The slices included the hippocampus, amygdala, entorhinal and perirhinal cortices and substantia innominata and were on the same plane as slices prepared for electrophysiology. Based upon ChAT immunohistochemistry, stimulation sites were determined for LTP, fEPSPs, and intracellular electrophysiological studies. Differences in fiber density observed between the BL and La were also measured using ImageJ.

ChAT immunohistochemistry showed a clear separation between the BL and La nuclei based upon cholinergic fiber density (Fig. 6.1.1). The stria terminalis also displayed reactivity against the ChAT antibody. Cholinergic neurons were observed caudal medial to the BL nucleus of the amygdala corresponding with the area of the substantia innominata which is also the location of the nucleus basalis of Meynert. ChAT positive fiber tracts could be observed between that of the SI and the BL nucleus; fibers of the External Capsule were also distinguishable and showed limited ChAT reactivity.

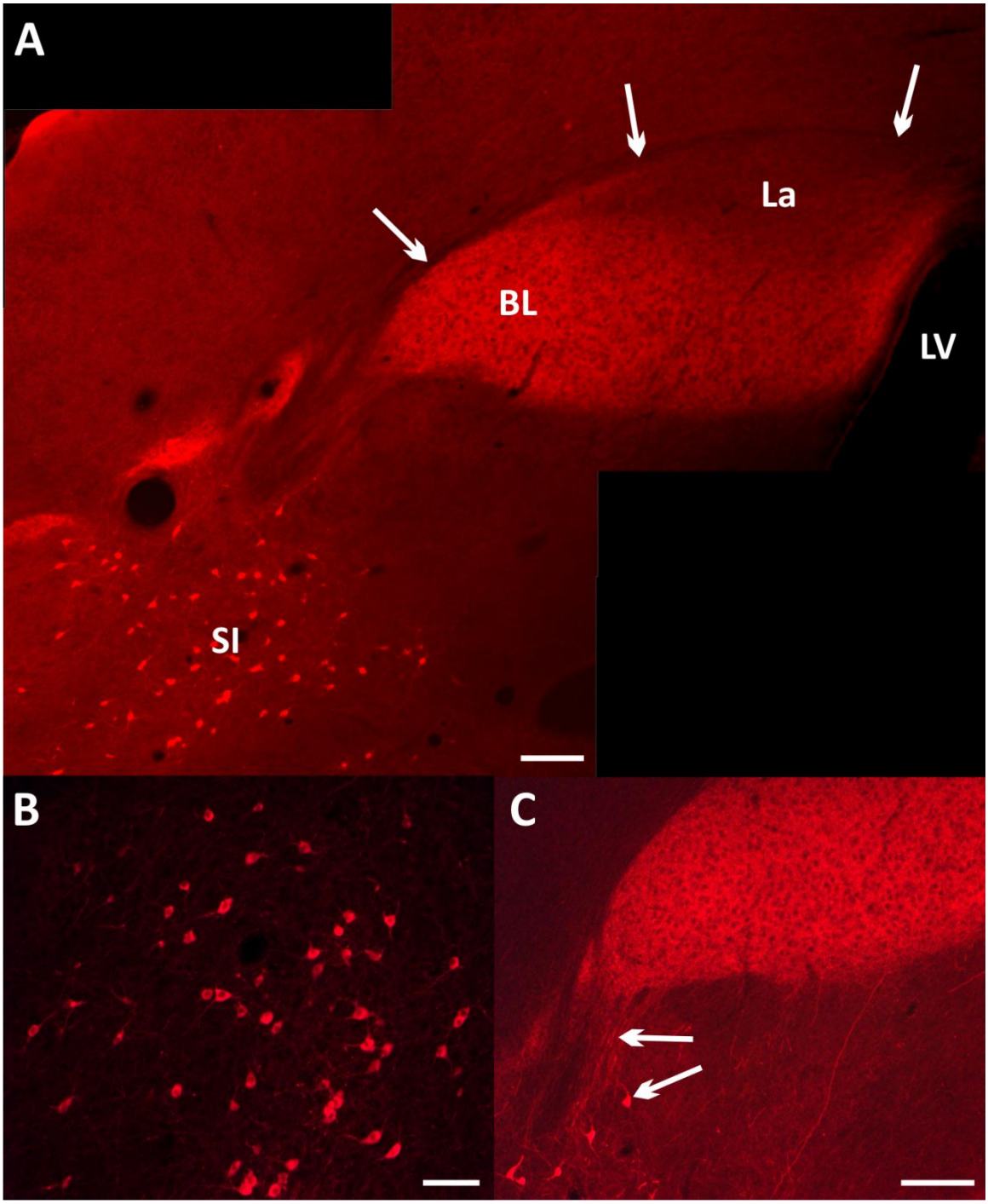


Figure 6.1.1 | ChAT Immunohistochemistry Immunohistochemistry in 25 µm horizontal slice preparation. (A) 4 X collage of horizontal slice showing SI, substantia innominata; BL, basolateral; La, lateral; and LV, lateral ventricle. Arrows indicate the fiber tract of the external capsule. (B) 10 X of cholinergic neurons located in the SI, source of amygdala cholinergic innervation. (C) 4 X showing cholinergic fibers as well as cholinergic neurons originating in the SI and projecting to the amygdala. Arrows indicate cholinergic afferents and neuron. Scale bars for A and C are 100 microns and 50 microns for B.

Measurement of intensity using ImageJ software revealed a highly significant difference for ChAT reactivity between that of the BL and La amygdala nuclei as revealed by the Mann-Whitney test (Fig. 6.1.2). The mean intensity for the BL and La were 34.60 ± 1.057 and 27.47 ± 0.8166 ($p \leq 0.001$) respectively.

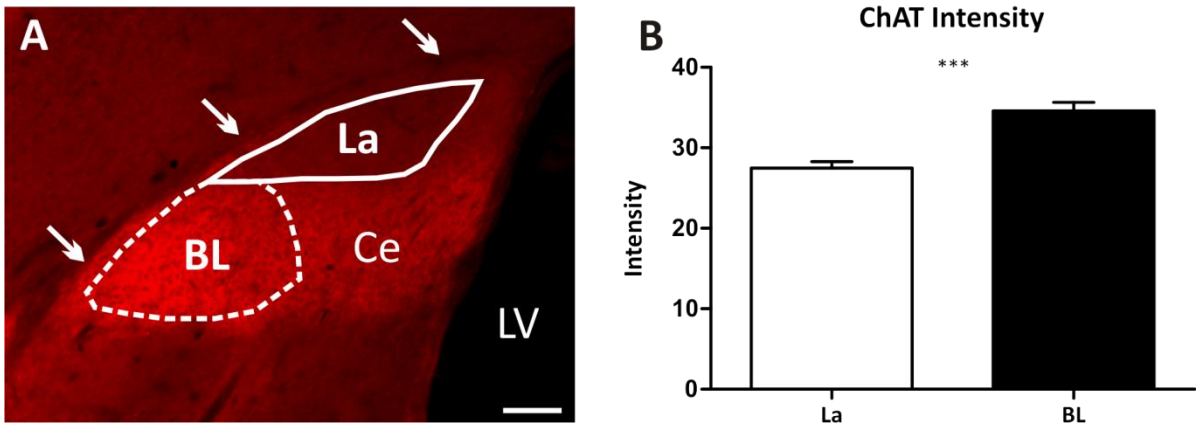


Figure 6.1.2 | ChAT Intensity (A) 25 µm horizontal slice showing areas measured for intensity using ImageJ software based on RGB color vectors and the formula V=(R+B+G)/3 with standard weightings of RGB. Arrows depict fibers of the EC. Scale bar equals 100 microns (B) Mann-Whitney test between La and BL of the BLA. Data is presented as mean ± sem.
La: **mean** 27.47; **sem**, 0.8166 **|** BL: **mean** 34.60; **sem** 1.057 ***p < 0.001 **N:** 5

6.2 Electrophysiology

Based upon ChAT immunohistochemistry, it was decided that for field potential recordings stimulation would be applied to the fiber tract of the external capsule while recording electrodes would either be positioned in the La or BL of the amygdala. Control experiments were then conducted to determine the best placement of the stimulating electrode in relation to the recording electrodes in order that a stable and reproducible signal could be

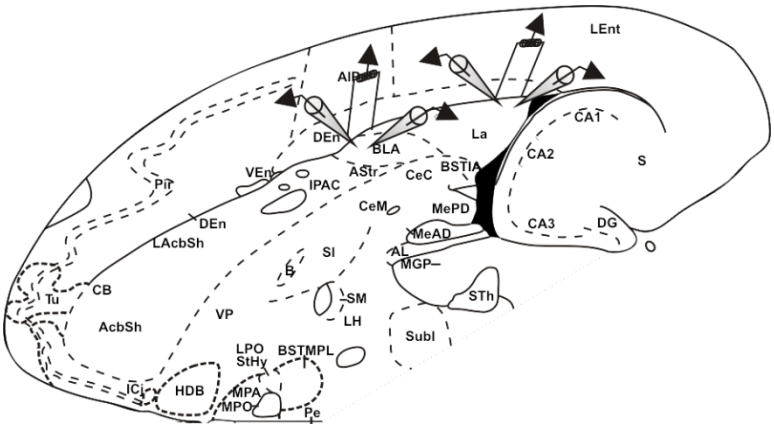


Figure 6.2.1 | Horizontal Slice Diagram depicting the plane of the horizontal slice preparation used for field recordings, PPF, and LTP and the approximate placements of stimulator and microelectrodes. Modified from Paxinos 1998 [186]

obtained. When the desired field excitatory postsynaptic potentials were sufficiently reproducible, preliminary experiments were then performed to determine if EC fiber stimulation could elucidate a cholinergic modulation of the fEPSPs.

6.2.1 Effect of Atropine and Eserine on fEPSPs

Since ChAT immunohistochemistry revealed a significant difference between the BL and La of the amygdala for cholinergic innervation, fEPSPs were recorded in both nuclei during cholinergic challenge in order to show a physiological difference as well. Excitatory postsynaptic field potential recording did not reveal any differences between the BL and La with or without cholinergic challenge (Fig. 11.1.1, appendix); therefore, data from both nuclei were combined. Cholinergic challenge did however significantly alter the field potential as can be seen in figure 6.2.2.

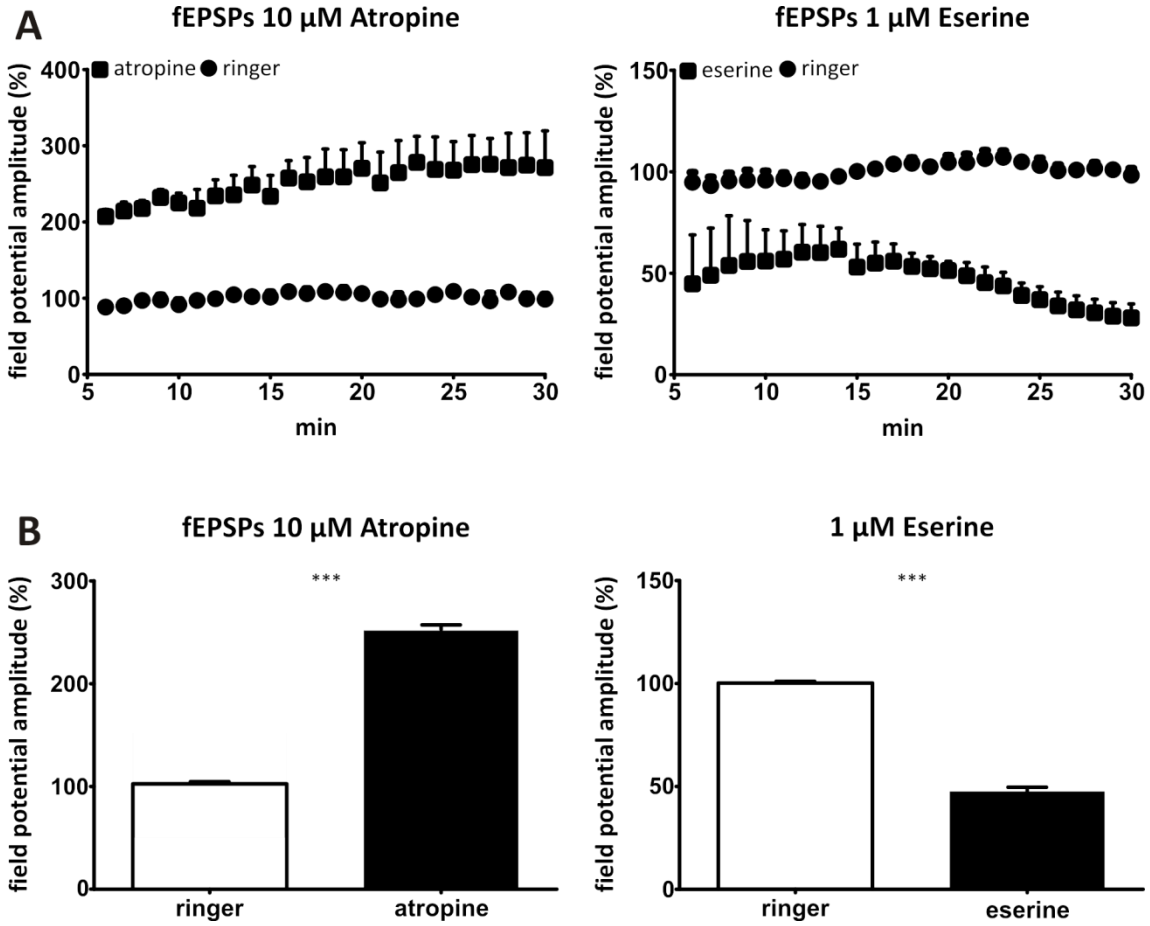


Figure 6.2.2 | Cholinergic challenge of fEPSPs Field potentials initiated with a stimulus which produced an amplitude 50% of maximum. (A) 10 µM atropine caused a potentiation of the fEPSP which reached a maximum at 20±5 minutes. 1 µM eserine inhibited the fEPSP reaching a maximum at 20±10 minutes. (B) Mann-Whitney analysis revealed a significant effect of atropine and eserine on the fEPSP. All drugs were bath applied. Data shown mean ± sem. Atropine: **mean** 250.6 ± 4.468 **|** Eserine: **mean** 47.48 ± 2.114 ***p ≤ 0.001 **N:** 5

Bath application of 10 µM atropine, a competitive selective antagonist of mAChRs, and 1 µM eserine, a slowly degraded inhibitor of AChE, were able to significantly potentiate or inhibit fEPSPs respectively. EC fiber stimulation was given at a strength elucidating a postsynaptic potential equal to that of 50% of the maximum amplitude. Both atropine and eserine demonstrated a time dependent action on the fEPSPs (Fig. 6.2.2). Application of atropine resulted in a gradual potentiation of the field potential and reached a maximum at approximately 15 to 20 minutes. Eserine inhibition of the field potential began at approximately around 5 to 15 minutes while a maximum effect was seen between 20 and 30 minutes. Eserine application of greater than 20 minutes eventually resulted in the complete inhibition of the field potential signal. A physiological cholinergic difference for fEPSPs between the BL and La was not observed; however, cholinergic antagonization or agonization did significantly either enhance or diminish the field potential respectively.

6.2.2 Paired Pulse Facilitation

Control experiments for PPF were first performed to determine the optimal inter stimulus interval that should be used for subsequent cholinergic challenge. The ISI was varied from 10 to 100 milliseconds which elucidated a maximal facilitation at 40 ms (Fig. 6.2.3). A significant difference was not observed between the lateral or basolateral nuclei of the amygdala and therefore data from both sites were combined. An ISI greater than 100 ms resulted in an exponential decrease in the paired pulse ratio and therefore were not conducted after initial pilot studies. Based upon the results obtained, it was decided that all subsequent experiments would be performed

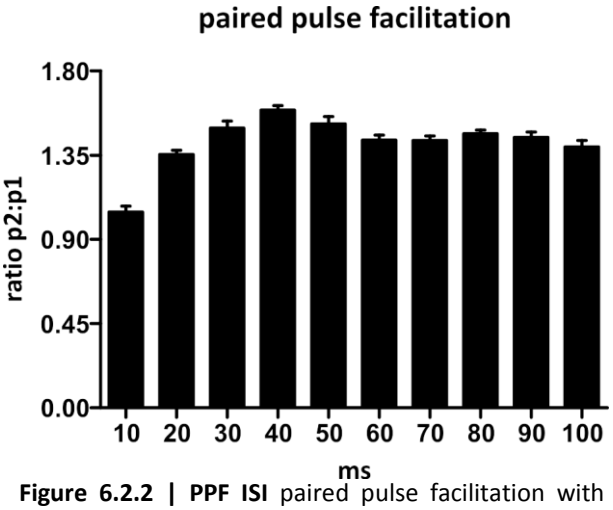


Figure 6.2.2 | PPF ISI paired pulse facilitation with varying inter stimulus intervals from 10 to 100 ms. at 50% maximum amplitude. **N:** 3

ISI (ms)	mean	sem	ISI (ms)	mean	sem
10	1.046	0.03114	60	1.431	0.02653
20	1.353	0.02357	70	1.429	0.02347
30	1.494	0.03729	80	1.465	0.01876
40	1.591	0.02436	90	1.445	0.02988
50	1.517	0.03851	100	1.394	0.03496

using an ISI of 30 ms and a stimulation intensity which induced an amplitude for the first pulse consisting of 50% maximum. The chosen ISI and stimulus intensity would allow for facilitation/depression to occur but would not produce a ceiling/floor effect when challenged.

Initial experiments demonstrated that eserine application was able to inhibit the field potential induced by EC fiber stimulation. Eserine inhibits the degradation of acetylcholine in the synaptic cleft by blocking acetylcholinesterase and thereby allowing the accumulation of ACh. During fiber stimulation in the presence of eserine, the amount of ACh in the synaptic cleft depends upon the frequency, duration and intensity of the stimulation; due to this, it was decided to test the effect of carbachol (CCh), a non-selective AChR agonist which is resistant from AChE, on the field potential. This allowed for a more stable level of ACh at the synaptic cleft which could be manipulated with the concentration of CCh used. Paired pulse facilitation was implemented to determine if the cholinergic effect was occurring pre- or post synaptically.

When paired pulses were delivered at a stimulus intensity of 50% maximum baseline it was observed with CCh as well as with eserine that the fEPSPs were abolished (Fig. 6.2.4 A). In order to test if this was a true inhibition or a change in threshold, stimulations of 2, 4 and 6 mV were given above initial baseline during CCh application (Fig. 6.2.4 A, Fig. 6.2.5 D). At all carbachol concentrations the threshold was increased corresponding in relation to the concentration of CCh and this increased threshold was reversed with the application of atropine; however, atropine could not reverse the baseline inhibition of the fEPSP at 50% maximum amplitude.

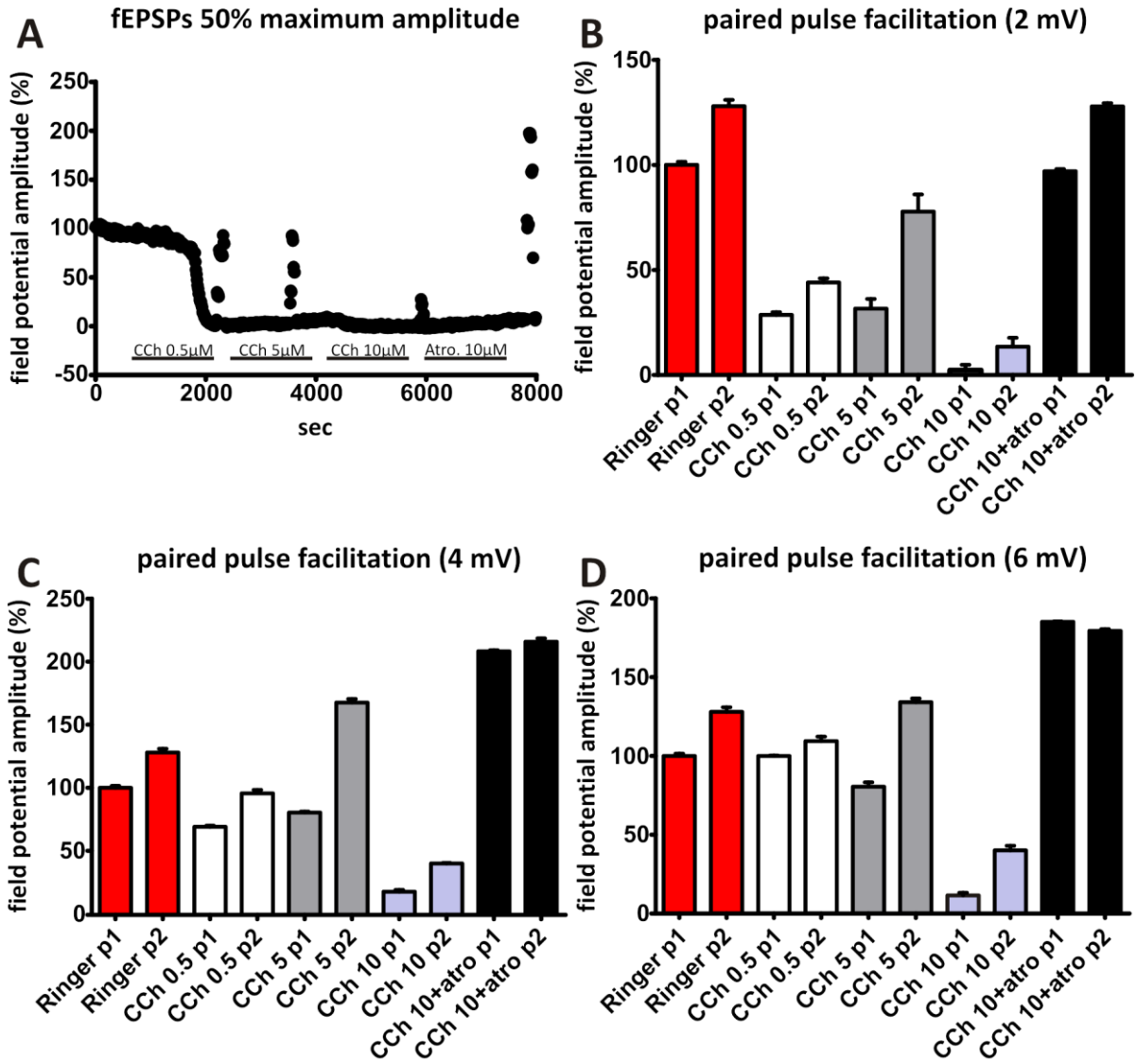


Figure 6.2.4 | PPF with Carbachol challenge (A) Time course showing CCh inhibition of the fEPSPs. Stimulations at 2, 4 and 6 mV above initial baseline were given to demonstrate an increase in firing threshold and not a complete inhibition of the field potential by CCh. Application of 10 µM atropine did not block the increased threshold. (B, C,D) PPF for stimulations at 2, 4 and 6 mV above baseline show that although CCh inhibited the fEPSP it increased the PPF ratio. CCh 0.5, 5 and 10 µM and atropine 10 µM were all bath applied. BL and La combined. **N:** 3

Since carbachol was able to severely inhibit the field potential at a baseline of 50%, which was irreversible with atropine, it was decided to use a baseline of 80% maximum amplitude. Carbachol was still able to significantly reduce the overall fEPSP amplitude but at the new baseline level atropine application was able to reverse this inhibition and induce potentiation (Fig. 6.2.5 A). The potentiation is in agreement with fEPSPs studies when atropine is applied alone. The reversal of the CCh induced inhibitions at 80% but not at 50% maximum amplitude also evidences an increased threshold as with the stimulus challenges in figure 6.2.4 A which showed significant differences with respect to CCh concentration as well as stimulus intensity as revealed by Two-way ANOVA with Bonferroni posttest (Fig. 6.2.5 D). The paired pulse ratio was also increased with CCh in a dose dependent manner indicating a presynaptic inhibition via mAChR activation as seen in figure 6.2.5 C. Paired pulse facilitation shows that the enhanced field potential in the presence of atropine is due to the disinhibition of the presynapse while eserine and carbachol increase the PPF ratio by augmenting presynaptic inhibition as well as enhancing transmitter release for the second pulse.

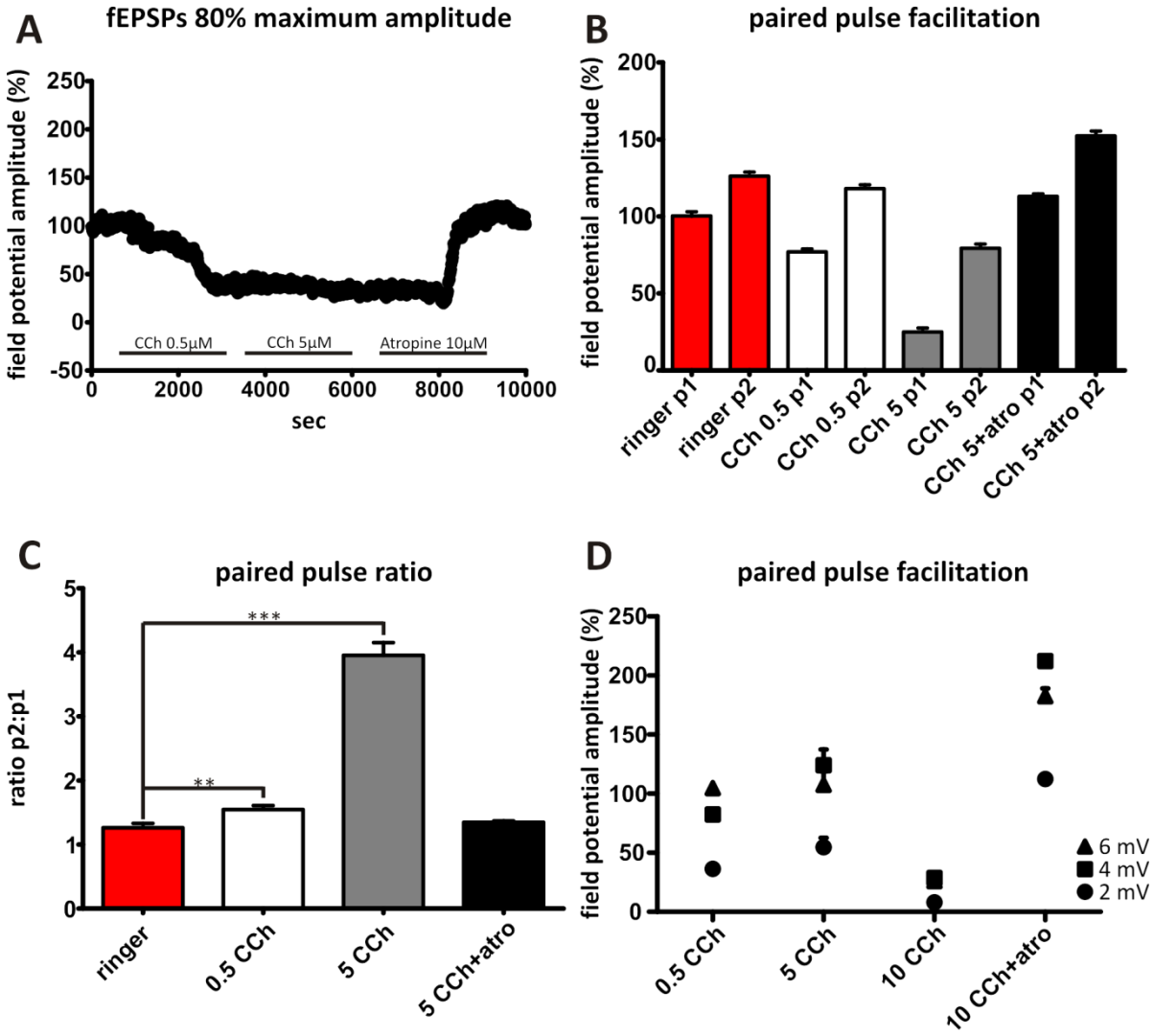


Figure 6.2.5 | PPF with CCh challenge at 80% maximum (A) Time course showing CCh inhibition of the fEPSPs. Application of 10 µM atropine was able to reverse the CCh inhibition and cause potentiation. (B, C) Kruskal-Wallis analysis with Dunns Multiple Comparison Test showed a very significant and highly significant difference in the PPF ratio for CCh concentrations at 0.5 and 5 µM respectively N: 3. Atropine blocked the increased ratio. (D) Stimulations of 2, 4 and 6 mV above baseline to test effect of CCh on threshold baseline was at 50% maximum amplitude Two-way ANOVA with Bonferroni posttest revealed a highly significant difference between CCh and stimulus ***p < 0.001 N: 3 Bonferroni summary can be seen in table below (A, B, C) Baseline stimulation was at 80% maximum amplitude All drugs were bath applied. BL and La combined.

C	Ringer	0.5 CCh	5 CCh	5 CCh+Atro
mean	1.262	1.548	3.953	1.346
sem	0.02125	0.02200	0.1986	0.02269
p	n/a	**	***	Ns
N=3				

D	2 mV	4 mV	6 mV	2 v 4 mV p	2 v 6 mV p	4 v 6 mV p
0.5 CCh	36.35	82.31	104.7	***	***	*
5 CCh	54.74	124.0	107.4	***	***	ns
10 CCh	7.922	28.71	25.86	Ns	ns	ns
10 + atro	112.4	212.1	182.3	***	***	**
N=3						

6.2.3 Characterization of BL and La neurons

Only neurons which had characteristics of typical projection neurons were measured [70]. Some cells were filled with biocytin (10 from 82) for morphological characterization.

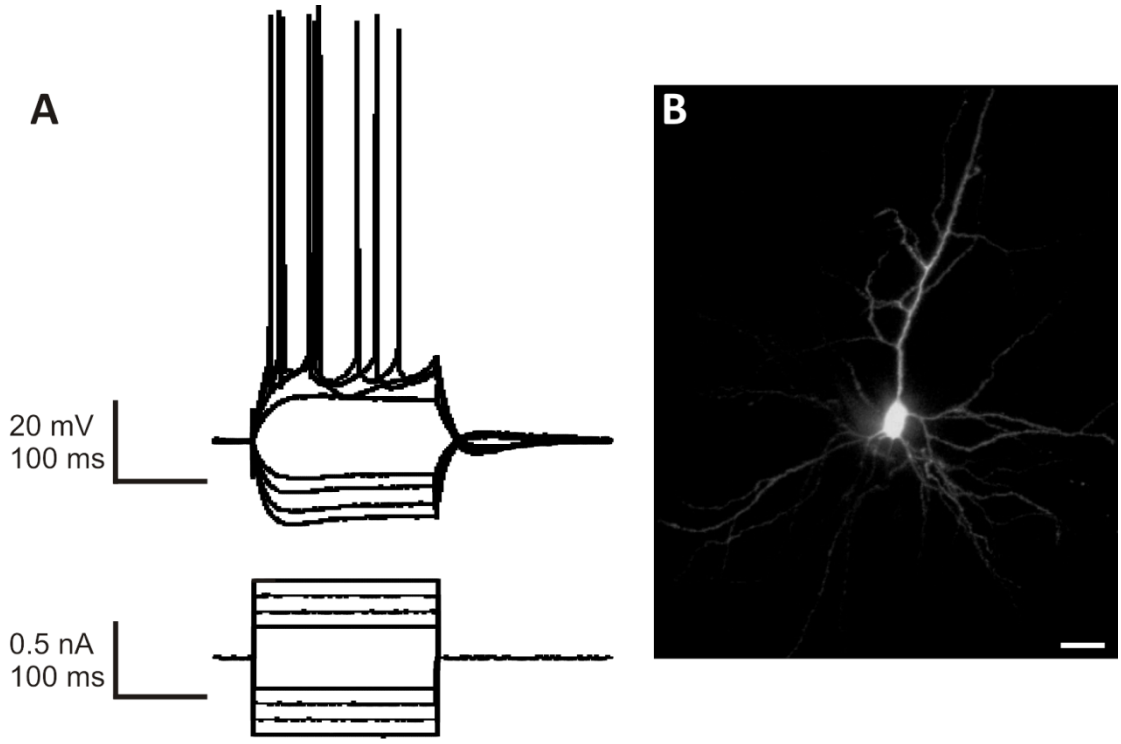


Figure 6.2.6 | Characterization of neurons (A) Sample characterization of a typical BL neuron negative and positive current injection was given at 1,2, 3 and 4 nA 200 ms duration V_M 76 mV (B) Biocytin filled neuron developed with a Streptavidin-Cy3 protocol. Picture displays only the R channel (RGB) and is adjusted for brightness and contrast. Location of neuron was superficial in slice and found in BL nucleus. Scale bar is 25 microns

6.2.4 BL neurons show persistent firing

As it was already shown that neurons in the lateral amygdala nucleus were capable of producing persistent activity during cholinergic activation [9], it was decided to test this phenomenon in the BL which displays greater cholinergic fiber density. Initial experiments were first conducted in the presence of 10 µM carbachol and direct current injection to hold the cell membrane near firing threshold. A four second pulse was then delivered to induce plateau firing. In the presence of CCh using standard techniques, BL neurons showed persistent activity. Stimulation of cholinergic SI afferents in the presence of 1 µM eserine was then used to determine if persistent firing could be induced in BL neurons via cholinergic synaptic activation. As in

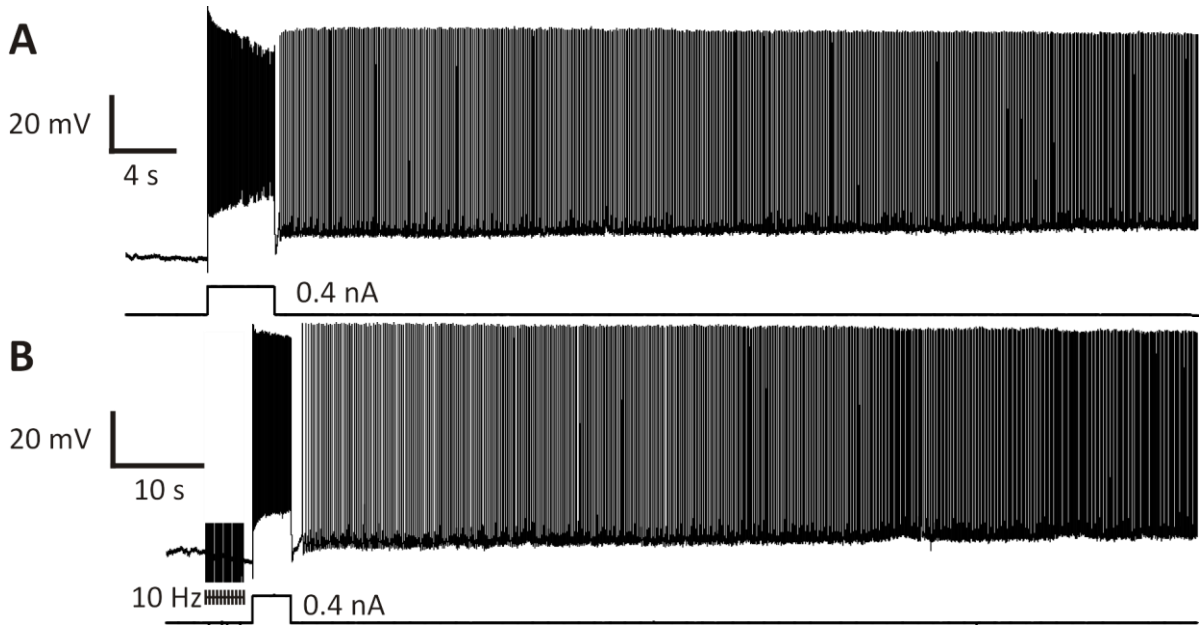


Figure 6.2.7 | Persistent firing in BL neurons (A) Persistent activity induced with continuous DC injection to just below threshold and a 4 s 0.4 nA pulse in the presence of 10 µM CCh. N=27 (B) Persistent activity induced with DC injection to just below threshold and cholinergic fiber stimulation at 10 Hz followed by a 4 s 0.4 nA pulse in the presence of 1 µM eserine. N=23 (A,B) lower traces show direct current injection. All experiments were done during glutamatergic and gabaergic blockade using 2 mM kynurenic acid and 100 µM picrotoxin respectively. All drugs were bath applied. Starting V_M for A and B was 71.2 and 73 mV respectively.

CCh experiments, the cell was held just below firing threshold with direct current injection. A four second, 10 Hz fiber stimulation was given to release ACh followed by a four second, 0.4 nA pulse to induce plateau firing. All experiments were done during glutamatergic and gabaergic synaptic blockade using kynurenic acid, a non-selective antagonist at NMDA and AMPA/kainate receptors, and picrotoxin a $GABA_A$ antagonist. As already shown in La neurons, induction of plateau firing in BL neurons was possible with a standard carbachol paradigm and by synaptic release of ACh via activation of SI afferents in the presence of eserine as shown in figure 6.2.7.

6.2.5 SI afferent stimulation exhibits a biphasic effect in BL neurons

During testing for plateau firing in BL neurons of the amygdala, it was observed that a small cohort of these neurons (8 out of 23) displayed an inhibitory effect following stimulation of cholinergic SI afferents. All of these neurons displayed a hyperpolarization following afferent stimulation and afferent activation was able to fully or partially inhibit 4 second DC injection which normally induced action potential firing (6 from 8). Experiments were performed during synaptic blockade by application of kynuren-

ic acid and picrotoxin as well as ACh augmentation with eserine. As with persistent firing studies, afferent stimulation was always given at an intensity that did not produce action potentials. The stimulus intensity and frequency was varied between 8 and 14 mV and 10 to 50 Hz respectively. Duration of the stimulus was either 2 or 4 seconds and the inter stimulus interval was 30 seconds or greater. It was observed that SI afferent activation with an ISI that was less than 30 seconds would lead to epileptic like firing and loss of cell.

Figure 6.2.8 demonstrates a cell which did not exhibit a strong deflection at resting V_M following afferent stimulation but did demonstrate inhibition of a 4 second 0.4 nanoampere DC pulse delivered closely after afferent activation. A 2 second 10 Hz stimulation with an ISI of 30 seconds was able to almost fully inhibit the DC pulse; stimulation of 40 Hz frequency was able to completely inhibit the direct current injection. A lapse of at least 45 seconds between stimulation negated cholinergic afferent activation. Change in stimulation intensity, duration or membrane potential (less negative) did not have an observed effect on DC pulse inhibition.

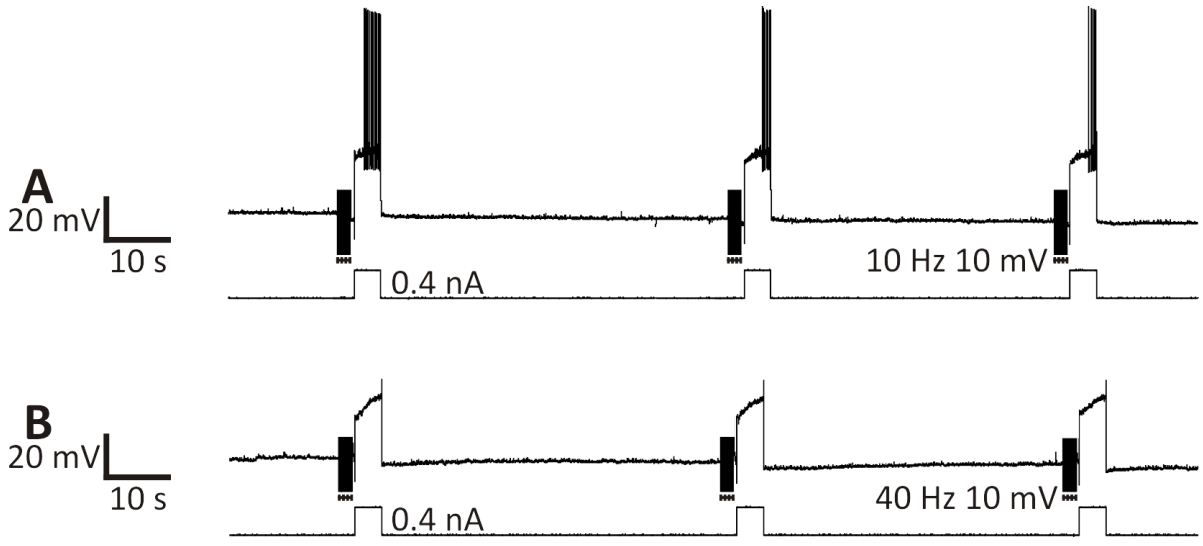


Figure 6.2.8 | Cholinergic afferent inhibition in BL neurons Stimulation of cholinergic afferents was able to induce inhibition of DC current injection AP fringing in BL neurons. (A) At V_M rest, a subthreshold 10 mV 10 Hz stimulation of cholinergic afferents was able to partially block a 4 s 0.4 nA suprathreshold current injection. Repetition of the stimulation every 30 s was able to elicit greater inhibition but never a full block of the current injection. (B) A 10 mV 40 Hz stimulation of cholinergic afferents was able to fully inhibit the suprathreshold DC injection. 2 mM kynurenic, 100 µM picrotoxin and 1 µM eserine were all bath applied. Starting V_M 70.2 mV

Atropine was added to the bath to determine if activation of cholinergic afferents was indeed responsible for the observed inhibition in some BL neurons. Figure 6.2.9 shows the effect of atropine on afferent stimulation and DC injection. A 2 second 10 Hz pulse with an intensity of 8 mV elicited a hyperpolarization in V_M. Upon atropine application this hyperpolarization was completely abolished. Increasing the stimulation duration to 4 seconds, the frequency to 50 Hz and the intensity to 14 mV did not cause a change in the atropine block of V_M deflection by cholinergic afferent activation. Inhibition of 4 second direct current injection was also abolished in the presence of atropine. The observed hyperpolarization induced by SI afferent activation could be reversed at a more negative V_M which was between 20 and 25 mV more negative than V_M rest.

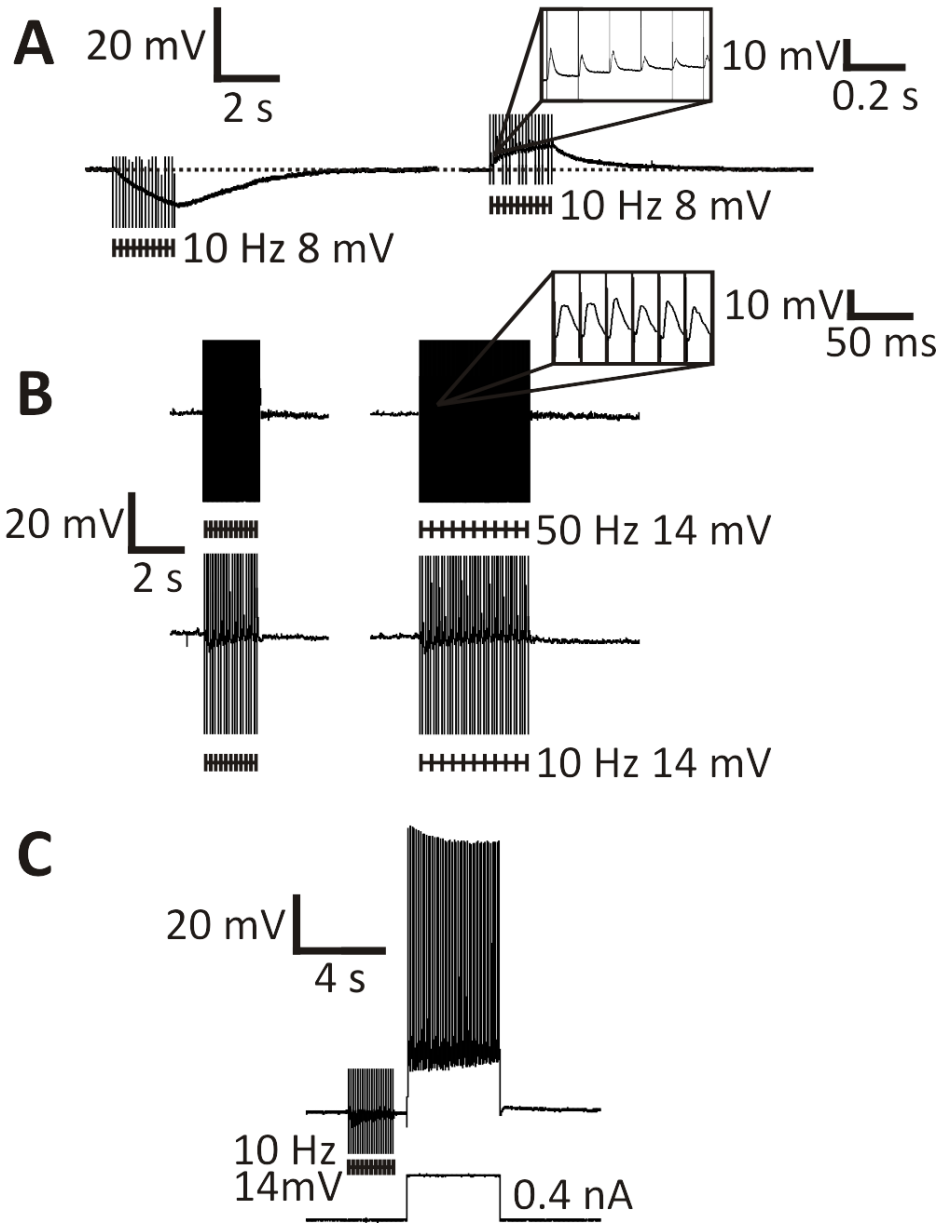


Figure 6.2.9 | Atropine block of cholinergic inhibition (A) A subthreshold stimulation of cholinergic afferents at 10 Hz 8 mV induced a hyperpolarization of V_M at rest which was reversed at a more negative V_M. (B) Bath application of 10 µM atropine was able to suppress cholinergic afferent induced membrane deflection at stronger stimuli of 14 mV and 10/50 Hz. (C) Inhibition of suprathreshold 0.4 nA 4 s DC injection by a 10 Hz 14 mV afferent stimulation was also blocked in the presence of 10 µM atropine. 2 mM kynurenic, 100 µM picrotoxin, 1 µM eserine and 10 µM atropine were all bath applied. Starting V_M was at 70.5 mV **N:** 3

Hyperpolarization and the inhibition of 4 second DC pulse following cholinergic activation, by stimulation of SI afferents, did not interfere with the ability of these BL neurons to induce plateau firing. Figure 6.2.10 displays a BL neuron which showed hyperpolarization as well as inhibition of direct current following SI afferent activa-

tion. Acetylcholine release by 10 Hz stimulation at 13 mV was able to completely inhibit a 0.2 nA 4 s DC pulse which without prior SI afferent stimulation was able to induce action potentials for the duration of the pulse. Following repeated ACh release the 0.2 nA DC pulse showed an increase in firing rate when it did not immediately follow SI activation and came within a time window of less than 45 seconds of the last 10 Hz stimulus. In order to test the effect of SI afferent stimulation on plateau firing, 10 Hz 2 second stimulation at 13 mV was given every 30 seconds. This caused a gradual depolarization of membrane potential and eventual plateau firing. Initial plateau firing lasted for 65 s before subsiding and V_M was allowed to return to rest. SI afferents were then activated as before and again the cell displayed plateau firing which lasted until continues DC injection was given to drive the cell to resting membrane potential.

A total of 82 neurons were measured (BL: 66 La: 16) of which 62 showed persistent firing (BL: 50 La: 12). 23 of the 50 BL neurons were tested for SI stimulation induced plateau firing in the presence of eserine. Interestingly, 8 of these neurons showed a hyperpolarizing membrane deflection which was not observed in the La following cholinergic afferent stimulation. 6 of the 8 neurons also demonstrated partial or full inhibition of a DC pulse which normally elicited AP firing following SI stimulation; however, this inhibition did not affect the induction of plateau firing. Results are summarized in table 6.2.1 below.

Table 6.2.1 | Summary of intracellular recordings

	BL	**La**
Total Number	66	16
Membrane potential (V_M)	71.2 ± 4.5	70.2 ± 5.2
afferent stimulation deflection (mV)	9.44 ± 3.38	n/a
Persistent firing	50	12
In CCh/eserine	27/23	7/5
Membrane deflection	8	n/a
Pulse inhibition	6	n/a
Biocytin filled	7	3

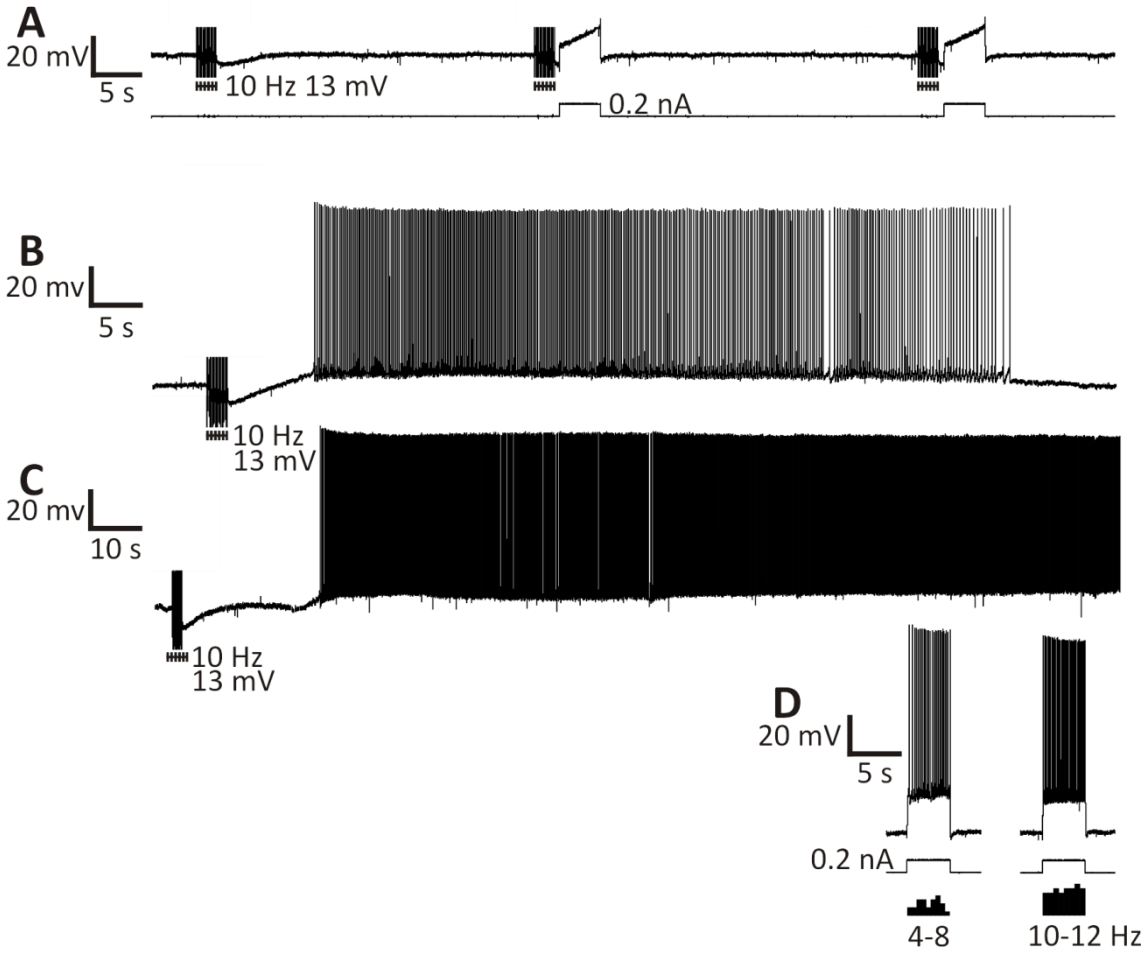


Figure 6.2.10 | Induction of plateau firing from VM rest (A) A 13 mV 10 Hz stimulation of cholinergic afferents was able to fully block a 0.2 nA 4 s suprathreshold direct current injection. (B) Afferent stimulation every 30 s was able to initiate plateau firing which ceased after 65 s. (C) Further stimulations were able to re-induce plateau firing that lasted until quenched by continuous hyperpolarizing DC injection. (D) The firing frequency of suprathreshold 0.2 nA current injection was increased when given between 10 and 20 s following multiple afferent stimulations revealed by peristimulus histogram analysis. 2 mM kynurenic, 100 µM picrotoxin and 1 µM eserine were all bath applied. Starting V_M 67.8 mV

6.3 p75^{NTR} mice

6.3.1 Genotyping

p75^{EXIV} animals were genotyped as described in methods. The wild type primer mixture (2590/Mp75Ex4-S3) revealed a band consisting of 345 bp while the ko primer mixture (2590/Pgk-2) had a band at 475 bp. Heterozygous animals displayed both bands as seen in figure 6.3.1.

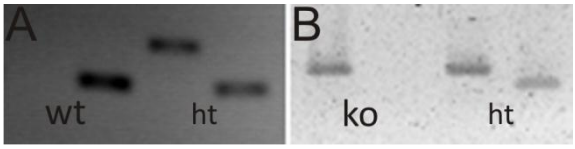


Figure 6.3.1| p75^{EXIV} genotyping (A) gel showing wt animal and ht animal (B) PCR bands corresponding to a ko animal and a ht animal.

6.3.2 Effect of Atropine and Eserine on fEPSPs

Atropine and eserine significantly potentiated or inhibited the field potential in wt animals. Since it is known that p75^{EXIV} animals show a substantial increase in cholinergic innervation bath application of 10 µM atropine and 1 µM eserine was given during fEPSP induction using a stimulus which produced a field potential amplitude 50% of maximum to determine if p75^{EXIV} animals showed differences compared to their wild type (wt) counterparts. The maximum effect of atropine on fEPSPs was observed between 15 and 20 minutes from onset which coincides with wt animals. Unlike wt mice, p75^{EXIV} animals showed no potentiation in the presence of atropine. Application of eserine was able to inhibit the field potential in p75^{EXIV} mice but to a lesser degree as compared to wt mice (Fig. 6.3.2 A, B). The effect of eserine began around 5 to 15 minutes following initial application also corresponding to observations in wt mice, however, unlike wt mice; p75^{EXIV} animals did not show a complete inhibition in the field potential after bath application of 30 minutes or more as was seen in wt animals as well there was only a negligible increase of fEPSPs threshold in p75^{EXIV} animals. In the presence of atropine, p75^{EXIV} animals in fact showed a significant depreciation of the field potential (Fig. 6.3.2 A) in contrast with wt mice which showed a highly significant potentiation (Fig. 6.2.2 B). Interestingly, atropine applica-

tion decreased the variability of fEPSPs in p75^{EXIV} animals allowing for this significance which otherwise would not have been seen. Unlike in wt animals, atropine did not reverse the effect of eserine inhibition back to baseline levels or above in p75^{EXIV} mice.

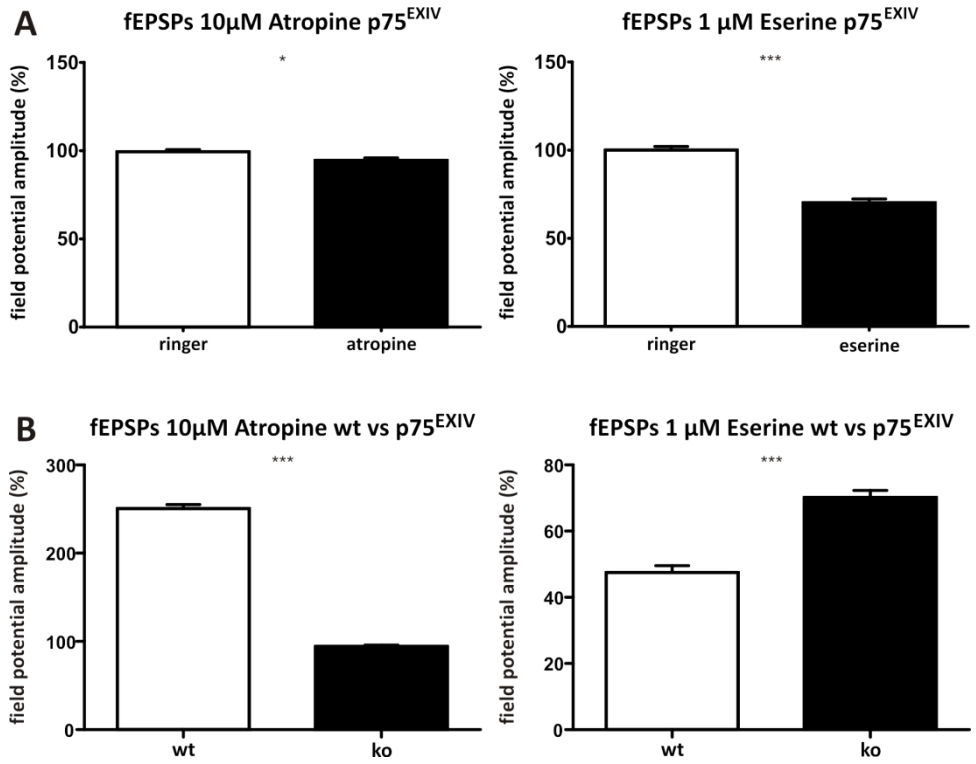


Figure 6.3.2 | Cholinergic challenge of fEPSPs in p75EXIV mice (A) Challenge of fEPSPs in p75EXIV mice showing significant and highly significant inhibition of the fEPSP for atropine and eserine respectively. (B) comparison of cholinergic challenge between wt and p75EXIV animals. Atropine was able to produce a highly significant potentiation in wt animals whereas p75EXIV showed a significant inhibition. In both wt and p75EXIV mice eserine showed a highly significant inhibition of the fEPSP, however; the degree of inhibition was greater in wt animals. 10 µM atropine and 1 µM eserine were bath applied.
Mann-Whitney test data showed as mean ± sem.

A	**atropine**	**eserine**	**B**	**atropine wt**	**atropine ko**	**eserine wt**	**eserine ko**
mean	94.48	70.17	**mean**	250.6	94.48	47.48	70.17
sem	1.403	2.161	**sem**	4.468	1.403	2.114	2.161
p	*	***	**p**		***		***
N=3			**N=3**				

As compared to wt animals, p75^{EXIV} animals display an attenuated response to eserine as well as to atropine. The inhibitory effect of the fEPSP in the ko animals is significantly reduced and the ability of atropine to reverse this inhibition is not present nor does atropine cause any potentiation of the fEPSP when applied alone. Wild type and ko animals do not show a significant difference in the field potential response during application of normal ringer (wt: 2.378 ± 0.2266 ko: 2.578 ± 0.2805, p=ns, N=5). The

p75 receptor seems to play a crucial role in cholinergic mediated presynaptic inhibition; however, a reduced response to cholinergic inhibition in p75^{EXIV} mice does not cause an increase in normal field potentials.

6.3.3 Paired Pulse Facilitation in p75^{EXIV} animals

Building on the cholinergic challenge of fEPSPs, paired pulse facilitation was performed in p75^{EXIV} animals. In order to determine if there was a difference in the ISI for the p2:p1 ratio, preliminary test were done as before using a stimulus which produced a field potential amplitude of 50% maximum for pulse one and an ISI ranging from 10 to 100 milliseconds. p75^{EXIV} animals showed a maximum ratio at 30 ms (Fig. 6.3.3) as compared with wt animals which displayed the maximum p2:p1 at 40 ms (Fig. 6.2.3). To eliminate the possibility of a ceiling effect, p75^{EXIV} animals were tested at an ISI of 20 ms. Since eserine was able to increase the PPF ratio at 30 ms, in order to maintain congruence with wt animals, PFF test were also performed with an ISI of 30 ms. During preliminary PPF trials, wt animals showed a relative stable p2:1 between 50 to 100 ms and an exponential decrease following 100 ms (Fig. 6.2.3), p75^{EXIV} animals, however began to decline after 70 ms (Fig. 6.3.3). Eserine increased the instability in the PPF ratio after 40 ms whereas atropine induced stability (Fig. 6.3.4). Two-way ANOVA with Bonferroni posttest however revealed that the eserine instability was insignificant, whereas atropine had a significant affect by preserving the increased p2:p1 ratio for ISIs 50 to 100 ms. This is in contrast to wt animals where atropine returned the PPF ratio back to baseline levels. At 20 and 30 ms interstimulus intervals, p75^{EXIV} mice, to a lesser extent, resembled their wt counterparts.

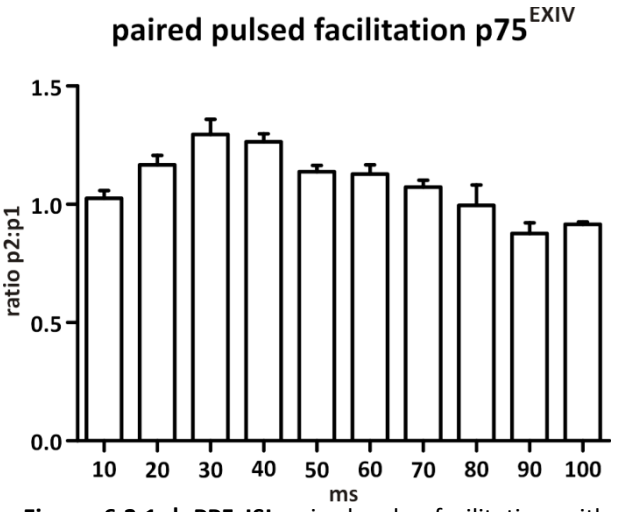


Figure 6.3.1 | PPF ISI paired pulse facilitation with varying inter stimulus intervals from 10 to 100 ms. at 50% maximum amplitude. **N:** 5

ISI (ms)	mean	sem	ISI (ms)	mean	sem
10	1.025	0.03211	60	1.128	0.03832
20	1.167	0.04039	70	1.073	0.02864
30	1.295	0.06380	80	0.9957	0.08622
40	1.264	0.03393	90	0.8771	0.04509
50	1.138	0.02555	100	0.9155	0.009926

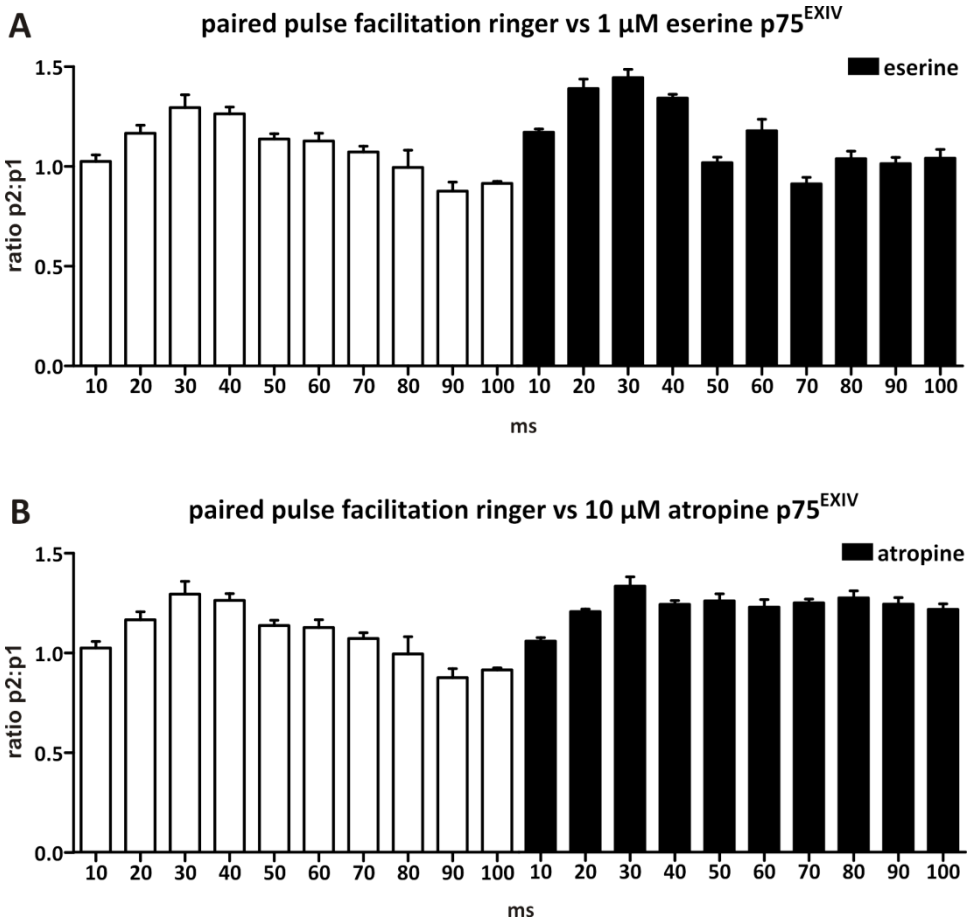


Figure 6.3.4 | Paired pulsed facilitation p75EXIV ISIs PPF in p75^{EXIV} mice at ISIs between 10 and 100 ms. (A) application of eserine insignificantly disrupted the p2:p1 ratio (B) atropine did not fully restore p2:p1 to base-line levels but did significantly maintain a stable level similar to ISI 40 in baseline conditions for ISIs 40 to 100 ms. Data are presented as mean ± sem summary of Two-way ANOVA with Bonferroni posttests are in table below

	ringer	eserine	atropine	ringer vs eser	ringer vs atro	eser vs atro
10	1.025±0.026	1.172±0.050	1.056±0.024	ns	ns	ns
20	1.167±0.036	1.391±0.036	1.203±0.029	***	ns	**
30	1.295±0.053	1.445±0.051	1.331±0.040	ns	ns	ns
40	1.264±0.032	1.343±0.028	1.239±0.018	ns	ns	ns
50	1.138±0.021	1.020±0.029	1.257±0.026	ns	ns	***
60	1.128±0.047	1.179±0.046	1.226±0.028	ns	ns	ns
70	1.073±0.042	0.913±0.026	1.247±0.030	*	*	***
80	0.988±0.060	1.040±0.068	1.272±0.030	ns	***	***
90	0.877±0.048	1.014±0.053	1.241±0.027	ns	***	***
100	0.915±0.020	1.042±0.043	1.215±0.023	ns	***	*
N=5						

As previously stated PPF experiments in wt and ko animals were conducted using an ISI of 30 and 20 ms respectively to avoid a celling effect. To compare ko and wt animals, an ISI of 30 ms was also utilized in the ko animals. Two-way ANOVA analysis with Bonferroni posttest did not reveal any differences between $p75^{EXIV}$ and wt animals at either 20 or 30 ms ISIs (Fig. 6.3.5). Both groups displayed significant differences in the PPF ratio between eserine and atropine except at ISI 30 ms for ko animals. The overall effect of eserine or atropine on the PPF ratio was not as strong in $p75^{EXIV}$ mice as in wt coinciding with field potential results. Wild type animals showed an overall general increased PPF ratio and reach a maximum PPF at a longer ISI than ko mice indicating that the loss of the p75 receptor interferes with normal transmitter release. A summary of the results are found in the corresponding table below Fig. 6.3.5.

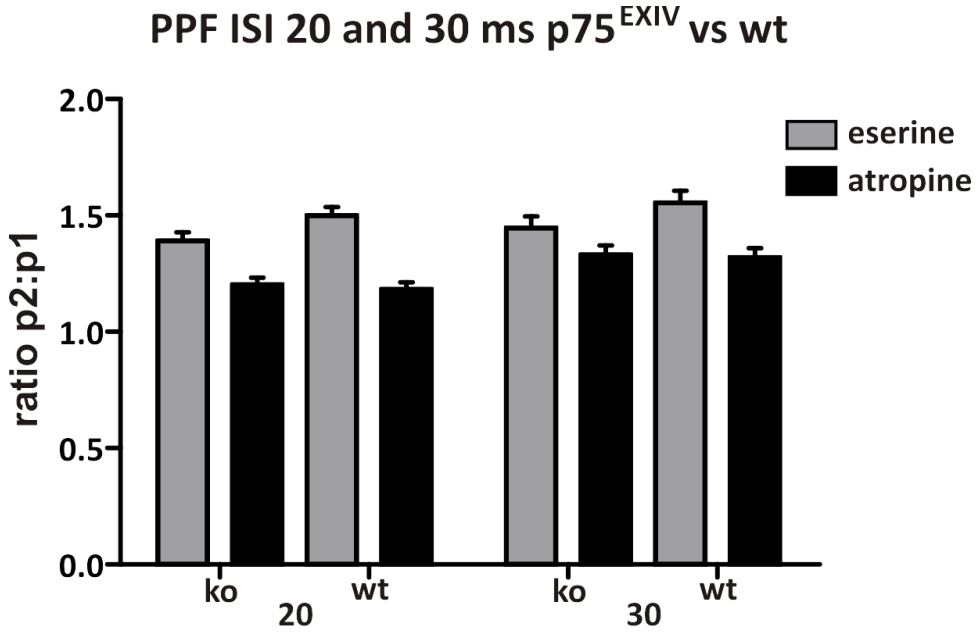


Figure 6.3.5 | Paired Pulse Facilitation p75EXIV mice vs wt PPF was carried out at 20 ms to eliminate the chance of a ceiling effect and at 30 ms to compare with wt animals. Data: Two-way ANOVA with Bonferroni posttests, plotted as mean ± sem.

	wt eserine	**wt atropine**	**ko eserine**	**ko atropine**
20	1.500±0.036	1.183±0.029	1.391±0.036	1.203±0.029
30	1.554±0.051	1.320±0.040	1.445±0.051	1.331±0.040
20	vs wt atro ***	vs ko atro ns	vs ko atro **	
30	vs wt atro ***	vs ko atro ns	vs ko atro ns	
20	vs ko eser ns			
30	vs ko eser ns			
N=5				

6.4 LTP

6.4.1 LTP induction in wt animals

LTP indcution in wt animals was performed using a standard high frequency stimulus paradigm consisting of a stimulus that induced 50% amplitude of the maximum for field potentials and induced via external capuslue stimulation (Fig. 6.2.1). Wild type animals showed a stable, higly significant LTP (159.9 ± 0.6127 ***$p < 0.001$; N=5) that could be meausered for at least 60 minutes or longer (Fig. 6.4.1 A). Baseline and LTP was stable through out recordings and induction of LTP occurred in approximately 60% of induction attempts. As no differences were observed between the La and BL, data was combined (Fig. 11.1.1, appendix).

6.4.2 LTP induction in $p75^{EXIV}$ animals

Induction in ko animals occurred in only about 35% of attempts and did not last for longer than 35 ± 10 minutes (Fig. 6.4.1 B). The standard paradigm as used in wt mice had to be modified in that the induction baseline of 50% maximum amplitude did not induce LTP and therefore, the baseline induction amplitude was between 50 and 60% of the maximum field potential induced with EC stimulation. Baseline as well as LTP showed greater variability and it was often the case that a stable baseline of 20 minutes could not be obtained. However $p75^{EXIV}$ did show a significant potentiation compared to baseline on the same level as wt animals (161.4 ± 1.100 ***$p < 0.001$; N=5) Input/output curves did not reveal any differences between $p75^{EXIV}$ and wt mice (Fig. 6.4.2). In all attempts, it was never possible to achieve a stable LTP in $p75^{EXIV}$ animals for 60 min or longer as in wt mice, indicating that the p75 neurotrophin receptor may play an important function in LTP maintenance.

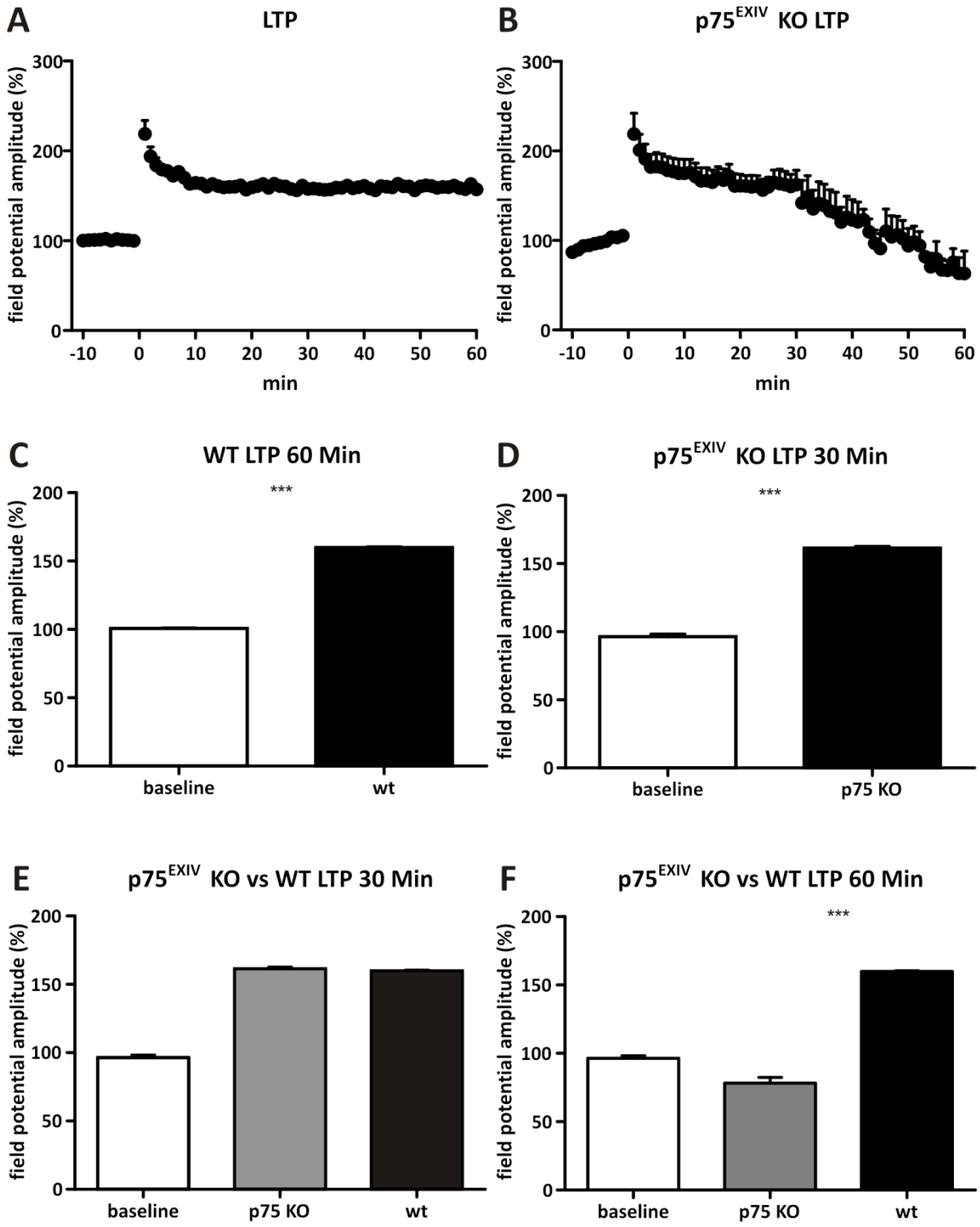
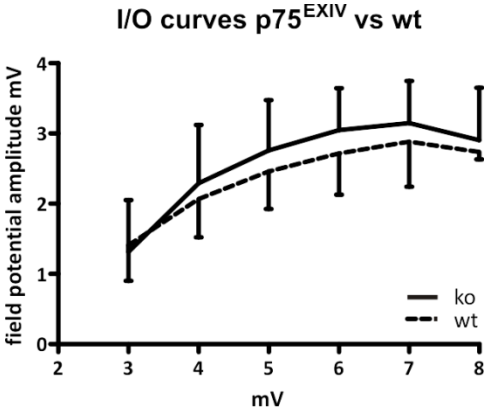


Figure 6.4.1 | LTP LTP in wt and p75EXIV mice induced by HFS stimulation (A,B) Time course for LTP in wt and ko (C,D) Mann-Whitney analysis of last 10 min for wt and min 20 to30 for ko reveals highly significant LTP potentiation in both wt and ko mice (E,F) Kruskal-Wallis analysis with Dunns Multiple Comparison Test shows no significant difference between wt and ko mice at 20-30 min (E) and highly significant differences for 50 to 60 min (F). It must be noted that for B time 30-60 min and for F the N for ko animals is only 2.
(C,D) wt: 159.9 ± 0.6127 | ko: 161.4 ± 1.100 ***p < 0.001 n: 5 (E) wt: 159.9 ± 0.6127 | ko: 161.4 ± 1.100 ns n: 5 (F) wt: 159.9 ± 0.7012 ko: 78.22 ± 4.193 wt n: 5 ko n: 2



	wt	ko	p
3	1.409 ± 0.179	1.316 ± 0.260	ns
4	2.063 ± 0.192	2.293 ± 0.293	ns
5	2.460 ± 0.189	2.757 ± 0.254	ns
6	2.715 ± 0.208	3.048 ± 0.211	ns
7	2.882 ± 0.227	3.149 ± 0.211	ns
8	2.736 ± 0.073	2.904 ± 0.528	ns

Figure 6.4.2 | I/O curves I/O curves for p75EXIV mice and wt mice Two-way ANOVA with Bonferroni posttests reveals no differences between wt and p75EXIV mice **N:** 5

6.4.3 Cholinergic challenge of LTP

Bath application of either 1 µM eserine or 10 µM atropine before HFS induction was used to determine a cholinergic effect on LTP in wt animals. As expected based upon fEPSPs, eserine prohibited LTP induction. Since eserine application caused an increase in threshold, a higher intensity HFS stimulus (80% of maximum amplitude) was also given resulting in either no LTP or short lived LTP lasting 2 to 5 minutes and in rare occasions, induction of LTD. As all observations were not readily reproducible with higher intensity HFS data are not shown. Also in congruence with fEPSPs, atropine blocked LTP formation as it already created potentiation greater than that normally reached by standard HFS induction. Due to lack of animals, experiments were not repeated in p75^{EXIV} mice.

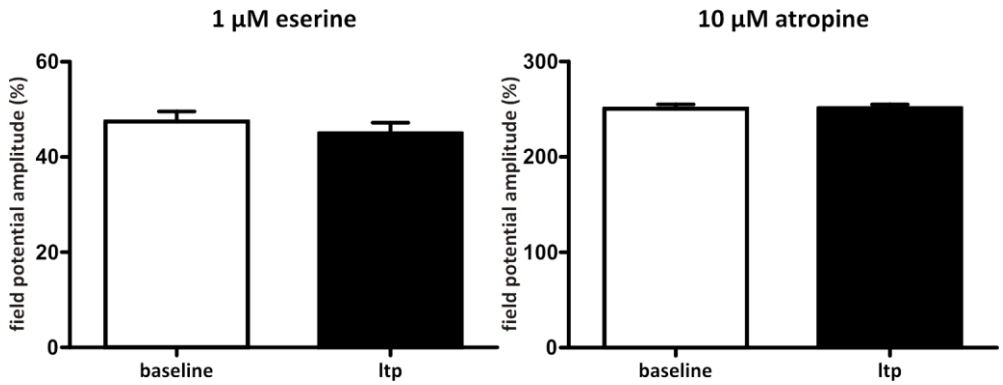


Figure 6.4.3 | Cholinergic challenge of LTP Challenge of LTP by either esering or atropine show no effect. Mann-Whitney **N:** 5

7 Discussion

7.1 Immunohistochemistry

7.1.1 ChAT Staining

As was expected, in control animals, ChAT immunohistochemistry showed a significant difference between the amygdala nuclei BL and La. This corresponds with what has been previously reported in the literature and provides a basis for the relative few differences observed between the two nuclei during electrophysiological recordings. ChAT staining also provided strong support that the horizontal slice preparation was a suitable means for subsequent electrophysiological studies in that the BL and La nuclei were readily accessible and identifiable, cholinergic SI afferents could easily be stimulated and both nuclei received SI innervation on this plane, the fiber tract of the EC was also intact providing a stimulation site for cortical afferent input for both the BL and La nuclei. What could also be seen in the horizontal slice preparation, but was not investigated, was the clear difference in cholinergic innervation of the amygdala as compared to other brain areas as previously reported [187].

Cholinergic projections to the amygdala from the SI appear to be the only known cholinergic pathway in the basal forebrain that do not necessarily express the p75 neurotrophin receptor as elucidated with IgG-saporin injections; suggesting at least two types of cholinergic projections to the amygdala [67, 68]. Although the BL showed significantly higher intensity for ChAT as compared to the La when measured with ImageJ, the subsequent electrophysiology deployed here failed to elucidate a strong cholinergic difference between the two nuclei except for intrinsic studies. This also corresponds with what has already been reported where differences are readily seen in patch studies but not field studies despite the known difference in cholinergic innervation of the BL and La nuclei and resulting in reference given only to the full BLA and no distinction being made between the individual BL and La nuclei.

7.1.2 Cholinergic challenge of fEPSPs

Bath application of 1 µM eserine or 10 µM atropine was able to inhibit/block and potentiate the field potential in both the BL and La of wt animals respectively. In $p75^{EXIV}$ animals, the effect of eserine or atropine on the fp was attenuated. Atropine in $p75^{EXIV}$ actually resulted in a slight depreciation in sharp contrast to the high potentiation in the wt animals. The attenuation of the inhibitory effect of eserine in ko animals can be attributed to a lower release of ACh as p75 depleted cells do show a 3.7 fold decrease in ACh levels [188]; however, the lower release did not affect the fp for $p75^{EXIV}$ animals in normal ringer. A possible explanation for this is discussed in the next section as it relates to differences seen in paired pulse facilitation.

Activation of mAChRs inhibits presynaptic transmitter release through activation of gabaergic interneurons and this is the most obvious explanation for the seen decrease in fESPs for wt mice. As well, in the case of atropine potentiation in wt animals, it can also be argued that the effect is due to blockade of mAChRs by atropine allowing an increase of the fp by greater transmitter release [33, 69, 189]. The observed inhibitory effect of acetylcholine agonist is in agreement in studies elsewhere. Yajeya et al. [190] showed that carbachol dose dependently suppressed the excitatory postsynaptic potential in BL and La neurons using a sharp microelectrode protocol and stimulation of the external capsule to elicit orthodromic EPSPs. This carbachol induced suppression of the EPSP was completely blocked by application of atropine which is in agreement with the field studies presented here. The ability of atropine to potentiate the fESP as shown here is implied in the study by Yajeya et al. but he makes note of the necessity for further investigation on the intrinsic effect of atropine on the ESP as his report did not look at atropine applied alone. As it is known that the cholinergic system plays a vital role in information processing for the amygdala, these data here suggest possible learning/memory impairment for $p75^{EXIV}$ mice as they have a significant change in their response to cholinergic challenge.

7.1.3 Cholinergic challenge of paired pulse facilitation

The p2:p1 ratio reached a peak in wt animals at an ISI of 40 milliseconds while $p75^{EXIV}$ mice showed a peak at 30 ms indicating a difference in transmitter release probability for the two groups as such; wt animals show a greater release probability as compared to ko animals. This coincides with fEPSPs results in that the wt mice demonstrate stronger fp inhibition during eserine application as compared to ko animals as a result of stronger mAChR activation. It also provides a possibly explanation for the differences between $p75^{EXIV}$ mice and wt mice with regard to atropine application. Since wt animals have greater transmitter release probability, it can be suggested that 1: the basal level of ACh in $p75^{EXIV}$ animals is lower (due to release probability) giving rise to a larger fp in baseline, (due to diminished presynaptic inhibition via mAChRs) which was not significant to wt animals, resulting in a lower effect of atropine and 2: since the stimulation site of the EC is not exclusively cholinergic, it can also be suggested that release probability as a whole for $p75^{EXIV}$ is diminished so that in the presence of atropine and muscarinic block, glutamate release is also lower than that of wt animals resulting in no potentiating effect for ko animals as in wt. It has been shown that lesioning of neurons which express $p75^{NTR}$ results in reduced basal levels of acetylcholine as well as release [191].

The data here evidence that both ACh levels and release probability are most likely working together. I/O curves for $p75^{EXIV}$ display a more exponential curve and reach plateau faster than that of wt animals indicating stronger field potentials; however, there were no significant differences in I/0 curves between ko and wt animals. The p2:p1 ratio is increased significantly more in wt animals as ko mice in the presence of eserine indicating greater presynaptic inhibition for the wt mice and indirectly a greater release probability of ACh. Application of atropine returns the PPF ratio back to baseline levels for wt mice at all ISIs whereas in $p75^{EXIV}$ animals, atropine does not fully return the ratio to baseline levels. This is especially true for ISIs greater than 40 ms where the ratio stays elevated which could also indirectly hint at a stronger fp for $p75^{EXIV}$ mice due to a lack of presynaptic inhibition via mAChR activation which was

not seen during fp induction in normal ringer. Finally, it must also be noted that NGF and ACh have a synergetic relationship in that NGF release augments the release of ACh as well as the reuptake of choline for greater ACh production. Likewise, ACh release enhances the release of NGF creating a positive feedback loop [192]. Most importantly, it has recently been reported that the enhanced release of ACh by NGF is dependent upon the p75 neurotrophin receptor [193]; providing the best explanation for the observed cholinergic differences seen here which indicate lower ACh release/levels in $p75^{EXIV}$ animals. Coinciding with data from cholinergic challenge of the field potential, the discrepancy in PPF between wt and $p75^{EXIV}$ animals also gives credence to a disruption in the normal cholinergic response for these animals as well as a high probability for problems associated with learning and memory. The PPF data here are in agreement with reports that carbachol increased the paired pulse ratio in the entorhinal cortex via presynaptic inhibition [194] which was blocked by atropine. A CCh induced increase of the PPF ratio was also observed in the hippocampus [195].

7.1.4 Plateau firing

It was possible to initiate plateau firing in neurons of the basolateral and lateral amygdala nuclei by two standard methods that have been previously reported [9, 124]. During bath application of carbachol, in both nuclei plateau firing was induced by holding V_M to just below threshold (10 to 12 mV more negative) with direct current injection and eliciting plateau firing with a 0.2 to 0.4 nA 4 second suprathreshold pulse. As well, persistent activity could also be induced in both nuclei in the presence of eserine, using the same V_M hold technique and stimulating cholinergic substantia innominata afferents directly before the suprathreshold pulse. However, unlike what has been reported for La neurons [9] as well as the data presented here, it was also possible to induce plateau firing in BL neurons by continuously stimulating SI afferents every 30 seconds from V_M rest.

A possible explanation for the differences in plateau induction between the BL and La is the greater cholinergic innervation of the BL. This would provide for greater ace-

tylcholine release resulting in a possible larger rise in postsynaptic calcium levels by mAChR activation of the IP_3 cascade. This in turn, leads to a gradual depolarization and eventual action potential firing and is in line with a non NMDAR form of LTP which has been coined LTP_{IP3} [110]

Another phenomenon that was present in BL neurons but absent in La was the ability of SI afferent stimulation to cause a hyperpolarizing membrane deflection. When a suprathreshold DC pulse was given immediately following this deflection it was blocked. Neurons that displayed this V_M hyperpolarized deflection were still able to produce plateau firing following repeated 30 second ISI SI afferent stimulation. Again greater cholinergic innervation of the BL could account for this observed difference. Muscarinic acetylcholine receptors also activate SK channels causing a brief hyperpolarization of membrane potential, yet this only occurs when ACh acts on soma and proximal dendrites [70]. Greater cholinergic innervation of the BL would provide greater probability that ACh could act at somatic and proximal dendritic sites as well as distal dendritic sites, where in the La, most ACh activity would only occur distally. The data here also show that the ability of SI stimulation to inhibit a suprathreshold pulse was dependent upon stimulation frequency and not duration or intensity providing evidence this inhibition could be related to the frequency of SK channel activation. Stronger SI stimulation intensities resulted in greater membrane depolarization and a higher probability of AP firing most likely due to greater ACh release and stronger induction of the IP_3 pathway. It has been shown that IP_3 pathway blockade prevents plateau firing in La neurons [9] and SK channel block increases mAChR induced membrane depolarization as well as AP firing [70].

In line with this study but using a LTP paradigm, Park et al. [196] showed that carbachol was able to induce a transient depression of the field potential in the lateral amygdala yet also induce a form of LTP both the depression and LTP were observed in the presence of picrotoxin. The initial transient depression did not interfere with the ability of CCh to induce a postsynaptic form of LTP which was dependent upon

the cAMP pathway. Both the initial transient depression as well as the induced L-LTP could be blocked by atropine. Inhibition of the cAMP pathway blocked LTP induction by carbachol but not the initial transient inhibition. This is in agreement with the data presented here that the membrane deflection seen following SI stimulation in BL neurons did not interfere with the ability of these neurons to induce plateau firing initiated by repeated SI stimulation every 30 seconds suggesting that the initial deflection is presynaptic and, coinciding with previous reports, the plateau potential is a postsynaptic event relying on second messenger cascades [9].

The cholinergic system appears to be able to modulate both network activity and intrinsic activity in the amygdala. Presynaptic inhibition controls network excitability while activation of SK channels can regulate the excitability of single neurons regardless of network activity. Still, even when individual neurons or the network are receiving substantial inhibition, acetylcholine can act postsynaptically via second messenger pathways to induce plateau firing in individual neurons or LTP in the network.

7.2 LTP

7.2.1 $p75^{EXIV}$ vs wt animals

Wt and $p75^{EXIV}$ animals did not differ in LTP potentiation as compared to baseline; however, LTP of 60 minutes or greater was not possible to achieve in ko animals with the standard HFS induction paradigm deployed here. The loss of the p75 neurotrophin receptor could disrupt postsynaptic calcium release and thereby hindering LTP maintenance which is virtually always a postsynaptic event. Still, with other induction paradigms such as TBS, or other experimental conditions such as GABAergic synaptic blockade, LTP duration of 60 minutes or greater might be achieved in these animals. HFS was selected here since it is generally considered to be a more robust induction paradigm and it has been shown that TBS fails to elicit LTP in the amygdala via EC fiber stimulation [197]. As it is generally agreed that L-LTP is dependent upon protein synthesis, it may also be that the lack of the p75 neurotrophin receptor disrupts protein synthesis in $p75^{EXIV}$ mice and therefore L-LTP. It has been shown in hippocampal

LTP that the lack of BDNF interacting with TrkB disrupts HFS induced LTP and prevents the formation of L-LTP [198]. Cleary the lack of the p75 receptor disrupts HFS LTP in $p75^{EXIV}$ animals as a higher intensity stimulus must be deployed of 50 to 60% maximum amplitude and L-LTP is lacking in these mice. So a synergy between NGF, $p75^{NTR}$ and TrkA could be necessary for L-LTP protein synthesis as well.

In the data here, $p75^{EXIV}$ animals have a clear disruption in LTP in that LTP was never induced for a period longer than 30 minutes and required a stronger induction as that for wt animals. However, LTP was not fully abolished in these animals suggesting that $p75^{EXIV}$ animals may show learning deficits based upon the type of learning as not all task would be dependent on L-LTP. This corresponds well with the field potential and PPF studies in that the $p75^{EXIV}$ animals are showing a clear impairment in normal ACh response but not a complete lack thereof.

There is currently little reported about these animals in the literature and what is available is conflicting. Catts et al. [199] showed that p75NTR -/- animals demonstrated impairment in the Morris water maze along with decreased neurogenesis and higher cell death of new cells. They suggested that the findings are most likely a result of altered circuitry due to the loss of p75NTR. Barrett et al. [200] showed conflicting results in that they reported p75NTR -/- animals demonstrated enhanced spatial memory as revealed by the Morris water maze and enhanced hippocampal LTP. The differences in the studies could be related to the background strain of the animals used. Barrett reports using a 129/Sv strain and the animals are $p75^{EXIII}$ mice which still possess a truncated p75NTR allele while Catts animals were backcrossed into a C57/BL6 strain and procured from Jackson Laboratories indicating the mice were also $p75^{EXIII}$. While the report from Barrett also contradicts the LTP data here, it must also be noted the strain difference, that the animals here were $p75^{EXIV}$ which do not possess the truncated allele and experimental design. Despite the early conflicting reports it is clear that animals lacking the p75NTR have disruptions in learning and memory.

7.2.2 Cholinergic challenge of LTP

Eserine applied before induction was able to block LTP induction by increased presynaptic inhibition whereas atropine application preceding induction showed no effect. Following LTP induction, application of either eserine or atropine did not disrupt LTP indicating that LTP maintenance is most likely postsynaptic and it has been shown that LFS stimulation following LTP induction via the EC fiber pathway also did not affect LTP [197]. Of interesting note, LFS induction of LTP was also possible in the amygdala only via stimulation of EC fibers which resulted in a slow onset, greater than 60 minutes and dependent upon protein synthesis further indicating postsynaptic maintenance [201]. Unfortunately due to a lack of $p75^{EXIV}$ animals, cholinergic challenge of LTP was not performed. It has been shown that in the hippocampus atropine will either enhance or suppress LTP induction depending on the induction site although the reported enhancement was not significant [202, 203].

8 Conclusion

8.1 Plateau firing

Intrinsic plateau firing differed between the BL and La amygdala nuclei as greater cholinergic innervation of the BL allows stronger ACh release at somatic and proximal dendritic sites which gives rise to a robust biphasic effect of ACh which is not observed in La neurons where ACh primarily only acts at distal dendritic sites. Distal dendritic activation causes an inhibition of the after hyperpolarization current allowing excitation, whereas proximal and somatic activation creates a competition between small conductance, calcium activated, potassium channels, which cause hyperpolarization, and the inhibition of the AHP. BL neurons in the presence of eserine displayed this biphasic effect of ACh seen as a hyperpolarization membrane deflection following cholinergic SI afferent stimulation. As well, repeated cholinergic afferent stimulation every 30 s was able to induce plateau firing in BL neurons during eserine application but not in La neurons. As with the initial hypothesis, the higher cholinergic innervation of the BL, compared to that of the La, causes a difference in intrinsic firing properties of projection neurons of both nuclei.

8.2 Cholinergic Challenge

Unlike sharp recordings, extracellular field recordings did not elucidate a difference between the BL or La nuclei. Application of eserine or carbachol always caused a time dependent reduction of the field potential which could be blocked and reversed by atropine. Eserine also increased the threshold properties for the field potential. When atropine was bath perfused alone, it induced a substantial potentiation of the fp.

Contradictory to the initial hypothesis, $p75^{EXIV}$ did not demonstrate a greater response to cholinergic challenge as was expected due to the increased cholinergic fiber density for these animals. These mice showed an attenuation of the presynaptic inhibitory effect of eserine as well as no potentiation to atropine alone in fEPSPs ex-

periments. Paired pulse facilitation revealed that the ko animals have a lower release probability as well as reduced presynaptic inhibition as evidence by a smaller PPF ratio as compared with their wt counterparts.

8.3 LTP

It was also predicted that the $p75^{EXIV}$ mice would show enhanced LTP when initiated in a way that the cholinergic system would be activated. Compared to wt animals, $p75^{EXIV}$ showed a disruption in LTP induction as the HFS paradigm had to be carried out at higher stimulus intensity in these animals. They also only showed LTP in essentially 34% of induction attempts as compared to 60% for wild types. The level of LTP reached was comparable to wt when correlated at 20 to 30 minutes; however, p75 mice never showed LTP for 60 minutes or more. In fact, only 2 out of 5 animals tested, demonstrated LTP for greater than 30 minutes and these two quickly fell below baseline levels.

Agreeing with fEPSPs studies, there were no differences seen between the BL or La nuclei. Unlike fEPSPs experiments, cholinergic challenge did not affect LTP induction or maintenance in wt animals. It was not seen if this was the case in $p75^{EXIV}$ animals due to insufficient mice.

8.4 Summary

Projection neurons of the BL and La show intrinsic firing differences due to higher cholinergic innervation of the BL.

The differing cholinergic innervation of the BL and La does not affect fEPSPs or LTP.

$p75^{EXIV}$ mice have lower release probability resulting in an attenuated cholinergic response.

$p75^{EXIV}$ mice show a disruption in LTP induction as well as a lack of L-LTP in a HFS LTP induction paradigm.

9 Out Look

It would be necessary to further study LTP in the $p75^{EXIV}$ animals as the numbers here are insufficient to draw proper conclusions. However, the evidence of a lack of L-LTP is quite interesting and this should be followed up along with at least western blot analysis.

Intracellular recordings in the $p75^{EXIV}$ animals would also need to be done as well with blockade of SK channels to fully understand intrinsic differences underlying cholinergic responses between wt and ko animals.

Finally, behavior experiments that coincide with the amygdala LTP paradigms presented here such as fear conditioning or learned helplessness would be of great interest.

10References

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11Tables and Figures

12Appendix

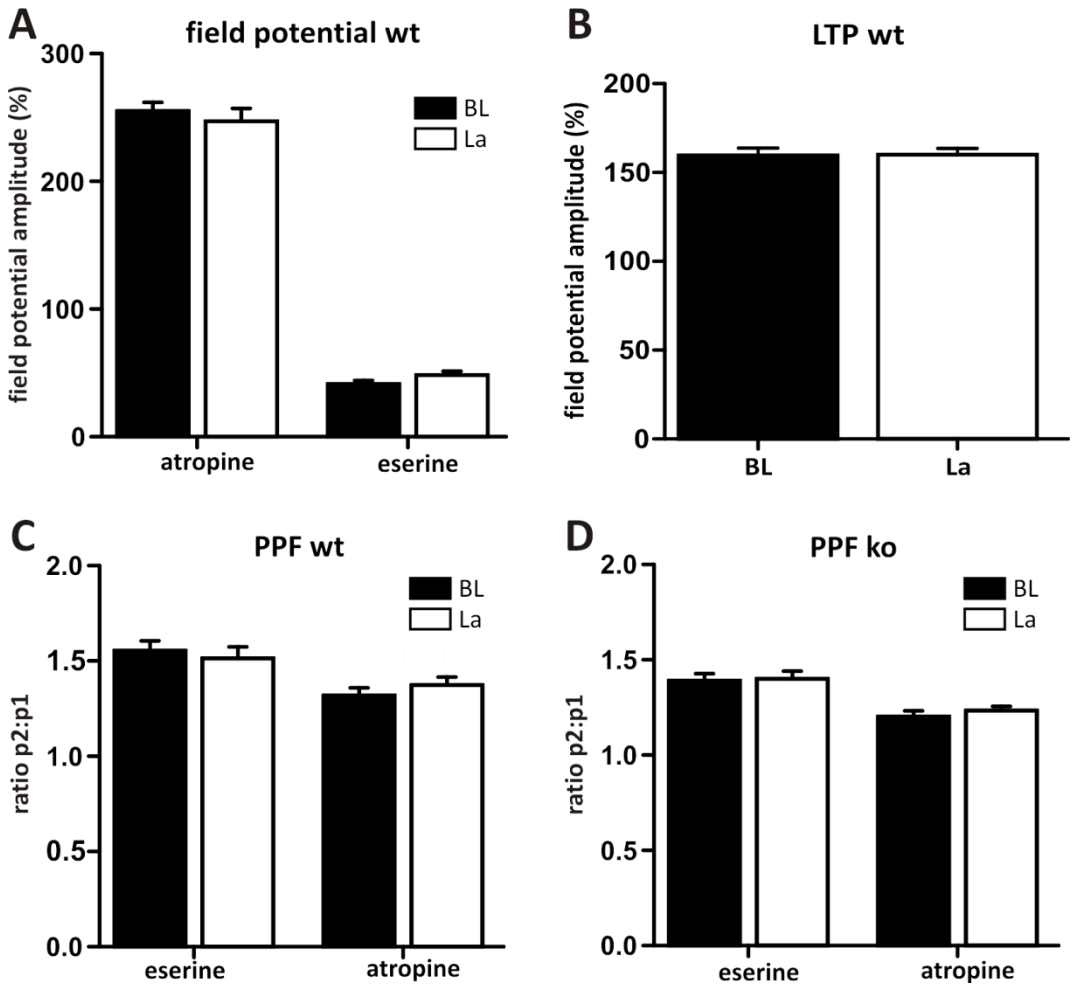

Figure 8.4.1 | BL vs La control Control studies to test differences between the BL and La nuclei of the amygdala. (A) Field potential recordings revealed no differences between the BL and La for normal ringer (not shown) or challenge with either 10 µM atropine or 1 µM eserine. (B) No difference in LTP magnitude was seen between the BL or La. (C,D) Paired Pulse Facilitation did not elucidate a difference between the BL or La in normal ringer (not shown) or with cholinergic challenge (10 µM atropine, 1 µM eserine) Drugs were bath applied. All data were analyzed using Mann-Whitney and presented as mean ± sem. (A,C,D) Were graphed together for simplicity.

	A		B	C		D	
	eserine	**atropine**	**n/a**	**eserine**	**atropine**	**eserine**	**atropine**
BL	41.35 ± 2.677	255.0 ± 6.857	159.6 ± 4.185	1.554 ± 0.051	1.32 ± 0.04	1.391 ± 0.036	1.203 ± 0.029
La	48.4 ± 2.96	247.0 ± 10.05	160.2 ± 3.407	1.514 ± 0.060	1.375 ± 0.041	1.401 ± 0.039	1.234 ± 0.022
p	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**
n	**5**	**5**	**3**		**5**		

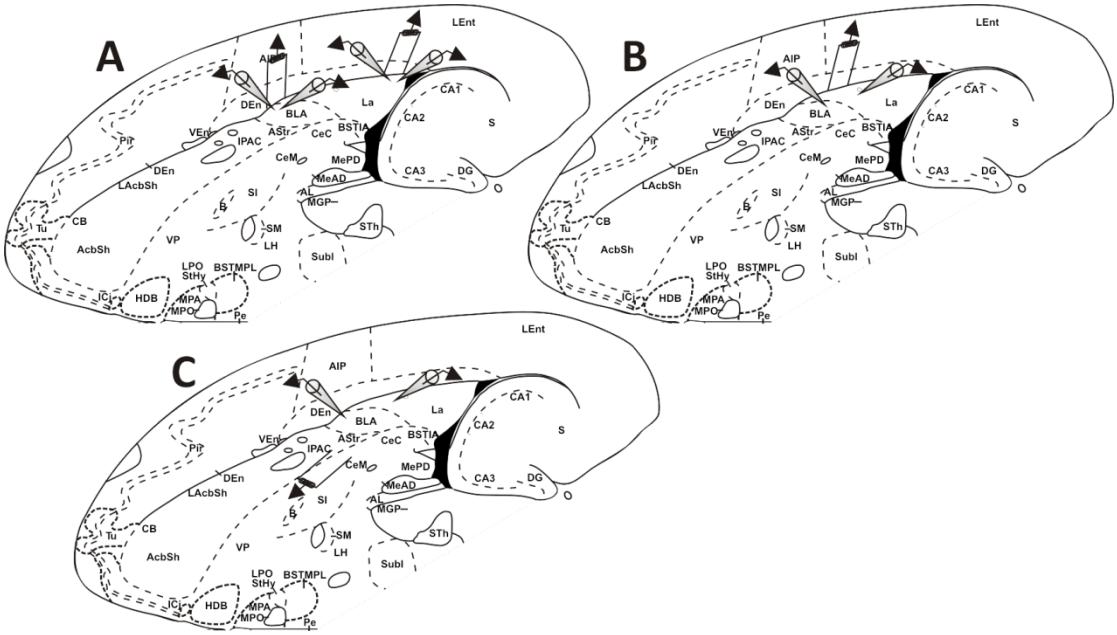

Figure 8.4.2 | Stimulator and Electrode Placements (A) Diagram depicting the plane of the horizontal slice preparation used for field recordings, PPF, and LTP and the approximate placements of stimulator and microelectrodes. (B) Placements of stimulator and microelectrodes for simultaneous recording in BL and La during fp, PPF, and LTP paradigms. (C) Placements during intracellular recording in either La or BL. Modified from Paxinos 1998 [186]

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