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CHOLINERGIC MODULATION OF SYNAPTIC TRANSMISSION AND PLASTICITY

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ABSTRACT

The physiological and cognitive states of the brain are influenced by variations in the activity of the cholinergic systems. For example, changes in the levels of ACh have been associated with arousal/sleep cycle, sustained and focal attention. Moreover interfering with cholinergic transmission affects learning and cellular plasticity. Despite cholinergic system exerts its action by modifying the extracellular cortical concentration of acetylcholine (ACh) few investigations have until now tested if and how variation in ACh concentrations could influence neuronal synaptic efficacy and plasticity in acute brain preparation. In order to investigate this aspect we have used a quantitative experimental approach (variations in the levels of cholinergic activity) rather than a simply qualitative (absence or presence of cholinergic activity) on rodent visual cortex slices. We found that the extracellular ACh concentration affected in opposite way cortical synaptic efficacy, producing either an enhancement or an inhibition of evoked field potentials (FPs) respectively with low or high concentrations of exogenously applied ACh. The versus of ACh modulatory action was dependent on the activity of AChE and relayed on specific subtypes of muscarinic acetylcholine receptors (mAChRs), thus linking the action of ACh to the activation of particular receptor subtypes. The demonstration of a synaptic-pathway specificity of ACh modulatory action, suggests that cholinergic release could control, in a dynamic way, the flow of cortical information. Moreover, we showed that ACh concentration in cortical tissue contributes to modulate long term changes of synaptic efficacy, such as LTP or LTD induced by specific patterns of afferent neuronal activity. We found that: 1) in the absence of muscarinic receptors activation LTP is not inducible as shown in slices treated with atropine, 2)cholinergic action on cortical LTP depends on the activation of the even (M_2, M_4) mAChRs. In addition, the sign of long term change, whether LTP or LTD, appears to be depend on the endogenous level of ACh; indeed, we reported that burst stimulation of afferent neurons, in rats with reduced cortical cholinergic innervation, induces an LTD instead of LTP. These results suggest that the degree of activation of cholinergic system could control cortical the direction of synaptic plasticity in visual cortex.

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ABBREVIATIONS

AC = nucleus accumbens

ACh= acetylcholine

AChE= acetylcholinesterase

AMP =adenosine monophosphate

cAMP= cyclic AMP

ChE= cholinesterase

ChAT= choline acetyltransferase

CNS= central nervous system

DAG= diacylglycerol

DR = dark rearing

E12 = embryonic day 12

FP= field potential

GABA= γ-aminobutyric acid

GMP= guanosine monophosphate

IC = intra-cortical

192 IgG-saporin= 192 immunoglobuline G-saporine

IP_3= inositol 1,4,5-trisphosphate

LC= Locus Coeruleus

LGN= lateral geniculate nucleus

LTD= Long-Term Depression

LTP= Long-Term Potentiation

M_1= Muscarinic acetylcholine receptor 1

mAChRs= muscarinic acetylcholine receptors

MD= monocular deprivation

NGF= nerve growth factor

P1= postnatal day 1

PI= phosphatidylinositol

PLC = phospholipase C

PPT= pedunculopontine tegmental nucleus

RT-PCR = reverse transcriptase polymerase chain reaction

TBS= theta burst

TC= thalamo-cortical

TTX= tetradotoxine

WM= white matter

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1.0 INTRODUCTION

1.1 Cortical organization

The cerebral cortex is divided in many areas, which can be distinguished by the appearance of the cells (cytoarchitecture) and fibres (myeloarchitecture). Based on their physiological function these cortical areas can be categorized as sensory, motor or associative. Sensory areas receive and process information about the external environment and the internal state of the organism. Each sensory modality (auditory, gustative, olfactory, somatosensory and visual) is analysed by a specific sensory area that is denominated auditory, gustative, olfactory, somatosensory and visual cortices. Within each of these areas further subdivisions in primary, secondary, etc. can be made based on hierarchical processing of sensory information. Primary sensory areas constitute the first cortical station for sensory inputs. In these areas the basic features of afferent signals triggered by external stimuli are extracted and then are sent to higher order sensory cortices where more complex analysis is computed. Motor cortical areas control voluntary movement through muscle contraction. Even in this case a hierarchical organization is present in which primary motor cortex constitute the final output. Associative areas support high cognitive functions and constitute a link between sensory and motor areas. Despite such specific functional competence, a common pattern of cortical organization is present not only among animals of the same species but also through the mammalian class. In particular a stratification of cortical cells of similar morphology determines the formation of cytoarchitectonically distinct layers. Starting from the external surface of the cortex these are numbered using progressive roman number. For example, sensory cortices are composed by six layers, from I to VI (fig.1.1). In sensory cortices, following the flow of information layer IV represent the principal station for the afferent fibres. Due to the presence of many small cells it is also called granular layer. This layer is particularly expanded in primary sensory areas that receive thalamic fibres bearing sensory information (Lund 1984; Martin and Whitteridge

1984; Levitt et al 1996). Here the principal cellular types are the spiny stellate neurons that are excitatory and are distinguished by their radially arranged star-like dendrites. In associative and higher order sensory cortices layer IV receive inputs from preceding cortical areas that synapse onto small pyramidal neurons (Lund 1984). The axon of layer IV excitatory neurons project to layer II/III of the same cortical area making synapses onto pyramidal neurons. In the primary sensory cortical areas these neurons also receive direct projections from thalamic nuclei. Pyramidal cells in layer II/III in turn provide the onward cortico-cortical projection with their axons that synapse onto neurons of next cortical areas (layer IV of non-primary sensory and in layer II/III of motor and associative cortices). The axons of superficial cells of layer II/III also have collaterals terminating in layer V of the same cortical area (Martin 1984). Layer V constitutes the principal output station of the cortex; indeed, there are large pyramidal neurons that project to subcortical sites (Martin and Whitteridge 1984). Some of the axon collaterals of these cells project to the layer I of cortical areas of lower hierarchical level forming a back-propagating system that is thought to have an essential role in the computational activity of the cortex (Rolls and Treves 1998). Layer VI pyramidal neurons receive excitatory inputs from thalamic fibres through their dendritic arborization in layer IV (Martin 1984). In turn they project back to the thalamic nuclei controlling the flow of information that reaches the cortex (Martin and Jessel, 1998). They also form back projections to layer I of preceding cortical areas (Jones and Peters 1984; Peters and Jones 1984). Layer I is thin and composed mainly by glial cells, fibres and dendrites from pyramidal neurons located in the other layers. Within the cortex there is also a heterogeneous population of GABA releasing inhibitory interneurons that, due to the absence of spines on their dendrites, present a smooth morphology. Basket cells that are distributed throughout all the layers with the highest concentration in supragranular layers represent the first type of inhibitory interneurons; their dendrites and axons can extend laterally from the cell body for several hundred micrometers producing a quite extensive lateral inhibition (Somogyi et al 1983). They receive synapses from many extrinsic afferents to the cortex and contribute

to a feedforward as well as feedback inhibition (Martin 1984). The chandelier cells have axo-axonic synapses with the initial segment of pyramidal cells in layer II/III (Peters 1984). For these reasons they are believed to prevent the generation an output spikes from this layer (Roll and Treves 1998). Third types of inhibitory cells are the double bouquet. These neurons are located in layer II/III and present a vertically oriented axon often confined to a narrow column of 50 μm in diameter (Somogyi and Cowey 1981). They receive mainly inhibitory synapses and synapse on pyramidal neurons. Such double inhibitory circuitry may emphasize activity within a narrow vertical column (Roll and Treves 1998). Another type of GABAergic neuron is the sparsely non-pyramidal neurons with local axonal plexuses (Saint Marie and Peters 1985). Underneath the cortical layers there is the white matter (WM), which is composed by myelinated fibres that go towards or come from the cortex; among these are the thalamic axons and the cortical projections from the modulatory systems with the parent cell bodies located in the forebrain, midbrain and brainstem such as cholinergic, noradrenergic and serotoninergic nuclei.

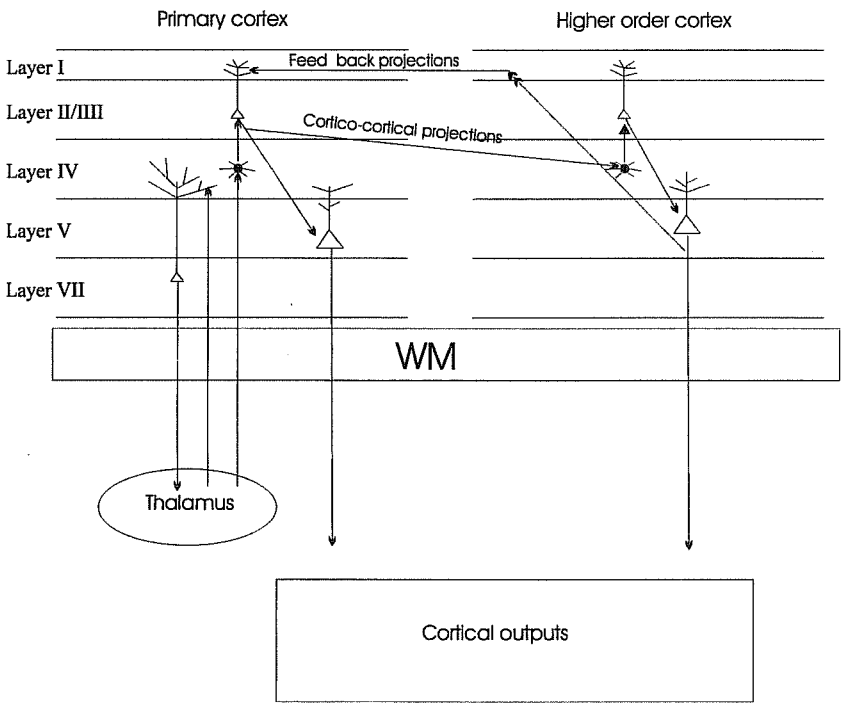

Fig 1.1 Cytoarchitectonic organization of the cerebral cortex

1.2 Cortical cholinergic system

A) Anatomical organization

In the cerebral cortex, there is an extensive cholinergic innervation that originates mainly from neurons whose cell body are located in the basal forebrain region and forming well characterized basal forebrain cholinergic nuclei (BFB) (Mesulam et al 1983; Rye et al 1984, Butcher 1992); despite cholinergic markers were also observed in a small number of neurons present within the cortex the real cholinergic nature of these cells is still debated (Houser et al 1983; Eckenstein and Baughman 1984; Levey et al 1984). Cholinergic neurons and their projections are detected by means of histochemical techniques and/or mRNA probes as markers for choline acetyltransferase (ChAT) and acetylcholinesterase (AChE). The former is the enzyme that, in the cholinergic terminals, synthesizes acetylcholine from acetyl-coenzyme A and choline and the latter the one that, in the extracellular space, degrades ACh to acetic acid and choline. The combined use of immunohistochemical and in situ hybridization techniques with retrograde tracing allowed identifying the topology of cholinergic system that project to the cortex (Rye et al 1984). In particular, based on their projection area, the BFB population gives rise to at least four different pathways: 1) the basalo-olfactory bundle, projecting to the olfactory bulb and associated nuclei; 2) the basalo-hippocampal bundle, innervating the hippocampal formation and nearby limbic cortex; 3) the medial cortical pathway, originating mainly in the vertical and horizontal limbs of diagonal band, nucleus basalis, and partially in the magnocellular preoptic area and substantia innominata (these nuclei project to medial cortical regions, including the medial frontal, cingulate, retrosplenial and medial occipital cortices); 4) the lateral basalocortical and basaloamygdalar tracts, supplying afferents to the remaining allocortex, to the lateral isocortex, and to the amygdala (Mesulam et al 1983, Butcher and Semba 1989). Cholinergic cells in the basal forebrain form concatenated constellations of large (18–43 μm) multipolar and isodendritic neurons with long axons (Butcher and Woolf 1984). These are intermingled with a heterogeneous population of non-cholinergic projecting neurons and interneurons forming a complex local circuitry (Zaborszky et al 1999) moreover some neuronal projections appear to be closely associated with blood vessels. This anatomical

arrangement suggests that cholinergic neurons can interact among themselves both synaptically and in an endocrine fashion.

Cholinergic nuclei receive afferent inputs from the cortex, striatum, hypothalamus and brainstem (Semba et al 1988; Cullinan and Zaborszky 1991; Henderson 1997; for a review see Sarter and Bruno 2000). Afferent projections can be classified according to the used neurotransmitter: glutamatergic, gabaergic and noradrenergic afferent pathways. Glutamatergic afferents of the basal forebrain arise primarily from cortical and amygdaloid areas (Fig. 1). Brainstem regions, including the pedunculopontine tegmental nucleus (PPT), may also send glutamatergic projections to the basal forebrain. Telencephalic projections, including afferents from the amygdala, converge on basal forebrain neurons and modulate the excitability of basal forebrain neurons (Fig. 1.2).

Several studies have focused on the regulation of cortical ACh efflux by GABAergic inputs to basal forebrain cholinergic neurons, presumably originating in the nucleus accumbens (AC) (see Fig. 1.2). These studies were based on the facts that cholinergic neurons in the basal forebrain bear GABAA receptors and that acting on GABAergic transmission by the use of agonists or antagonist modifies cortical ACh release.

Noradrenergic projections to basal forebrain cholinergic neurons originate in the Locus Coeruleus (LC) and the A5 group in the ventrolateral tegmentum (Fig.1.2). The cholinergic neurons of the basal forebrain receive a particularly dense noradrenergic input and the distal segments of their dendrites are repeatedly contacted by noradrenergic fibers.

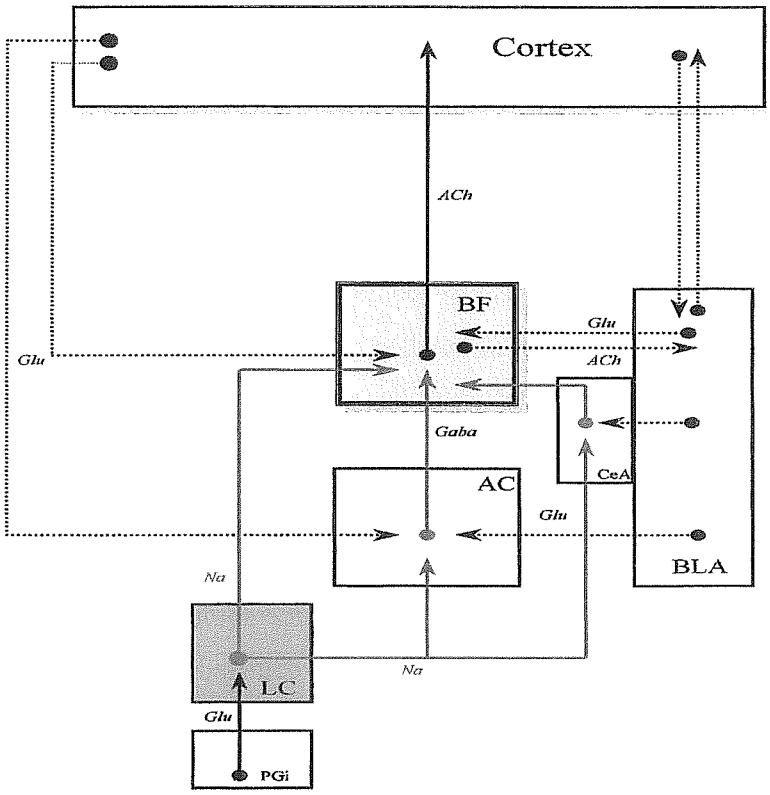

Fig. 1.2 Nucleus paragigantocellularis (PGi), is a sympathoexcitatory structure projecting to Locus Coeruleus (LC). Noradrenergic projection comes from LC. Glutamatergic pathways originate in the cortex and baso-lateral amygdale (BLA). Nucleus accumbens (AC) sends Gabaergic projections to BF nuclei. From Sarter and Bruno (2000).

Moreover BFB nuclei receive brainstem cholinergic projections from peduncolopontine and laterodorsal tegmental (PPT/LDT) cholinergic nuclei (Steriade 2001). PPT/LDT neurons appears to activate BF nuclei through glutamate (Rasmusson et al 1994) that colocalized with ACh in these cells (Lavoie and Parent 1994)

Neurogenesis of the rat cholinergic neural population begins at embryonic day 12 (E12) and is completed by E17 (Samba et al 1988). Retrograde labelling of neurons from the cortex to the BFB showed that already at postnatal day 0 (P0) the number and the pattern of distribution is similar to the adult. However, at this time the neurons are significantly smaller and faintly stained. Marked changes occur by P14 when the morphological features of BFB neurons became similar to adult ones (Dinopulos et al 1989, Calarco and Robertson 1995). For what concerns the investigation of developmental cortical cholinergic innervations different methods have been employed.

The use of anterograde tracing of BFB axons showed that cortical innervations appears at P0 in the subcortical WM under the occipital cortex; at this age the occipital cortex comprises the subplate, layer VI, an emerging layer V, an undifferentiated cell dense cortical plate and a marginal zone. At P3, when layer IV starts to differentiate from the cortical plate, BFB axons are seen in layer VI and V, while by P4 they reach layer IV. At P6, all layers of the occipital cortex are differentiated and the BFB axons are seen throughout all layers, although the vast majority of labelled axons are still confined in the infragranular layers. During the second postnatal week the axons continue to develop to reach a mature pattern of distribution by P11 (Calarco and Robinson 1995). Despite retrograde labelling allows to precisely identifying the projecting fibres it lacks specificity for the type of neuron labelled. A different method to identify cholinergic projections is by detecting the markers that are involved in the metabolism of ACh (i.e. ChAT and AChE). ChAT activity in rat visual cortex cannot be detected until P8 when a low level of activity can be observed in all layers. Thereafter, the level of activity increased until P24 (Mc Donald et al 1987). In the adult cortical cholinergic projections appear to be evenly distributed throughout all layers of motor cortex while in sensory cortex layer specificity seems to be present. In particular layer V is the most innervated while layer IV and VI are the less. The fibres display a vertical orientation in layer IV and a horizontal one in layer I where they can extend as far as 500 μm. No specific orientation is observed in the others layers (Houser et al 1985).

The cholinergic synaptic innervation of the cortex is diffuse with cholinergic terminals that show little target specificity. In particular, only a small fraction (around 17 %) of ACh containing varicosities on the cholinergic axons make synaptic contact with postsynaptic elements. In this case they make symmetric synaptic contacts primarily with dendritic branches and less frequently with spines and cell bodies of either glutamatergic or GABAergic neurons (Houser et al 1985,Wainer et al 1984). On the other end most of the cholinergic varicosities (around 83 %) terminate free in the extracellular matrix without being associated with any postsynaptic specialization (Umbriaco et al 1994, Descarries and Umbriaco 1995, Mrzljak et al. 1995). The a-synaptic varicosities seem to be randomly distributed in relation with the surrounding elements and show an incessant movement of translocation and re-shaping along the fibre (Descarries et al 1975). This

motility could constantly modify the exact position of neurons in relation to the targets and hence their functional influence. Such characteristic leads to the suggestion that ACh could act in a paracrine fashion on cortical elements, with ACh that, following the release from the cholinergic terminals, diffuse in the extracellular space influencing a large number of cortical elements (Descarries et al 1997). This modality of release is consistent with the concept of volume transmission of neurotransmitter proposed by Fuxe and Agnati (1991). The concept of volume transmission is supported by the evidence of the small number of BFB cholinergic neurons compared with that of cortical neurons subjected to cholinergic modulation (Mesulam et al 1983). Descarries et al. (1997) also proposed that in brain regions that are densely innervated by cholinergic fibres it would exist a basal level of extracellular ACh which is maintained by spontaneous or evoked release from non-juctional varicosities. This level would fluctuate depending on the activity of cholinergic neurons (Descarries et al 1995; Descarries and Umbriaco 1995). Indeed cortical fluctuations of ACh level can be measured in vivo and they have been shown to correlate with variation of physiological state of the brain (see later).

Within a diffuse action of cholinergic release, an anatomical topographic specificity exists. This is due to the reduced cortical collateralization of the axons of BFB cholinergic neurons with a single neuron innervating a restricted cylinder of cortex with an approximate diameter of 1-2 mm^2 (module) (Bigl et al. 1982, Price and Stern 1983, Baskerville et al. 1993). The number of cholinergic neurons clustered to form a cortical projecting module is greater in primates (average nomber > 15) than in rats or cats (3-5). It was speculated that this could represent a structural feature accounting for the greater cognitive abilities of primates (Woolf and Butcher 1991). This modular anatomical organization of cholinergic projections could explain the independent regional variation in cortical ACh levels such as those that are observed when sensory modalities are activated (Collier and Mitchel 1966, Fournier et al 2004). In this specific case the stimulation of a single sensory modality determines an increased ACh level restricted to the correspondent sensory cortex (Fournier et al 2004).

The cerebral cortex receives also non-cholinergic projections from BFB nuclei that have been suggested to follow the same trajectory of cholinergic projection (Woolf et al 1986). Most of these have been shown to belong to GABAergic neurons (Fisher et al

1988, Beaulieu and Somogyi 1991). In vitro studies showed that both cholinergic and GABAergic cells in BF display an intrinsic pacemaker activity that allow them to discharge in rhythmic trains (Alonso et al. 1996). The complex anatomy, cellular composition and function of BFB region rendered extremely difficult the interpretation of results obtained stimulating BFB neurons with the aim of describing cholinergic effects. Indeed, these attempts resulted in the stimulation of several groups of neurons besides the cholinergic neurons making unclear the nature of the observed changes.

B) Cholinergic receptors

Acetylcholine can bind and activate two types of membrane receptors, namely the nicotinic and the muscarinic receptors. Neuronal nicotinic receptors are ionotropic receptors of pentameric structure composed by a wide variety of subunits (a2-a10, b2-b4), exhibiting specific anatomical and temporal expression patterns within the nervous system. Nicotinic receptors subunits can be assembled into homomeric or heteromeric channels, all exhibiting a low ionic selectivity; however, homomeric receptors have an extremely high relative permeability to calcium ions (Sargent 1993, Role and Berg 1996). Muscarinic acetylcholine receptors (mAChRs) are metabotropic, i.e. involving second messengers in their action. They are composed by single subunits with seven transmembrane-spanning region and a large cytoplasmic domain and they belong to the family of G-protein coupled receptors (Kerlavage et al 1986, Hulme et al 1990). Five different subtypes of mAChRs have been discovered. These are named by uppercase letters M_1 to M_5 if pharmacologically characterized or by lowercase letters m1 to m5 if genetically isolated (Bonner et al 1987, 1988, Buckley et al 1989). Activation of different subtypes of mAChRs leads to the activation of distinct intracellular cascades of second messengers. The even mAChRs receptors M_2, M_4 are preferentially coupled with the G_i family of G-proteins through which they inhibit adenylyl cyclase reducing the intracellular levels of cyclic AMP (cAMP). The odd mAChRs M_1, M_3, M_5 activate preferentially the phospholipase C (PLC), and consequently increase the levels of diacylglycerol (DAG) and inositol 3-phosphate (IP_3) second messengers, through the coupling with the Gq family (Nathason 1987, Wess 1996, Caulfield and Birdsall 1998). However, this specificity is not absolute and a cross talk between signalling pathways can

occur depending on cell type specific differences in signalling (Peralta et al 1988, Nathanson 2000). Acetylcholine has also been reported to increase the levels of cyclic GMP trough the activation of mAChRs (for review see De Vente 2004). This has been reported to be mediated by the production of nitric oxide (Castoldi et al 1993).

The cortical distribution of mAChRs follows a laminar pattern that reproduces that of the incoming cholinergic fibres. Also in this case layer V is the most densely labelled by mAChR antibodies and layer IV is the least, with intermediate levels of binding in layer II/III and VI (Van der Zee et al 1989, Buwalda et al 1995). Differences in the relative abundance of each mAChR subtype in the cortex have also been reported, where m1 receptor is the most represented and m2, m3 and m4 the less expressed (Levey al 1991). The cortical expression of m5 mAChRs is still debated; recent data obtained in our laboratory by using RT-PCR showed that in mouse visual cortex m5 trasncript is expressed at very low level (E. Aztiria, personal communication). Immunohistochemical labelling using the antibody M35 showed that around one-sixth of neocortical neurons express mAChRs (Wolf 1993), including both pyramidal and GABAergic neurons (Van der Zee and Luiten 1999). Also glial cells, in particular astrocytes express mAChRs (Holsi and Holsi 1993). In line with the theory of volume transmission the mAChRs were observed not only at the level of the junctional interfaces made by synaptic ACh varicosities but also more diffusely along the neuronal membranes (Hersch et al 1994, Van der Zee et al 1989). In addition to their postsynaptic localization mAChRs were also shown on the presynaptic terminals of both cholinergic (autoreceptors) and glutamatergic (heteroreceptors) fibres (Levey et al 1995, Rouse and Levey 1996, Mrzljak et al 1993); their local activation is supposed to inhibit the release of neurotransmitters (Williams and Johnston 1990, Zhang et al. 2002).

Considering the ontogenetic expression of the different mAChRs subtypes in rodents, the even receptors appear to reach the maximal adult-like levels earlier than the odd ones (around the second postnatal week for $m_{2,4}$ and the fourth postnatal week for $m_{1,3}$) (Lee et al 1990, Wall et al 1992). However, the second messenger cascade activation, i.e. PI turnover for odd mAChRs and adenyl cyclase suppression for the even receptors, follow the opposite pattern of development with the former being already present at birth while the latter starting from the second postnatal week (Lee et al 1990).

This suggests that the transduction system linked to the activation of m_1 and m_3 mAChRs develops earlier than that linked to the activation of m_2 and m_4.

Several reports suggested that neuronal activity regulates the expression of mAChRs during postnatal development. In particular, the work produced by the group of Schliebs in the visual cortex of monocularly deprived animals (Kumar and Schliebs, 1992; Rossner et al., 1993) showed a transient alteration of mAChRs expression following visual deprivation. More recently, Gu et al. (1998) reported that blocking the neuronal activity in the lateral geniculate nucleus by injection of tetrodotoxin (TTX) retarded the developmental expression of mAChRs in visual cortex. However, using TTX was not possible to discriminate between evoked and spontaneous activity. On the other hand, plastic modification in the expression of mAChRs in the adult cortex have been shown to occur associated with environmental and behavioural variations, such as seasonal changes, and learning associated with specific behavioural tasks (Jacobs 1996, Van der Zee et al 1994). These reports suggest that the pattern of expression of mAChRs can be considered plastic depending on sensory experience and possibly on the state of activation of cholinergic and/or other modulatory systems as well.

C) Cholinesterases

Differently from the other neurotransmitters, ACh is not removed from the extracellular space through a re-uptake system but by means of enzymatic cleavage catalysed by cholinesterases (ChE). These enzymes are serine hydrolase that promotes ACh hydrolysis by forming an acetyl-AChE intermediate with the release of choline, and the subsequent hydrolysis of the intermediate to release acetate. Among the cholinesterase family the most efficient in ACh degradation is acetylcholinesterase (AChE). Despite the fact that the catalytic site of AChE is located in the bottom of a narrow gorge which is not easily accessible to the ACh, this enzyme posses one of the fastest known catalytic activity, with a single protein able to degrade $3\text{-}7 * 10^3$ ACh molecules/sec (Vigny et al 1978, Massoulier et al. 1993). The AChE family comprises six molecular forms, three asymmetric ($A_{4,8,12}$) and three globular ($G_{1,2,4}$). The first three are all hydrosoluble while the latter are either hydrosoluble ($G_{1,2}$) or amphiphilic (G_4) having an hydrophobic tail which anchors them to the membrane (Gisiger et al 1978, Brzin et al 1982). However, in

the brain the globular forms are almost the totality with G_4 being the most abundant (60–90%) (Massoulié et al. 1993). Such difference in structure and solubility accounts for the specific location of AChE to different subcellular sites where they may regulate different specific physiological functions (Descarries et al 1997). From an immunohistochemical study it appears that, in the CNS, AChE is distributed in clustes along dendrites and axons without being associated to synaptic specialization (Rotundo and Carbonetto 1987). Several authors reported that AChE is secreted from both neuronal somatodendrites and axon terminals of different brain regions (Kreutzberg and Toth 1974, Skau and Brimijoin 1978, Llinas and Greenfield 1987). Moreover the extracellular levels of AChE can vary in a plastic manner as a consequence of neuronal stimulation, changes in neurotransmitter levels and drug treatments that modulate the secretion of the enzyme (Baregggi and Giacobini 1978, Skau and Brimijoin 1978, Greenfield and Smith 1979, De Sarno et al 1987).

Considering its ontogenic development in the mouse cortex, AChE appears on the first postnatal day (P0) increasing progressively until P30 remaining unchanged thereafter (Hohmann and Ebner 1985). In the adult, the pattern of AChE activity is high in layer I, in deep layer IV and layer V. AChE in the CNS seems to act mainly by regulating the levels of extracellular ACh more than simply inactivating ACh upon release (Damsma et al 1988, Taber and Fibiger 1994). It has recently been suggested that also butyrylcholinesterase (BuChE), another serine hydrolase, can have a physiological role in the degradation of ACh in the CNS (Darvesh et al 2003). Although its efficiency in ACh degradation is lower than that of AChE (Silver 1974) BuChE activity seems to be able to compensate for some of the functions of AChE, as shown in AChE KO animals (Li et al 2000, Mesulam et al 2002,). Since a dynamic equilibrium between continuous ACh release and inactivation (Messamore et al 1993) depends on the substrate saturation level of the ChE (Silver 1974), variations in the type of the expressed isoform, level of expression and site of localization of ChE might produce local differences in the concentrations of ACh having a functional impact. The alteration of the expression of AChE induces learning and memory deficits in mice (Beeri et al 1995); moreover reduced AChE activity was found in Alzheimer (Kuhl et al 1999) and Parkinson's (Shinotoh et al 1999) patients.

D) Release of ACh.

The cortical release of ACh is almost entirely due to the activity of basal forebrain cholinergic cells. Both spontaneous (Celesia and Jasper 1996; Jimenez-Capdeville et al 1997) and evoked (Casamenti et al 1986; Kurosawa et al 1989) activity in BFB region induce an increase of cortical cholinergic release. Both phasic and tonic release of ACh have been described in relation with phasic and tonic firing of BFB cholinergic neurons. For example, basal forebrain cholinergic neurons receive noradrenergic projections from LC being predominantly depolarized via a1 receptors upon noradrenaline release; noradrenaline drives cholinergic cells into a tonic mode of firing increasing their rate of repetitive spike discharge.

The use of microdialysis techniques to measure the levels of ACh release in living animals allowed correlating the transition between different behavioural and functional states of the brain with the variation of local ACh concentrations. These studies showed an increase of cholinergic activity, particularly in hippocampus and neocortex, associated with either spontaneous and acquired behaviours such as exploration of a new environment (Thiel et al 1998,Giovannini et al 2001), locomotor activity (Dally et al 2001, Ceccarelli et al 1999), visual attention and arousal (Acquas et al 1996, Passetti et al 2000), chronic stress (Mizoguchi et al 2001), sensory stimulation (Fischer 1991, Acquas et al 1998), working memory and spatial learning (Hinoraka et al 2001, Fadda et al 2000), operant behaviour (Orsetti et al 1996), visuospatial attentional task (Dalley et al 2001) and contextual fear conditioning (Nail-Boucherie et al 2000) (reviewed by Pepeu and Giovannini 2004). A recent study showed that differences in the release of ACh across brain areas, such as hippocampus and striatum, are responsible for the type of learning strategy adopted to solve a learning task (McIntyre et al 2003). Variations in hippocampal and cortical ACh levels are also associated with the wake-sleep cycle; an increase of cholinergic release is observed during active waking as compared to slow waves sleep where the levels of ACh decrease to less than one third and rise again during the REM sleep (Jasper and Tessier 1971, Kametani and Kawamura 1990). The increase of ACh release associated with the different behavioural and physiological states depends on the brain structure and the behavioural paradigm considered. Measure of the variations

in brain ACh levels showed that these can span from few percentage points until 800 % over basal ACh release. In absolute value, ACh concentration has been measured to vary in a nano-micromolar range.

1.3 Role of ACh in brain activity

Whereas classical neurotransmission is characterized by rapid, precise and specialized synaptic transmission that produces fast acting postsynaptic currents, cholinergic neurotransmission in the CNS is long lasting, widely diffused and with only few specialized synaptic contact, and produces slow and long lasting postsynaptic effects.
These characteristics associated with the capacity of ACh to influence the biophysical properties of the neurons and synaptic efficacy of other transmission systems supports the idea that the cholinergic system acts as a neuromodulator of brain functions (Krnjevic 2004). The activity of the cholinergic system has been proved to affect several aspects of cognitive functions spreading from learning and memory to arousal, attention and plasticity (Casamenti et al 1998, Steriade et al 1991, Leanza et al 1995, Everitt and Robins 1997, Sarter and Bruno 2000, Dalley 2001, Conner et al 2003). In particular damage in the basal forebrain region can result in global cognitive impairment; for instance, aneurysms of the anterior communicating artery that injured the basal forebrain are associated with amnesia and impairment of executive function in humans (Damasio et al 1985, Diamond et al 1997, Abe et al 1998). Moreover, pathological conditions that determine cognitive deficits such Alzheimer's disease correlate with the extent of deterioration of cholinergic neurons in the basal forebrain (Perry et al 1978, Bierer et al 1995). Experimental approaches designed to interfere with cholinergic transmission such as blocking muscarinic receptors with specific antagonist (Spencer et al 1985, Cox and Tye 1973, Aigner et al 1991), lesions of cholinergic afferent fibres (Deutsch 1971) or selective damage of cholinergic neurons located in the BFB by the use of the immunotoxin 192 IgG-saporin that binds P75, the low affinity receptors for NGF present on the cholinergic terminals, have confirmed the importance of cholinergic transmission in memory and attention (Leanza et al 1995, McGaughy et al 1996, Waite et al 1999).

The investigations on the physiological action of ACh in the CNS support the idea that cholinergic system influences cognitive functions exerting a modulatory action at three different levels: a) the modulation of neural excitability b) the modulation of synaptic transmission c) the modulation of neuronal plasticity.

A) Cholinergic modulation of neuronal excitability

When considering its action at the cellular level ACh modifies the biophysical properties of neurons resulting in a modulation of intrinsic excitability. In particular, application of ACh on hippocampal and cortical neurons determines membrane depolarization, an increase in membrane resistance and a reduction in the firing frequency adaptation with a consequent increase in the firing rate of the cells (Krnjevic & Phillis, 1963; McCormick & Prince, 1987). All these excitatory effects are mediated through the activation of muscarinic receptor and are due to the reduction of at least three types of potassium conductances: 1) the low-threshold slowly-activating, and non-activating M current (I_M) that is a voltage dependent outward potassium current activated by small depolarization close to the firing threshold; this current promotes membrane hyperpolarization following a spike episode (Krnjevic et al 1971); 2) the ongoing outward leak potassium current, whose blockade accounts for the slow depolarization elicited by muscarinic antagonists (Constanti and Sim 1987); 3) the calcium dependent potassium current (SK) that is responsible for the slow after-hyperpolarization (sHAP) that follows burst action potential. The cumulative hyperpolarization produced by successive sHAP prevents sustained firing in response to a depolarising input; the suppression of sHAP by ACh therefore promotes sustained firing (Cole and Nicoll 1984, Madison et al 1987). Moreover contribution to the excitatory action of ACh is also due to the activation of a non-selective cationic inward current that promotes prolonged action potentials (Fraser and MacVicar 1996, Klink and Alonso 1997).

B) Cholinergic modulation of synaptic transmission

Associated with the effects on neuronal excitability ACh has proven to influence the efficacy of synaptic transmission of other systems such as glutamatergic and GABAergic. This topic has been largely treated by several research works. However, the experimental

approach was rather heterogeneous differing for the brain region and the synaptic pathways investigated, the type and the concentration of the used cholinergic agonists and the techniques employed for the investigation. This caused a production of large literature with results that in some cases were contradictory leading sometimes to opposite conclusions supporting either a facilitatory or depressing action of the cholinergic system on synaptic efficacy. In the slices containing the CA1 region of hippocampus either exogenous application or endogenous release of ACh by stimulation of medial septum diagonal band region produced a facilitation of population spike, possibly due to reduction of inhibitory transmission (Krnjevich et al 1981, Krnjevich and Ropert 1981). In contrast, in stratum radiatum, a muscarinic induced depression predominated (Valentino and Dingledine 1981, Rovira et al 1983). In CA3 region of hippocampus bath application of muscarine depressed synaptic transmission of commissural fibers through direct reduction of presynaptic release and mossy fibres trough the indirect excitation of GABAergic neurons (Vogt and Regehr et al 2001). In 1987 Sato and co-workers in an in vivo experiment performed in the visual cortex, tested the effects of ionophoretic application of ACh on responses elicited by visual stimuli or electrical stimulation of the lateral geniculate nucleus (LGN). They found that the response was facilitated in 74% of the cells while it was depressed in 16%, in both cases through mAChR activation. Sillito and Kemp (1983) obtained a similar result. A cholinergic facilitatory effect on visual cortical response produced by the stimulation of LGN was also observed after mesopontine electrical activation (Lewandowski 1996). Also in primary somato-sensory cortex, iontophoretic application of ACh mainly enhanced the response to tactile whiskers stimulation while a small percentage of cells were depressed (Donoghue and Carroll 1987). In prefrontal cortex slices, Vidal and Changeux (1993) reported a differential role of nicotinic and muscarinic receptors on synaptic transmission. Iontophoretic application of nicotinic agonists increased the amplitude of excitatory postsynaptic potentials (EPSPs) mediated by glutamatergic receptors while muscarinic agonists (muscarine and acetyl-β-methylcholine) decreased EPSPs amplitude; in both cases, postsynaptic membrane potential and resistance were not modified, this suggesting a presynaptic action. Cox et al (1994) observed an increase of postsynaptic response elicited in neocortical neurons by puff application of glutamate in

the presence of ACh; the authors showed that this enhancement was mediated by muscarinic receptors. A similar result was obtained by Markram and Segal (1990) and Calabresi et al (1998a). The former showed that, in hippocampus, ACh increased the NMDA component of postsynaptic potentials elicited by ionophoretic application of glutamate while the latter reproduced a similar selective NMDA enhancement after muscarine application in the striatum. The cholinergic effects were mediated through activation of even mAChRs leading to the activation of the IP3 pathway in hippocampus while m1 mAChR activation was prevalent in the striatum. Kimura and Baughman (1997), using monosynaptically connected cortical cells from cultured neurons, investigated the modulatory action of ACh on both excitatory (EPSPs) and inhibitory (IPSPs) post-synaptic potentials. Their results showed an ACh dose-dependent suppression of transmission in both types of synapses. The suppression of EPSPs was obtained with low concentrations of ACh through the activations of m4 mAChR while the suppression of IPSPs was mediated by m1 mAChR; in both cases, the action of ACh was presynaptic. In slices containing the somatosensory cortex, Gil and colleagues (1997) tested the effects of cholinergic agonists on synaptic transmission elicited by the electrical stimulation of two separate pathways, the intracortical (IC) and thalamocortical (TC) pathway. They found a selective facilitation of TC pathway by nicotine while both pathways were depressed by bath application of muscarine. In addition a low dose of ACh enhanced TC synapses similarly to nicotine. The pathway-specificity for the type of input was also reported in entorhinal cortex by Hasselmo and Bower (1992). In particular, bath application of charbachol strongly reduced the amplitude of field potentials and single cell responses in layer I neurons elicited by stimulation of intrinsic fibres. In contrast, the responses elicited by stimulation of extracortical afferent fibres were almost unchanged by carbachol. Similar effects were produced by bath application of muscarine or ACh in the presence of the AChE inhibitor neostigmine. In somatosensory cortex slices, charbachol suppressed the synaptic transmission in layer I leaving unaffected the responses evoked by intracortical pathway connecting WM to layer IV (Hasselmo and Cekic 1996). Depression of intracortical synaptic transmission in entorhinal cortex was also observed in layer II/III and layer V after bath application of charbachol (Yun et al 2000, Cheong et al 2001). From the reported results emerges the

idea that ACh action does not symply result in facilitation or depression of synaptic transmission but rather ACh appears to regulate the balance between facilitation and inhibition of inputs from different sources.

C) Cholinergic system and neuronal plasticity

In neuroscience, neuronal plasticity is a term that refers to an interaction between the environment and the brain determining a series of neural modifications. These changes can span from structural changes of the number, dimension and distribution of neurons to modification of synaptic efficacy leading to change of spontaneous and evoked neuronal activity. This structural and functional plasticity characterise several neuronal processes spanning from re-organization of sensory maps to learning and memory. In order to study the cellular and biochemical mechanisms underlying brain plasticity several investigators addressed their efforts to settle experimental protocols inducing stable, reliable and consistent neuronal changes both in vivo and in vitro. In slices from nervous system modifications of synaptic efficacy that can be simply obtained using either electrical stimulations or pharmacological tools. The efficacy of synaptic transmission can be regulated over a wide range of temporal scales, ranging from a temporal modulation of milliseconds, to minutes and hours (pair-pulse facilitation, short-term plasticity, long-term plasticity). Concerning long lasting modifications, two forms of synaptic plasticity can be experimentally induced, long-term potentiation (LTP) and long-term depression (LTD). Because long-term changes of synaptic efficacy are supposed to occur during memory acquisition and consolidation, LTP and LTD can be considered models of learning at the cellular level.

The evidence that the cholinergic system is involved in memory acquisition and consolidation has pushed several researchers to investigate the action of ACh on LTP and LTD. Different approaches have been used showing the involvement of cholinergic system on synaptic plasticity in several brain regions. A pioneer study on CA3 region of the hippocampus showed that muscarine bath application blocked LTP induced by mossy fibres stimulation. This was obtained by interfering with the early phase of LTP since muscarine applied after the induction phase did not influence the LTP (Williams and

Johnston 1988). Application of muscarinic receptor agonists on the other hand facilitated LTP in dentate gyrus (DG) (Blitzer et al 1990), and in region CA1 of hippocampus (Burgard and Sarvey 1990). In the latter case, it was observed a concentration-dependent effect with only low concentrations of muscarine being effective in facilitation of LTP through the activation of m1 mAChR. In agreement with these results, muscarinic antagonists blocked LTP both in CA1 and dentate regions (Hirotsu et al 1989; Abe et al 1994). In the dentate gyrus, however, the interference of mAChR antagonists on LTP was dependent on the strength of stimulation, being ineffective in preventing LTP generated by strong tetani, thus suggesting a facilitatory but not regulatory role of the cholinergic system in DG's LTP (Abe et al 1994). A long-lasting facilitatory effect on CA1 Shaffer-commissural transmission was also observed after iontophoretical application of ACh. In this case the facilitation did not require any specific protocol of electrical stimulation. The enhancement of EPSPs was specifically due to the increase of the NMDA component and it was mediated by the activation of mAChRs (Markram and Segal 1990). A more direct evidence of the involvement of cholinergic system in hippocampal LTP was provided by selective damage of the cholinergic cells in rat BFB (Motooka et al 2001). Selective death of BFB cholinergic neurons was induced by the ventricular injection of the neuronal toxin saporine conjugated with an immunoglobulin G (192 IgG-saporin) that binds the low affinity NGF receptor P75. This receptor is located on hippocampal and neocortical cholinergic terminals and upon the binding NGF or 192 IgG-saporin is retrogradelly transported. When 192 IgG-saporin is taken up and internalised the neurons are killed (Leanza et al., 1996a). Using this method, the authors showed that LTP is impaired in the DG of hippocampus if the lesion was performed in adulthood but not in immature animals. The authors suggested that this rescue was due to the development of compensatory non-cholinergic neuromodulatory systems (serotoninergic and noradrenergic). A facilitatory effect of ACh on LTP was also observed in the piriform cortex (Hasselmo and Barkai 1995) and striatum (Calabresi et al 1998b, Calabresi et al 1999).

In the neocortex, homogeneous results were reported in assigning a relevant role to cholinergic innervations in modulating plastic phenomena. Depletion of cholinergic afferents to the somatosensory cortex of cats prevented the expansion of topographic

maps that normally occurs after removal of one digit (Juliano et al 1991) In the barrel cortex, selective damage of cholinergic cells of the BFB induced by 192 IgG-saporin leads to reduced activity-dependent plasticity (Baskerville et al 1997, Zhu and Waite 1998) and retards the development of the barrel cortex (Zhu et al. 2002). Similarly, BFB cholinergic neurons modulate the reorganization of the sensory map in auditory cortex (Kilgard and Merzenich 1998).

In the visual cortex most of the studies on factors and mechanisms regulating neuronal plasticity were performed during postnatal development. This is in line with the evidence that the brain is characterized by a high degree of neuronal plasticity during development with the visual cortex (VC) that represents the most studied area for investigating mechanisms underlying developmental plasticity. In this region, manipulation of visual input during a restricted time-window of early postnatal development (the so called critical period), causes a dramatic change in cortical connectivity (Hubel and Wiesel 1979, 1998). Since the pioneering studies of Hubel and Wiesel, it is well known that, in kittens and baby monkeys, if one eye is deprived of vision by closing the eyelids for several days or weeks, during early postnatal development, the ocular dominance distribution of neurons in visual cortex is drastically shifted: most of the neurons are monocularly driven, only by the stimulation of the open eye. This is particularly evident in animals that present the pattern of ocular dominance columns such as monkey, cat and ferret. Monocular deprivation results effective and irreversible if performed during the critical period, which corresponds to the first postnatal weeks. Sensory experience is a strong determinant for the duration of critical periods: lack of visual experience (dark rearing) usually prolongs critical periods for monocular deprivation. Thus, dark rearing induces two clear effects: i) delays the end of the critical period for monocular deprivation; ii) delays the postnatal development of the functional properties of visual cortical neurons, as reported in the previous chapter.

In developing visual cortex, combined destruction of the cortical noradrenergic and cholinergic innervations reduces the shift in ocular dominance induced by monocular deprivation, although the alternate lesion of either system is ineffective (Bear and Singer 1986). Pharmacological blockade of cholinergic transmission by muscarinic antagonists infused into the visual cortex is by itself sufficient to suppress ocular dominance changes

(Gu and Singer 1993). In agreement with these studies both LTP and LTD appears to be modulated by the cholinergic system. Brocher et al (1992) showed that LTP in layer II/III cells was facilitated by concomitant application of muscarinic with noradrenergic agonists. However, no effects were observed when each of them was administrated individully. Pesavento et al. (2002) using a transgenic mouse model that presented reduced cortical cholinergic innervation, found that LTP was impaired in visual cortex slices of such animals. Exogenous application of ACh however rescued LTP suggesting the essential role of this neurotransmitter in cortical synaptic plasticity. In the somatosensory cortex of *in vivo* rats a long-term potentiation of neuronal discharge evoked by glutamate application was induced by single iontophoretic application of muscarinic agonists (Lin and Phillis 1991). A biphasic action of the cholinergic system on cortical synaptic plasticity is suggested by the work of Kirkwood and colleagues (1999) who reported that, in visual cortex, also LTD is favoured by charbachol.

D) Modelling of cortical cholinergic function

From the results reported on synaptic transmission and plasticity it appears that ACh has several functions on different neuronal networks. A theoretical model was proposed by Hasselmo (1996,1999, 2004) with the aim to explain how different physiological effects of ACh could interact to define a specific functional property of the cortex. This model is based on the following evidences: a) the involvement of cholinergic system in cognitive processes such as attention and memory; b) the diffuse modulatory activity of ACh; c) the variations of cortical cholinergic levels associates to different physiological states; d) the pathway specificity of cholinergic modulation. Hasselmo proposed that variations in cortical ACh levels promote either encoding of sensory information or memory consolidation. He suggested a two-stage model of memory formation. In the first step extracortical afferent fibres bearing relevant sensory information are favoured respect to intracortical synaptic activity by high concentration of ACh, in this way producing a sustained attention on the external world. During memory acquisition a decrease in cortical ACh release favours intracortical over extracortical synaptic activity allowing consolidation of previously acquired information within cortical circuitry.

1.4 Introducing rat and mouse visual cortex

In rat and mouse, the region of both occipital cortical poles is occupied by the primary visual cortex (area Oc1, area 17) and is flanked by cytoarcitectonic areas 18a and b, considered secondary visual areas (Coogan and Burkhalter, 1993). In rat and in mouse, as well as in other mammalian species, the primary visual cortex receives the visual input from the lateral geniculate nucleus (LGN). LGN is a thalamic nucleus that can be subdivided in two regions, according to the segregation of retinal afferents (Reese, 1988); using Cresyl Violet staining associated with anterograde tracers injected into one eye, it results that each region receives inputs from the contralateral retina or ipsilateral retina. In particular, the region receiving from the contralateral eye is much larger than that receiving from the ipsilateral eye (Domenici et al., 1994). Both regions project to the primary visual cortex (area 17). In area 17, two sub-areas can be individuated, a medial area (Oc1m) receiving the visual input exclusively from the contralateral eye (therefore OC1m receives from the contralateral region of the LGN) and a large lateral area (Oc1b) receiving the visual input from both eyes and, consequently innervated by both regions of LGN (see Paxinos and Watson 1986, Zilles et al., 1984).

LGN fibers directed to area 17 terminate predominantly in cortical layer IV. From layer IV visual information is transferred to layer II-III neurons and, in part, to infragranular layers. In the white matter underlying the visual cortex, together with afferents from LGN and feedback projections from the visual cortex to LGN, superior colliculus and thalamic nuclei, there are also other afferent fibers coming from different nuclei. In particular, basal forebrain cholinergic neurons (Mesulam et al., 1983; Saper, 1984; Eckstein et al. 1988) and a small group of GABAergic neurons in the basal forebrain regions (Gritti et al., 1997) send well defined projections to visual cortex, as well as other cortical areas. The same is true for noradrenergic neurons from locus coeruleus (Levitt and Moore, 1978) and for serotoninergic neurons from raphe nuclei (D'amato et al, 1987). These projections, in particular cholinergic projections from basal forebrain, are not restricted to layer IV and distribute throughout all visual cortical layers, as described before.

The functional characteristics of visual cortical neurons in the rat and mouse are similar to those described in other mammals. Briefly, visual cortical neurons respond to

different portions of the visual field, having well characterized receptive fields, respond to one or the other eye (monocular cells) or both eyes (binocular cells) (area OC1b) and to different characteristics of visual stimulus such as orientation and direction of movement (Fagiolini et al., 1994). Functional postnatal development has been fully characterised in rodent visual cortex (Fagiolini et al 1994; Siciliano et al 1997). In particular, during postnatal development the responses of visual cortical neurons are maximally sensitive to manipulation of vision (developmental visual cortical plasticity). The critical period spans three to four postnatal weeks, from eye opening (postnatal day 14-15) to P30-40 (Fagiolini et al., 1994). During the critical period monocular deprivation of the type introduced by Wiesel and Hubel (1963) produces irreversible alterations in the visual cortex, with minor differences between rat and mouse; visual cortical neurons stop to respond to deprived eye and as a consequence binocular units are reduced (Maffei et al., 1992; Fagiolini et al., 1994; Hensh et al., 1998; Hanover et al., 1999), geniculate neurons receiving from the deprived eye and sending projections to the primary visual cortex reduce their volume (shrinkage, Domenici et al., 1993).
Neuronal plasticity is not limited to the critical period. Indeed, if we exclude neuronal plasticity of the type tested by monocular deprivation, there are other forms of neuronal plasticity associated to visual learning and changes of the topographic sensory map induced by a peripheral lesion (for example retina, Gilbert and Wiesel, 1992; Obata et al., 1999). All these results indicate that rat and mouse visual cortices are well-defined cortical circuitries and represent good models to study synaptic transmission during development and in adulthood.

1.5 AIMS

The aim of the present thesis was to investigate the role of cholinergic system and, in particular, ACh in regulation of synaptic transmission and synaptic efficacy in the CNS.

Indeed, despite bulk of results suggest that several brain functional states and cognitive functions are influenced by variations in the activity of the cholinergic system no investigations have until now established a link between ACh local concentration and neuronal synaptic transmission in acute brain preparations. In order to achieve these results we used the rat and mouse visual cortex, i.e. two animal models and an area widely used to address the study on factors and mechanisms controlling synaptic transmission and efficacy. Concerning the visual cortex, this represents a well-known structure both at the anatomical and functional level. In addition, mouse and rat visual cortical slices express long term forms of synaptic plasticity such as LTP and LTD both in adulthood and during postnatal development rendering affordable to investigate the mechanisms and factors involved in synaptic plasticity.

By means of electrophysiological recording in visual cortex slices we evaluated the effect produced by the variation of extracellular concentration of ACh, on evoked synaptic response. In order to study the role of ChE in the modulatory actions exerted by the cholinergic system we compared these results with that produced by cholinometic compound resistant to ChE activity or by bath application of ACh in the presence of the ChE inhibitor edrophonium. The involvement of the different subtype of muscarinic receptors in the modulatory process exerted by ACh was investigated either pharmacologically or genetically by the use of selective muscarinic antagonist and single or double knockout mice for the different muscarinic receptor subtypes. Moreover developmental changes of the modulatory action of ACh were considered and analysed in function of variation in AChE and second messenger systems. We were also particularly attracted by the supposed role of ACh in modulating differentially extracortical and intracortical inputs. Following the hypothesis made by Hasselmo on the role of ACh in memory acquisition and consolidation, we thought that differential cholinergic action could represents a necessary requisite to modulate the flow of information through sensory cortices. In order to investigate such hypothesis we compared the modulatory action of ACh on three cortical synaptic pathways, two purely intracortical (layer IV-layer II/III and layer II/III-layer II/III synaptic connection) and one (WM-Layer II/III pathway) that can be considered mainly extracortical because of the presence of incoming thalamo-cortical fibres.

Finally we looked at the role of cholinergic system on cortical synaptic plasticity using two different approaches: a) deprivation of cholinergic input achieved by using the immunotoxin 192 IgG-saporine that selectively damages BFB cholinergic neurons through the binding with the low affinity receptors for NGF present on the cholinergic terminals; b) the use of KO mice for the different mAChRs.

2.0 MATERIALS AND METHODS

2.1 Animals

Wistar rats (Harlan; Milan, Italy), SLJ mice crossed with C57BL/6J mice, (SJL-C57BL/) and M_1-M_5 mAChR KO mice were used. Except the developmental studies all the animals were oolder than 6 weks.

M_1-M_5 single receptor KO mice (genetic background: 129/SvEv x CF1 [M_1, M_3, M_4, and M_5] or 129J1 x CF1 [M_2]) described in (Gomeza et al., 1999a, b; Yamada et al., 2001a, b; Fisahn et al., 2002); M_1/M_3 receptor double KO mice described by Gautam et al. (2004) (genetic background (129SvEv [50%] x CF1 [50%]), were produced in Jourgen Wess's laboratory, and were purchased from Taconic (Germantown, NY, USA) This mutant mouse had the following mixed For each KO strain, WT mice of the same mixed genetic background were used in parallel as controls. The animals were maintained under controlled environmental conditions with an artificial 12/12 h light/dark cycle with free access to water and food. If not state otherwise the experiments were carried out in adult animals.

2.2 Slice preparation

Primary visual cortex slices were prepared. Animals were deeply anaesthetized by intraperitoneal injection of urethane (0.7 ml/100 mg in 20% physiological solution) and then decapitated. The brain was rapidly removed and 400-μm-thick coronal sections of the occipital poles were sliced with a vibratome. All steps were performed in ice-cold ACSF solution (mM: NaCl, 119; KCl, 2.5; $CaCl_2$, 2.5; MgSO4, 1.25; NaH_2PO_4, 1; $NaHCO_3$, 26.2; and glucose, 11) bubbled with 95% O_2/5% CO_2. Prior to recording, slices were stored for at least 1 h in a recovery chamber containing oxygenated ACSF solution, at 33 ± 1 °C. During electrophysiological recordings, slices were perfused at 3-4 ml / min with oxygenated ACSF, at 33 ± 1 °C.

2.3 Electrophysiological recordings

Extracellular field potentials (FPs), sharp electrode intracellular and patch clamp recording were obtained from layer II/III visual cortex slices. FPs were evoked via a tungsten concentric bipolar stimulating electrode placed in three different sites: WM/layer VI border, layer IV and layer II/III. In order to isolate the horizontal from the vertical synaptic pathway a vertical cut under the stimulating electrode was made when the stimulating electrode was placed in layer II/III. To investigate the effects of endogenous ACh on FP amplitudes, we used a particular arrangement of stimulating electrodes. The first stimulating electrode was placed in layer I to stimulate horizontal cholinergic fibers with the second electrode placed in WM as above described. The recording electrode was filled with ACSF solution and placed in layer II/III.

Changes in the amplitude of FPs mirror changes in the slope of negative potential and correlate with variation in the magnitude of monosynaptic current (Mitzdorf and Singer, 1978; Domenici et al., 1995). FPs were shown to depend on glutamatergic transmission by using bath application of CNQX (10 μM, Sigma, USA).

Baseline responses were obtained with a stimulation intensity that yielded 50-60% of maximal amplitude. All FPs had peak latency from time of stimulation ranging from 4 to 7 ms, and maximal amplitude of at least – 0.5 mV. If not state otherwise FP amplitudes were monitored every 20 s and averaged every three responses by an on-line data acquisition software.

At least ten minutes of stable basal FP amplitude were recorded before any type of treatment. To check for involvement of the cholinergic system we employed different concentrations of ACh (10 μM to 1 mM), agonists of cholinergic receptors (10 μM muscarine, 50 μM carbachol, 100 μM nicotine) and antagonists of cholinergic receptors (10 μM atropine, 3 μM mecamylamine). In addition, we used edrophonium (1-10 μM) to block acetylcholinesterase (AChE) activity. All compounds were purchased from Sigma (St. Louis, USA). The role of mAChRs in synaptic transmission was investigated by using different antagonists purchased from Tocris (Bristol, UK); M_1 receptors were preferentially blocked by using pirenzepine (10 nM-2 μM); M_4 receptors were preferentially blocked by PD102807 (0.5-1 μM). Cholinergic drugs were dissolved in ACSF solution and delivered through slice perfusion.

Drug effects on FP amplitudes were measured by averaging the FP amplitudes of the last three minutes of drug application normalized with respect to the average of FP amplitudes of the last three minutes of basal stimulation (relative amplitude with respect to the baseline). In the particular experimental condition designed to evaluate the contribution of endogenous ACh, a stimulation train at 50 Hz (T50) was given layer I. In this case FP elicited by WM stimulation 2-6 seconds after T50 was normalized with respect to the average of FP amplitudes of the last two minutes of baseline. Relative change FP amplitudes due to T50 were measured either in absence or presence of atropine (10 μM) in the bath solution. The muscarinic contribution to T50 FP modifications was calculated by subtracting the relative change of FPs in the presence of atropine from the relative change of FPs in ACSF solution.

LTP and LTD were elicited respectively by theta burst stimulation ((TBS; 5-10 bursts composed by 5 pulses at 100 Hz, 200 ms interval between burst) and low frequency stimulation (LFS; 900 pulse at 1 Hz) applied to layer IV. LTP and LTD magnitude were measured as the average of FP amplitude of the last 10 minutes of recording normalized respect to the whole 10 minutes baseline.

Intracellular sharp electrodes recording were done in the current clamp mode with 2 M K-acetate-filled electrode pulled from thin walled borosilicate tubes (outer diameter 1.5 mm, Hilgenberg). When filled with 2 M K-acetate the resistance of electrodes ranged from 70 to 120 (MΩ). Current-clamp studies were performed with an Axoclamp-2B amplifier (Axon Instruments, USA). Selected traces were stored on a PC for data analysis using the LTP software (Anderson and Collingridge, 2001). Several criteria were used to accept cells for experiments: typical firing pattern of pyramidal neurons, stable resting membrane potentials of at least –60 mV, no spontaneous firing of action potentials, no sudden drops in the input resistance (indicating cell damage) and constant amplitude of the spike (>70 mV) obtained by direct activation of the cell.

Patch clamp recordings were obtained using 1.5 mm borosilicate glass electrodes (3-5 MΩ) containing: (mM: K-gluconate, 130; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 11; HEPES, 10; Mg-ATP, 2; Na-GTP, 0.3; pH 7.3, with KOH). Whole-cell current-clamp experiments were carried out with an Axopatch 200B amplifier (Axon Instruments, USA), filtered at 1 kHz and digitized (10 kHz). Statistics

Statistical comparison between FP amplitudes measured during baseline and FP amplitudes measured during bath application of pharmacological compounds was performed by applying Student's *t* test. The *t* test was also used for statistical comparisons among different groups. Differences were considered significant with $P < 0.05$.

2.4 BFB cholinergic neurons selective lesions:

Fourteen 4-day old (P4) rat pups, from two different litters were anesthetized by hypothermia and subjected to bilateral injections of 192 IgG-saporin (Advanced Targeting Systems; San Diego, CA, USA) into the lateral ventricles (0.4 μg/pup in 10 μl PBS; 5 μl each ventricle). Using a 10 μl microsyringe, the toxin was injected using the following stereotaxic coordinates: AP –0.6, L ±0.8, V –2.1; relative to bregma and the outer skull surface. The injection was made in a total time of 1 min and then the needle was kept in situ for an additional minute in order to allow for diffusion. Prior to being returned to the mother, the pups were recovered by warming them up to normal body temperature. Twelve other pups, siblings of the same litters that were chosen at random, were left uninjected serving as controls. Animals were then returned to normal housing conditions until postnatal day 16 when the functional analyses started to be conducted. In previous experiments no difference was detected between uninjected and vehicle-injected rats on either histochemical parameters (Leanza et al., 1996a); therefore we consistently used intact animals as controls.

2.5 RT-PCR for mAChRs expression:

Anesthetized animals were decapitated and the visual cortex was immediately removed, frozen in dry ice, and kept at -80°C until being processed. Total RNA was extracted using the Trizol reagent (Gibco BRL), treated with DNAse I (Ambion), and tested by PCR in order to ensure the absence of genomic DNA in the sample. Five micrograms of

total RNA were reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to manufacturer's indications, using random hexamers to start the reaction. Amplification of specific domains of the different muscarinic receptor cDNAs were carried out using the primers described in table 1 for rat sample and table 2 for mouse sample specific for each gene: Two different programs were used to amplify these sequences: PCR program A to amplify the m1, m3, and m5 cDNAs (consisting in an initial denaturing step at 94°C for 10 minutes, followed by 30 cycles of: 94°C for 30 seconds, 55°C for 30 seconds and, 72°C for 2 minutes, which were followed by a final step at 72°C for 8 minutes to finish all incomplete strands), or program B for the m2 and m4 cDNAs (essentially as program A, except that the 30 amplification cycles consisted of 94°C for 30 seconds, 60°C for 30 seconds and, 72°C for 1 minute). The specificity of the amplified bands was assessed by restriction endonuclease digestion.

Table 1:

mAChR subtype	primer sequence	annealing site (*)
M_1	**5'-cca aca tca ctg tct tgg cac-3'**	**30-228**
	5'-ggt gcc aat gat gag gtc agc-3'	
M_2	5'-gct att acc agt cct tac aag aca-3'	36 - 465
	5'-cca gag gat gaa gga aag gac c-3'	
M_3	**5'-gta cca gca gag aca gtc-3'**	**1713 - 1920**
	5'-gga tca gta aac agg tag-3'	
M_4	5'-gga gaa gaa ggc cgc cca gac gc-3'	1452- 1908
	5'-agg tag ctt ctc tcc cgt gag gt-3'	
M_5	**5'-tcc gtc atg acc ata ctc ta-3'**	**715 – 841**
	5'-cct gtt gtt gag gtg ctt cta c-3'	

(*) Respect to ATG codon.

Table 2:

mAChR subtype	primer sequence	annealing site (*)
M_1	5'-cca aca tca ccg tct tgg cac-3'	**30-228**
	5'-agt gcc aat gat gag atc agc-3'	
M_2	5'-gct att acc agt cct tac aag aca-3'	36 - 465
	5'-cca gag gat gaa gga aag aac c-3'	
M_3	5'-aag acc aca gta gca gtg-3'	**1713 - 1920**
	5'-ctc tct aca tcc ata gtc cc-3'	
M_4	: 5'-gga gaa gaa ggc caa gac tct gg-3'	1452- 1908
	5'-ggc agt cac aca ttc act gcc tg-3'	
m_5	A7: 5'-tcc gtc atg acc ata ctc ta-3'	**715 – 841**
	5'-ccc gtt gtt gag gtg ctt cta c-3'	

(*) Respect to ATG codon.

2.6 Histochemical assays

The brains were extracted in cold ACSF solution and fixed by 6-8 hours immersion in ice-cold phosphate-buffered/4% paraformaldehyde, and then transferred to 30% sucrose/PBS solution at 4°C until they had sunk. Forty micron-thick coronal sections were cut on a freezing microtome from the level of the frontal cortex through the basal forebrain nuclei to the dorsal hippocampus, and collected into four series.

One series of sections containing the medial septum and diagonal band of Broca, and the nucleus basalis magnocellularis were processed for choline acetyltransferase (ChAT) staining using a goat anti-ChAT polyclonal antibody (1:100; Chemicon, Temecula, CA, USA). Immunohistochemistry was carried out following a standard avidin-biotin ABC procedure. Briefly, tissue sections were permeabilized in PBS/0.05% Tween-20 (PBST), and then quenched by 10 min incubation in 3% H_2O_2 in PBS in order to eliminate endogenous peroxidase activity. Unspecific binding of the antibodies was prevented by preincubation of the sections with 10% fetal calf serum in PBST for 1 h at room temperature. Sections were exposed to the primary antibody for 48 h at 4°C with gentle rocking, then washed with PBS and incubated in a 1:200 biotinylated rabbit anti-goat IgG

(Dako, Glostrup, Denmark) for 3 h at room temperature. After repeated washes, the sections were incubated in avidin-biotin complex (Vectastain Elite kit, Vector, Burlingane, CA, USA, 1 h), and then reacted with diaminobenzidine (0.4 mg/ml, Sigma) in 0.1% glucose/Tris buffered saline, pH 7.4, and 0.01% glucose oxidase. Negative controls, in which the primary antibody was omitted, were run in parallel.

Another series of sections from lesioned and control animals was processed for acetylcholinesterase (AChE) histochemistry (Heedren et al., 1985), in order to visualize the effects of the immunotoxin treatment upon the terminal cholinergic innervation in the neocortex and the hippocampus. Briefly, free floating sections were incubated at pH 6 in a medium containing sodium citrate, copper sulphate, potassium ferricyanide, and acetythiocholine iodide. Non-specific esterases were inhibited by the addition of ethopropazine (Sigma) at a final concentration of 10^{-4} M to the incubation mixture. The brown reaction product was finally intensified by brief incubations with ammonium sulphide and silver nitrate. Sections from control and immunolesioned rats were processed simultaneously in order to avoid artifacts in the estimation of the AChE-positive fiber density (see below). Finally, the sections were mounted on gelatinized slides and blindly analyzed under the microscope. Each section was photographed and pictures were digitalized and analyzed using the NIH Image software (Rasband and Bright, 1995) of public domain. The optical density of the corpus callosum, which normally contains no AChE-positive signal, was used as a blank and was therefore subtracted from that of the parietal cortex, the dentate gyrus and the CA1 and CA3 subfields of the hippocampus.

Group differences in the optical densities in each region were evaluated by one-way ANOVA followed by Fisher's Protected Least Significant Difference (PLSD) post-hoc test.

3.0 RESULTS

3.1 Modulatory action of ACh on synaptic transmission:

3.11 The amplitude of FPs evoked by stimulation of WM is modulated in opposing way by different concentrations of ACh

To understand the role of ACh in cortical synaptic transmission we measured the changes in amplitude of FPs evoked by stimulation of WM and recording in cortical layer II/III, using bath application of different pharmacological compounds. Application of 100 μM ACh for ten minutes through general perfusion induced a significant increase in amplitude of FPs (120 ± 3.5 % of baseline, n = 15; figure 3.11a). This facilitation reached a plateau within a few minutes and returned to basal values after washout of ACh. A longer application of ACh (20 minutes) led to similar results (121± 3.2%, n=11; $p<0.001$; data not shown). In contrast, when 1 mM of ACh was applied, a significant decrease in the amplitude of FPs was observed (72± 3.5% of baseline, n=15;figure 3.11b). Also in this case the amplitude of FPs returned to basal levels after washout.

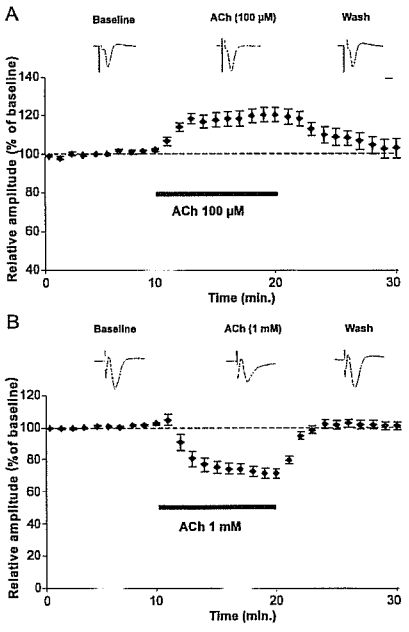

Figure 3.11: ACh modulates the amplitude of FPs in layer II/III of mouse (SLJ-C57BL/6J) visual cortex during stimulation of WM. (**A**) Bath application of 100 μM ACh induces an increase of FP amplitudes that return to baseline after wash out (n=15). (**B**) Bath application of 1 mM ACh induces a decrease in amplitude of FPs that returns to baseline after wash out (n=15). Horizontal bars depict the period of application of ACh. Inset: representative traces. Calibration bar = 0.2 mV /5 ms

In order to obtain a dose-response curve, we tested different concentrations of ACh. We found that 10 μM ACh did not induce any changes in the amplitude of FPs (99 ± 3.4 % of baseline, n=6; figure 3.12). Concentrations of 20, 50, 200 and 500 μM ACh induced a significant facilitation that was not significantly different from that obtained with 100 μM ACh (125 ±6, n=5; for 20 μM; 118±3.4, n=6; for 50μM; 118±2.6, n=6, for 200μM; 112 ± 2.6, n=7; for 500μM; figure 3.12). It should be noted that the ACh effects were due to modulation of glutamatergic transmission, since FPs evoked by stimulation of WM were completely blocked by CNQX (data not shown). Thus, ACh enhances or inhibits synaptic transmission depending on the concentration used.

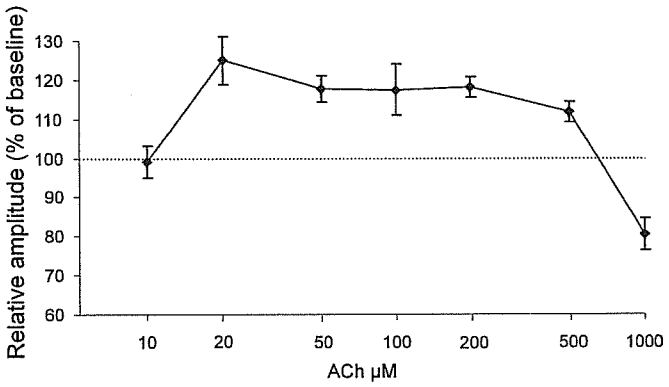

Figure 3.12: Dose-response curve of ACh bath application on FPs elicited by WM stimulation in visual cortex slices. Bath application of different ACh concentrations to visual cortex slices leads to an increase of FP amplitudes at ACh concentrations ranging from 20 to 500 μM (20 μM, n=5; 50μM, n=6; 100 μM, n = 15; 200μM, n=6; 500μM, n=7), a depression for 1 mM ACh (n=15), and no change for 10 μM ACh (n=6). Circles represent the mean amplitude of FPs during the last three minutes of ACh application normalized with respect to the mean FP amplitudes during the last three minutes of baseline.

In order to dissect the roles of the different types of cholinergic receptors in modulating cortical synaptic transmission, we employed nicotinic and muscarinic antagonists and agonists. The mAChR antagonist atropine (10 μM) completely prevented facilitation of FPs induced by 100 μM ACh (102 ± 3.5% of baseline, n=8;

figure 3.13a) and depression of FPs induced by 1 mM ACh (104 ± 3.3% of baseline, n=5; figure 3.13a). The nicotinic receptor antagonist mecamylamine (3 μM) did not modify ACh induced facilitation (114 ± 1.9 % of baseline, n=5; figure 3.13b) and, in addition, the basal amplitude of FPs was not modified by application of 100 μM nicotine (104 ± 1.3 %, n=7; figure 3.13c). These data suggest that the ACh induced modifications in the amplitude of FPs are mediated by muscarinic but not nicotinic receptors.

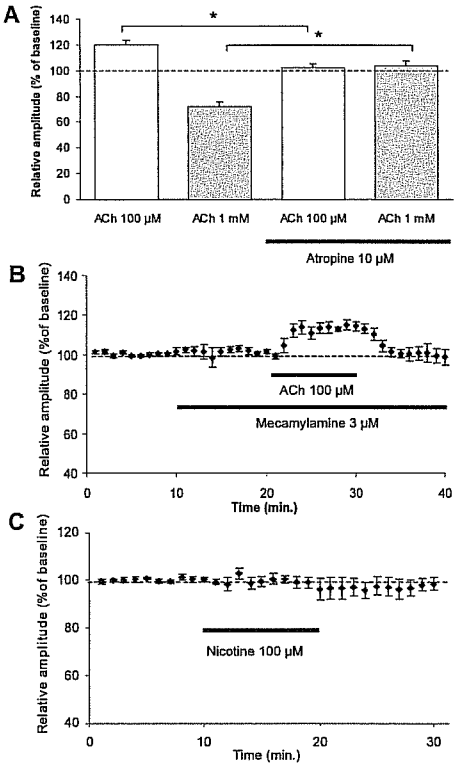

Figure 3.13: The modulatory effects of ACh in visual cortex slices are mediated by mAChRs.
(A) 10 μM atropine prevents both 100 μM ACh induced facilitation (n=8) and 1 mM ACh induced depression (n=5) of the amplitude of the FPs elicited by WM stimulation (n=5). Bars represent the mean FPs amplitudes of the last three minutes of drug application normalized with respect to the mean amplitude of FPs during the last three minutes of baseline. **(B)** 3 μM mecamylamine failed to prevent 100 μM ACh induced facilitation of the amplitude of the FPs elicited by WM stimulation (n=5). **(C)** 100 μM nicotine did not modify the amplitudes of FPs elicited by WM stimulation (n=7). * $p<0.05$.

3.12 Effects of endogenous ACh on synaptic transmission

An important question is whether modulation of FPs can be obtained under our experimental conditions, by stimulating the release of endogenous ACh. In particular, we asked whether depression of FPs could be evoked by endogenous ACh, since we applied a high concentration of exogenous ACh to induce synaptic depression. We took advantage of the morphological arrangement of the cholinergic pathway in the cortex (Houser et al 1985), with a contingent of cholinergic fibers passing through layer I. To stimulate the release of endogenous ACh and to study ACh effects on synaptic transmission, we used two stimulating electrodes (StE1, StE2,); StE2 was placed in layer I, where the cholinergic fibers elapse horizontally for several hundred microns (Houser et al., 1985), and StE1 was placed in WM (see figure 3.14a). The recording electrode was maintained in layer II-III. FPs were elicited by WM stimulation (StE1) using the standard protocol. To stimulate the local release of ACh sufficient to influence synaptic transmission, one train of 50 pulses at 50 Hz was delivered through the StE2 electrode, after ten minutes of FP recordings (baseline). Between two and six seconds after the train, a test pulse was delivered through the StE1 and the amplitude of recorded FPs was compared with the average amplitude during the last two minutes of baseline; we then bath-applied atropine (10 μM) and repeated the same stimulation protocol (figure 3.14b). By comparing changes in FP amplitudes induced by the train in either the absence or presence of atropine, we determined the mAChR-dependent component of these changes (see methods).

We found that ACh released by tetanic stimulation of layer I modulates the FPs elicited by WM stimulation in three different ways. In 7/10 slices a mAChR-dependent depression was found (-17, -14, -14, -9, -8, -7 and -6% with respect to atropine application; average: -11 ± 1.5 %, n=6; $p < 0.001$); in 2/10 slices a muscarinic dependent facilitation of FP amplitudes was induced (12 and 7 %) whilst in one slice no mAChR-dependent modification was observed. Thus, endogenous ACh acting on mAChRs can reproduce the depressing effect observed with exogenously_supplied ACh.

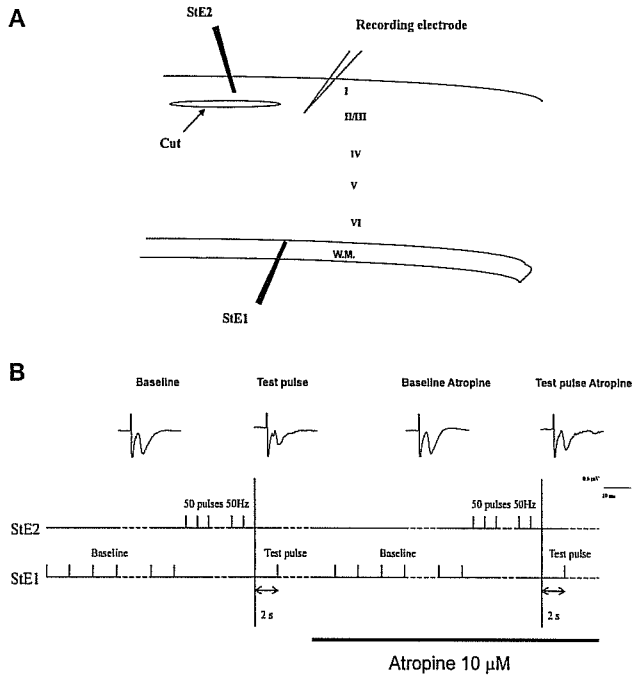

Figure 3.14: Modulation of FPs by endogenously released ACh.
(**A**) Schematic representation of experimental arrangement used to evaluate the effects of endogenous ACh on synaptic transmission in visual cortex slices. FPs evoked by WM stimulation (**StE1**) were recorded in layer II/III. High frequency stimulation of layer I (**StE2**) was used to induce endogenous ACh release. A vertical cut was made under StE1 in order to isolate the horizontal pathway from the vertical pathway. **StE1**= stimulating electrode 1; **StE2**= stimulating electrode 2. (**B**) Scheme of the experimental design used to evaluate the effects of endogenous ACh release on FP amplitudes. FPs were elicited through the stimulation of WM (**StE1**). After ten minutes of stable baseline, a tetanus (50 pulse at 50 Hz) was delivered to layer I (**StE2**). Two seconds after the tetanus, a test pulse was given through StE1 and tetanus dependent changes in the amplitude of FPs were evaluated. In order to isolate the muscarinic component of these changes in FPs, the same stimulating protocol was repeated in the presence of 10 µM Atropine. Inset: representative traces.

3.13 Acetylcholinesterase activity controls the cholinergic modulation of FPs

Application of two cholinergic agonists resistant to AChE degradation, carbachol (50 µM) and muscarine (10 µM), showed that both compounds induced a significant depression of FP amplitudes (carbachol: 68 ± 8% of baseline, n=4; muscarine: 66 ± 4.7 % of baseline, n=7; figure 3.15a). The relatively low concentration at which these agonists induced depression compared with that necessary for ACh prompted us to investigate the potential role of AChE in regulating ACh mediated

synaptic modulation. Specifically, we examined whether the ACh modulation of FP amplitudes was influenced by edrophonium, an AChE inhibitor. In the presence of 1-2 μM edrophonium, we observed a shift of the ACh concentration-response curve to the left (compare figure 3.15b with figure 3.12). Indeed, a significant depression of FPs was induced by 500 μM ACh (85 ± 2 %, n=5; figure 3.15b) while a significant facilitation was observed both with 100 μM ACh (135 ± 5 %, n=5; $p<0.01$; figure 3.15b) and 10 μM ACh (151 ± 7 %, n=5; figure 3.15b). A significant facilitation, although reduced in magnitude, was also seen with 1 μM ACh (112 ± 4 %; n=6, figure 3.15b), but was no longer observed with 0.5 μM ACh (102 ± 4 %, n =4; figure 3.15b). A dramatic change of the ACh dose-response curve was observed in the presence of 10 μM edrophonium. Indeed, during the last three minutes of ACh application, a significant depression of FP amplitudes was observed with 5-100 μM ACh (100 μM, 54 ± 9% of baseline, n=5; 10 μM, 80 ± 5% of baseline, n=11; 5 μM, 87 ± 6% of baseline, n=10; figure 3.15c). No statistically significant changes were found with 0.5-2 μM ACh (2 μM, 102 ±4% of baseline, n=9; 1 μM, 101 ± 4%, n=13; 0.5 μM, 97 ± 2%, n=5; figure 3.15c). During on-line acquisition we observed a transient increase of FP amplitudes immediately following bath application of ACh (figure 3.15d). This effect was present even in the absence of AChE but was masked by the averaging of the responses acquired every twenty seconds (see figure 3.11b). In order to study this transient increase of FP amplitudes we acquired the responses every ten seconds without averaging them; moreover, we triggered the stimulating pulse with the arrival of ACh-containing ACSF in the recording chamber. Using this approach, we were able to see a clear significant transient facilitation of FPs for different ACh concentrations, in the range of 1-5 μM (117 ± 5 %, n=10, for 5 μM; 114± 3 %, n=9, for 2 μM and 114 ± 4 %, n=8, for 1 μM; figure 3.15d, 3.15e). The duration and the delay of transient facilitation with respect to the arrival of ACh in the recording chamber increased with decreasing ACh concentrations; the time employed to reach the maximum value of transient facilitation was 30 s for 5 μM, 60 s for 2 μM, and 70 s for 1 μM ACh. At a concentration of 0.5 μM ACh, the transient facilitation was not observed (102 ±1 %, n=5; figure 3.15e). The transient facilitation was completely abolished by 10 μM

atropine (101 ± 2 %, n=4; see inset Figure 3.15d), indicating the involvement of mAChRs.

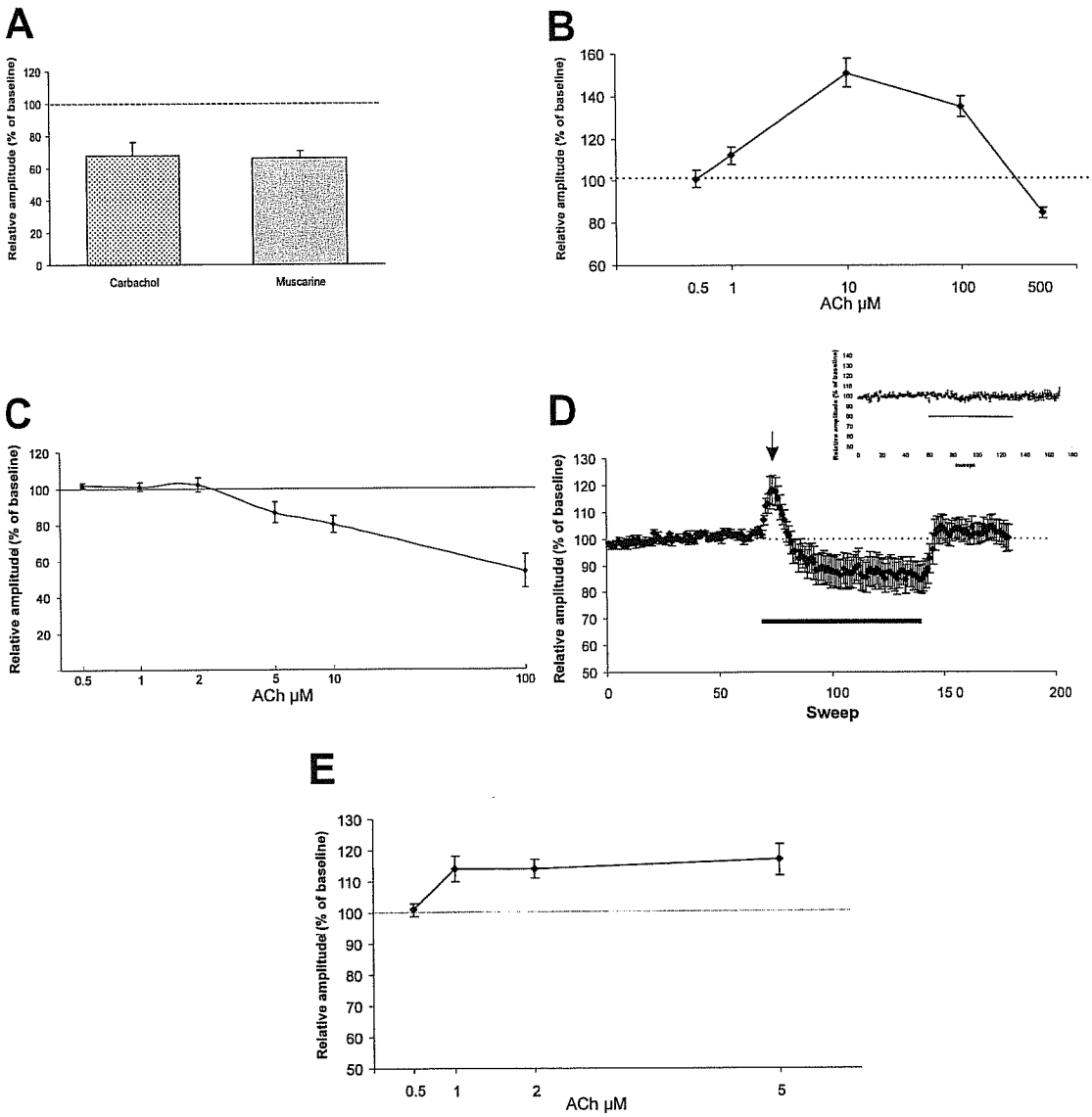

Fig. 3.15: AChE influences cholinergic modulation of synaptic transmission in visual cortex slices. **(A)** Muscarine (10 µM) (n=7) and carbachol (50 µM) (n=4) induce a decrease in the amplitude of FPs for stimulation of WM. Bars represent the mean FP amplitudes during the last three minutes of drug application normalized with respect to the mean amplitude of FPs during the last three minutes of baseline. **(B)** Dose-response curve of ACh effects on FP amplitudes is shifted towards lower values in the presence of 1 µM edrophonium (0.5 µM, n=4; 1 µM, n=6; 10 µM, n=5; 100 µM, n=5; 500 µM, n=5). **(C)** In the presence of 10 µM edrophonium, a depression of FP amplitudes was observed at ACh concentrations ranging from 5 to100 µM (5 µM, n=10; 10 µM, n=11; 100 µM, n=5) while no changes were found at ACh concentrations ranging from 0.5 to 2 µM (0.5 µM, n=5; 1 µM, n=13; 2 µM, n=9) at steady state (i.e. last three minutes of ACh application). **(D)** ACh (5 µM) induces a transient facilitation of FP amplitudes in the presence of edrophonium (10 µM) (n=10). Arrow shows the peak of transient

facilitation; horizontal bar indicates the period of ACh application. (E) In the presence of 10 μM edrophonium transient facilitation was observed for ACh ≥ 1 μM (0.5 μM, n= 5; 1 μM, n=8; 2 μM, n=9; 5 μM n=10) but not for 0.5 μM ACh. (F) Atropine (10 μM) prevented transient facilitation to occurs when 5 μM ACh was bath applied (n=4). Horizontal bar indicates the period of ACh application. In B and C, circles represent the mean amplitude of FPs during the last three minutes of ACh application normalized with respect to the mean amplitude of FPs during the last three minutes of baseline. In E, circles represent the mean amplitude of FPs over three sweeps (30 seconds) centered on the peak of transient facilitation and normalized with respect to the last three minutes of baseline.

These results suggest that AChE activity determines the range of ACh concentrations that induce enhancement or depression of FPs .

3.14 Role of mAChR subtypes in ACh induced modifications of FPs

By which mechanisms does ACh exert opposing actions on synaptic transmission? One possibility is that different mAChR subtypes differentially control facilitation or depression of FP amplitudes. In order to test this hypothesis, we made use of two different approaches. First, we studied preparations from M_1-M_5 receptor KO mice. Second, we used pharmacological tools with the aim to preferentially block specific mAChR subtypes.

M_1-M_5 receptor KO mice

M_1-M_5 mAChR single KO and M_1-M_3 mAChR double KO mice were used for the preparation of occipital slices. Wild-type control mice with the same genetic background as the individual KO mice were studied in parallel (for details, see 'Materials and Methods'). Mice prepared for electrophysiology were genotyped by PCR using DNA extracted from tail biopsies.

We found that 200 μM ACh induced a significant facilitation of FPs in both M_2 WT and M4 WT mice (M_4 WT, 115 ± 1 %, n=10, figure 3.16b; M_2 WT, 131 ± 6%, n=6, figure 3.16c). 1.5 mM ACh was necessary to obtain a significant depression of FPs in both control groups (69 ± 6%, n=7, for M_4 WT, figure 3.6b; 68 ± 6 %, n=6, for M_2 WT, figure 3.16c).

In M_1, M_3 and M_5 KO mice, 200μM ACh induced facilitation of FPs whose amplitude was not significantly different from that measured in control animals (126 ±

8 %, n=7, for M_1 KO; 116 ±12 %, n=7, for M_3 KO and 121 ± 4 %, n=8, for M_5 KO, figure 3.16a). In contrast, facilitation was absent or significantly reduced in M_2 and M_4 KO mice (M_2 KO, 112 ± 5%, n=8, $p<0.05$ compared to control, figure 3.16c; M_4 KO, 102 ± 5 %, n=7 $p<0.05$ compared to control, figure 3.16a). In all M_1-M_5 KO animals, 1.5 mM ACh induced a significant depression of FPs whose amplitude was not significantly different with respect to that measured in wild-type controls (M_1 KO, 65 ± 8%, n=6; M_3 KO, 82 ± 7 %, n=5; M_4 KO, 77 ± 6%, n=6; M_5 KO, 76 ± 8 %, n=7; M_2 KO, 69 ± 5%, n=7; figure 3.16 b, c). To further investigate whether a mixture of M_1 and M_3 receptors may modulate response to different ACh concentration we used M_1-M_3 double KO mice. Analysis of M_1-M_3 mAChR double KO mice showed that neither facilitation induced by 200 μM_ACh (121 ± 8 % in WT, n=10; 119 ± 4 % in M_1-M_3 KO, n=10) nor depression due to 1.5 mM ACh (71 ±6 % in WT, n=10; 71 ± 6% in M_1-M_3 KO, n=10) were affected (figure 3.16d).

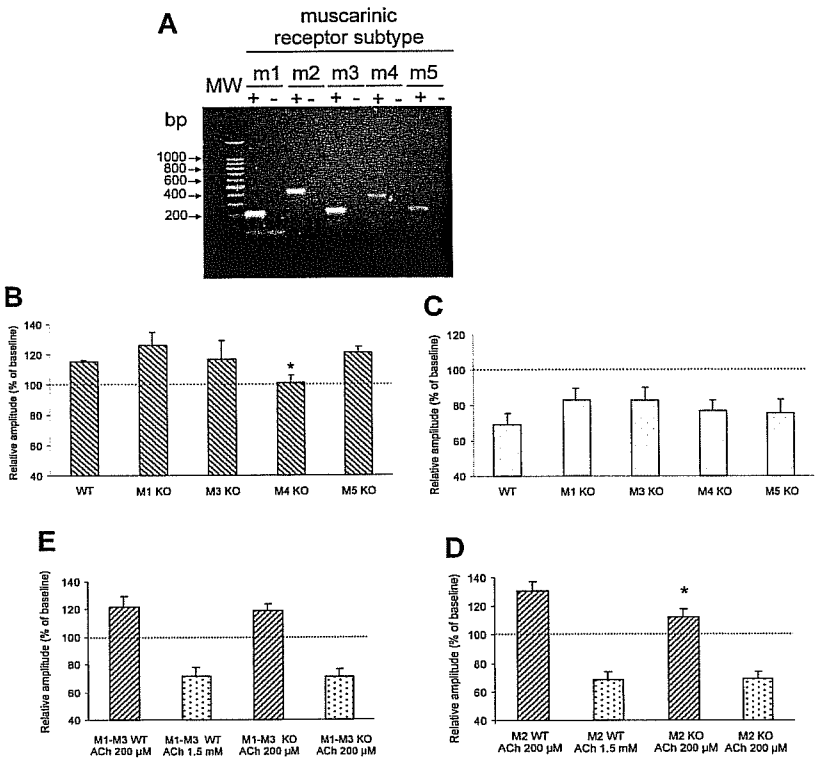

Figure 3.16: Modulatory effects of ACh on FPs elicited in visual cortex slices from M_1-M_5 mAChR KO mice.

(**A**) Agarose gel electrophoresis showing the products from reverse transcription and PCR of total RNA from visual cortex of SLJ-C57BL/6J wild type mice using M_1, M_2, M_3, M_4, and M_5 mAChR gene-specific primers. Amplification of cDNA from reverse transcription reactions is indicated as "+". Control reactions to check for contamination with genomic DNA were run without reverse transcribing the RNA samples (indicated with "-"). (**B**) Bath application of 200 μM ACh. Facilitation of FPs was absent in M_4 KO mice while it was retained in M_1, M_3 and M_5 KO mice (WT, n=10; M_4 KO, n=7; M_3 KO, n= 8; M_5 KO, n= 8; mean FP amplitudes were significantly different between M_4 KO and WT mice). (**C**) Bath application of 1.5 mM ACh. Depression of FPs was not affected in M_1, M_3, M_4, and M_5 KO mice (WT, n= 7; M_1 KO, n=6; M_2 KO, n=7; M_3 KO, n=5; M_4 KO, n= 6; M_5 KO, n= 7; mean FP amplitudes were not significantly different between KO and WT mice). (**D**) M_2 KO mice showed a significant decrease of facilitation induced by 200 μM ACh (WT, n= 6; M_2 KO, n= 8; mean FP amplitudes were significantly different between KO and WT mice). Depression of FPs induced by 1.5 mM ACh was normal in M_2 KO mice. (**E**) M_1-M_3 double KO mice showed normal facilitation and depression of synaptic transmission after bath application of ACh at different concentrations (200 μM and 1.5 mM) (M_1-M_3 WT, n=10; M_1-M_3 KO, n=10; mean FP amplitudes were not significantly different between KO and WT mice). Columns represent the mean amplitude of FPs during the last three minutes of ACh application normalized with respect to the mean amplitude of FPs during the last three minutes of baseline. (* $p < 0.05$).dulator

Use of muscarinic antagonists

We used the M_4 receptor-preferring antagonist PD102807 (0.5- 1 μM) to preferentially block M_4 receptors in SJL-C57BL/6J mice. The presence of PD102807 (0.5- 1 μM) did not modify depression induced by 1 mM ACh (79 ± 4% of baseline, n=7; figure 3.17). However, ACh (200 μM) induced only a small facilitation in the amplitude of FPs (107 ± 3.3 % of baseline, n=13; figure 3.17), which was significantly lower than that obtained in controls (p= 0.009, figure 3.17).

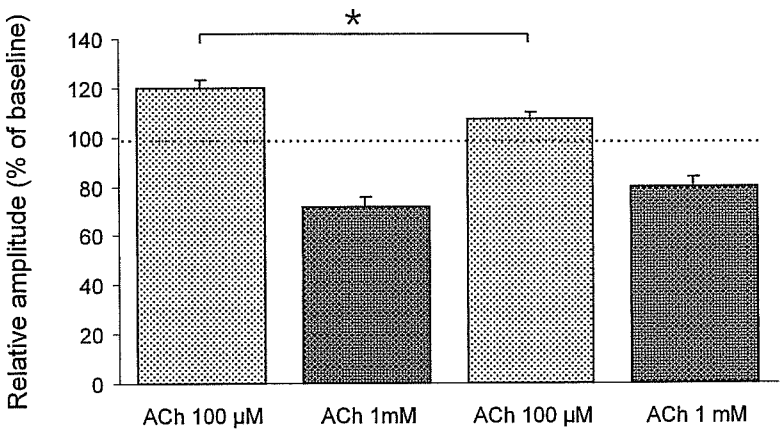

Figure 3.17: Effects of M_4 muscarinic receptor antagonists on ACh modulation of synaptic transmission in visual cortex slices of SLJ-C57BL/6J mice. The selective M_4 mAChR antagonist PD102807 (0.5-1 μM) reduced 100 μM ACh (n=13) induced facilitation without affecting 1 mM ACh induced depression in FP amplitudes (n=7).

To further investigate the contribution of different mAChRs to ACh modulation of synaptic transmission we used three different concentrations of pirenzepine (10 nM, 100 nM, and 2 μM). According to Caulfield and Birdsall (1998), 10 nM pirenzepine blocks about 50% of M_1 receptors, 100 nM pirenzepine blocks most M_1 and part of M_4 and M_3 receptors, and 2 μM blocks most M_1 and M_4 and a considerable portion of M_2, M_3, M_5 receptors. Facilitation induced by 100 μM ACh was not changed by 10 nM pirenzepine; higher concentrations of pirenzepine, 100 nM and 2 μM, slightly reduced ACh-dependent facilitation, although the level of reduction was not significant (124 ± 7 %, n=9, control SJL-C57BL/6J; 127± 5%, n=6, 10 nM pirenzepine; 117± 3 %, n=7, 100 nM pirenzepine; 113 ± 5%, n=6, 2 μM pirenzepine; figure 318). 1 mM ACh induced a significant depression of FPs (61 ± 8 %, n = 9, controls, figure 3.18) whose amplitude was not significantly reduced by 10 and 100 nM pirenzepine (67 ±4 %, n=8 and 81 ±7 %, n=7, respectively; figure 3.18). ACh-dependent depression was completely blocked only when pirenzepine was applied at the highest concentration (102 ± 5%, n=5, 2 μM pirenzepine; $p< 0.05$ compared to control, figure 3.18). Bath application of 2 μM pirenzepine alone did not modify FP amplitudes (100 ± 1.6%, n=9; data not shown).

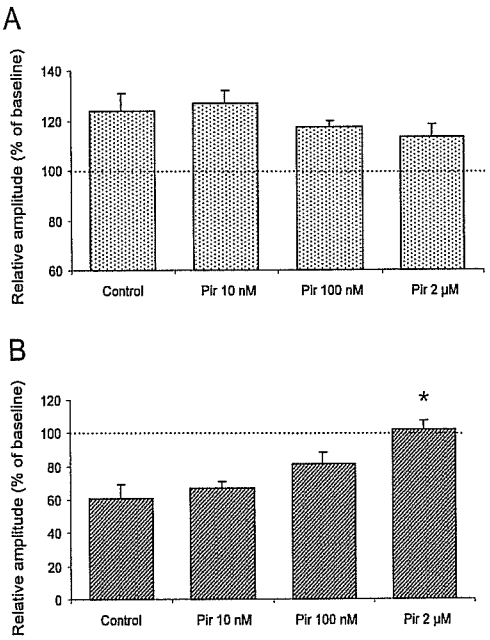

Fig 3.18: Effects of M_1 muscarinic receptor antagonists on ACh modulation of synaptic transmission in visual cortex slices of SLJ-C57BL/6J mice. (**B**) The selective M_1 mAChR antagonist pirenzepine was bath applied at three different concentrations (10 nM, 100 nM, and 2 μM). None of the pirenzepine concentrations used significantly changed the facilitation of FP amplitudes induced by 100 μM ACh. (**C**) Depression of FPs induced by 1 mM ACh was prevented by 2 μM but not by 10 and 100 nM pirenzepine (* $p< 0.01$).

The overall results obtained using mAChR KO mice and mAChR antagonists suggest that low concentrations of ACh facilitate synaptic transmission via activation of M_2 and M_4 mAChRs. Depression induced by high ACh concentrations appears to be mediated by multiple mAChRs.

3.15 Effects of ACh on cortical synaptic transmission upon stimulation of different cortical synaptic pathways

To study whether the modulatory action of ACh is input specific, we applied ACh while recording FPs induced by stimulation of different synaptic pathways of the primary visual cortex. The recording electrode was placed in layer II/III as usual, while the stimulating electrode was placed either in layer IV in order to stimulate layer IV-II/III vertical intracortical transmission, or in layer II//III, at the same level of the recording electrode, to stimulate horizontal intracortical connections (Domenici et al., 1995). We found that bath application of 10-50 μM ACh significantly facilitates FPs induced by stimulation of layer II-III (110 ±3 % of baseline, n=5, for 10 μM ACh; 114 ±2 % of baseline, n=5, 20 μM ACh; 116 ±5% of baseline, n=5, 50 μM; figure 3.19a.) and layer IV (110 ±4% of baseline, n=5, for 10 μM ACh; 114 ±3%, n=6, for 20 μM; 112 ± 5%, n=7, for 50 μM figure 3.19b). Application of 100 μM ACh did not modify either FPs induced by stimulation of layer II/III (105± 3.9 % of baseline, n=6; figure 3.19a) nor those induced by stimulation of layer IV (103 ± 3.2 % of baseline, n=8; figure 3.19b). When 500 μM and 1 mM ACh were applied, a significant depression in amplitude of FPs was observed for stimulation of both layers, i.e. layer II/III (88 ± 5 % of baseline, n=14, 500 μM ACh; 80 ±5.9 % of baseline, n=5, 1 mM ACh; figure 319a) and layer IV (78 ± 7.4% of baseline, n=6, 500 μM ACh; 75 ± 6.7% of baseline, n=5, 1 mM ACh, figure 3.19b).

These data show that different concentrations of ACh enhance or inhibit synaptic transmission for stimulation of intracortical pathways. However, ACh concentrations were shifted towards lower values when stimulating layer II-III and layer IV with respect to stimulation of WM (compare figure 3.19 with figure 3.12a), suggesting that cholinergic modulation of synaptic transmission is input specific.

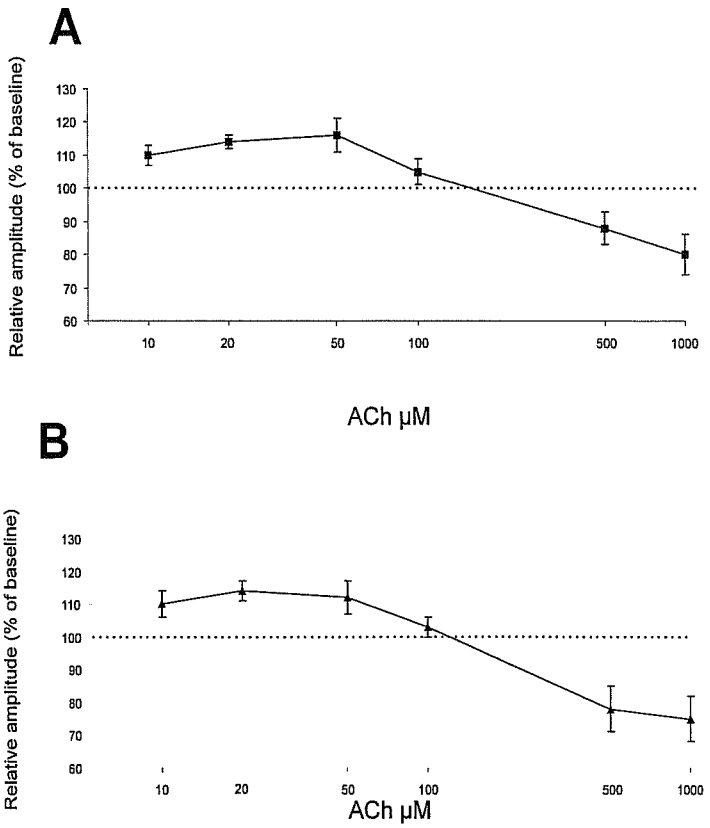

Figure 3.19: ACh modulation of FPs elicited in visual cortex slices via stimulation of intracortical pathways.
(A) Layer IV stimulation; facilitation of FPs is induced by 10-50 µM ACh. 100 µM ACh does not significantly modify FP amplitudes. High_concentrations of ACh, 500 µM and 1 mM, depress FP amplitudes (10 µM, n=5; 20 µM, n=6; 50 µM, n=7; 100 µM, n=8; 500 µM, n=6; 1 mM, n=5). **(B)** Layer II/III stimulation; ACh has the same effect as reported in (A) for stimulation of layer IV (10 µM, n=5; 20 µM, n=5; 50 µM, n=5; 100 µM, n=6; 500 µM, n=14; 1 mM, n=5). Symbols, filled squares and triangles, represent the mean amplitude of FPs during the last three minutes of ACh application normalized with respect to the mean FP amplitudes during the last three minutes of baseline.

3.16 Single cell recordings

Single cell recording of layer II/III neurons were made in order to evaluate the modulatory action of ACh on evoked post-synaptic potentials (EPSPs). Patch clamp in modality current clamp was done in visually identified neurons. Depending on the firings of the cell induced by an intracellular injection of current these neurons can be classified as regular spiking (putative excitatory neurons) or fast spiking (putative

inhibitory neurons). In both regular and fast spiking neurons bath application of 100 μM ACh produced a significant depression of EPSPs (68 ± 6% of baseline, n=7 for regular spiking; 71 ± 7%, n=2 in fast spiking; fig 3.111a). In a few cases, recordings of FPs and single cells were simultaneously performed within the same slice and at the same site using two distinct electrodes. The results confirmed that 100 μM ACh induces facilitation of FP amplitude and depression of single EPSP (fig 3.111b).

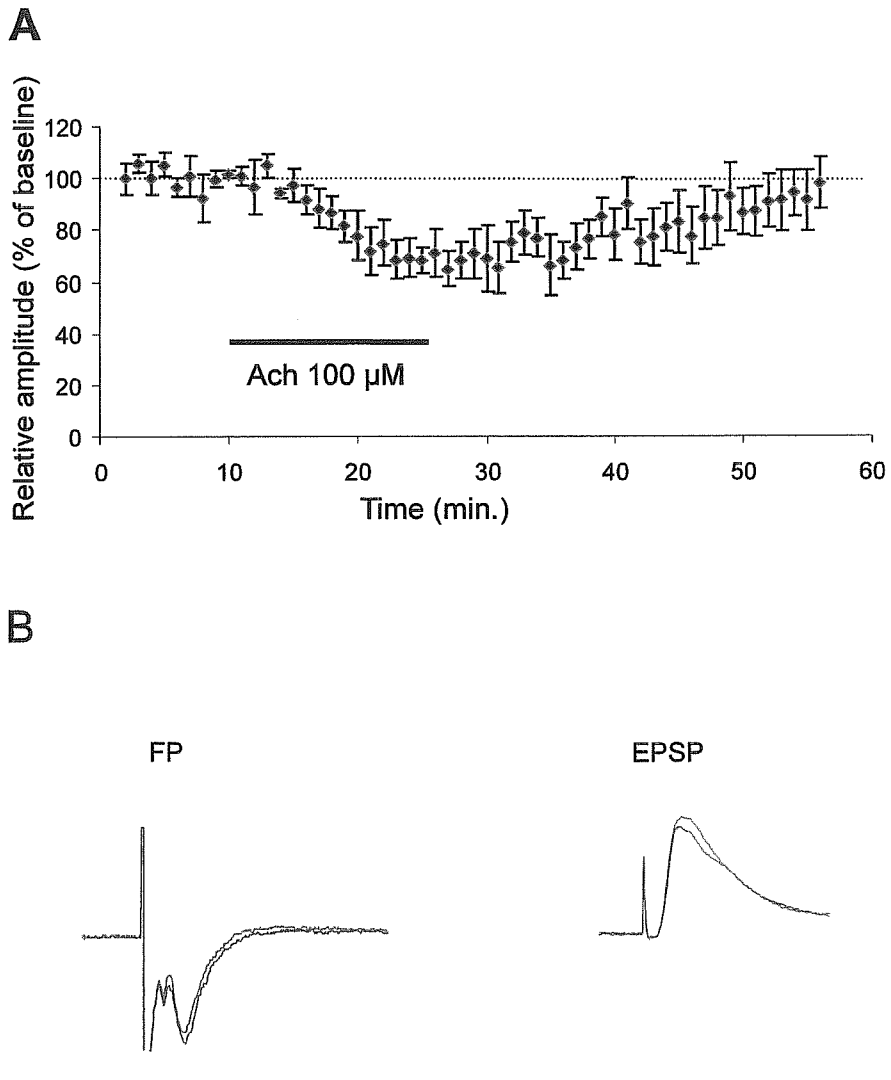

Fig 3.111 Patch clamp recording. Relative change in EPSP amplitude after ACh application.
(A) 100 μM ACh induce a depression of EPSPs. (B) Traces from simultaneous recording of single cell EPSP and FP showing depression of the former and facilitation of the latter after application of ACh 100 μM. Blue traces: Baseline responses; pink traces: responses in the presence of ACh.

To control for unspecific results due to intracellular dialysis during patch clamp we performed recordings from single cells using sharp electrodes (intracellular recordings, current clump mode). Also in this case, 200 μM ACh produced a small but significant depression of EPSP (85 ± 4 % of baseline, n=9; fig 3.112a) with a significant reduction of spike frequency adaptation (fig 3.112b) and increase of membrane potential (from -90 ± 4 mV to 85 ± 5 mV). When 1 mM ACh was bath applied, the depression of EPSP was significantly higher than that observed with 200 μM ACh (63 ± 6 % of baseline, n=5; fig 3.112a). Also with 1 mM ACh we observed a reduction of spike frequency adaptation (fig 3.112b) and significant depolarization of membrane potential (from –97 ± 4 mV was observed to –92 ± 4 mV). The membrane depolarization due to 1 mM ACh was, however, not significantly different from that produced by 100 μM ACh.

Thus, in adulthood, different concentrations of ACh enhance or inhibit synaptic transmission of vertical input from WM to a population of neurons localized in cortical layer II-III. Recordings from single cells showed that only depression was induced by different concentrations of ACh. However, low doses of ACh induced lower depression magnitude than high doses while, when considering ACh induced membrane depolarization, no differences were observed between the two concentrations. Thus the balanced in the inhibitory/excitatory action of ACh could explain the effects observed with FP recording.

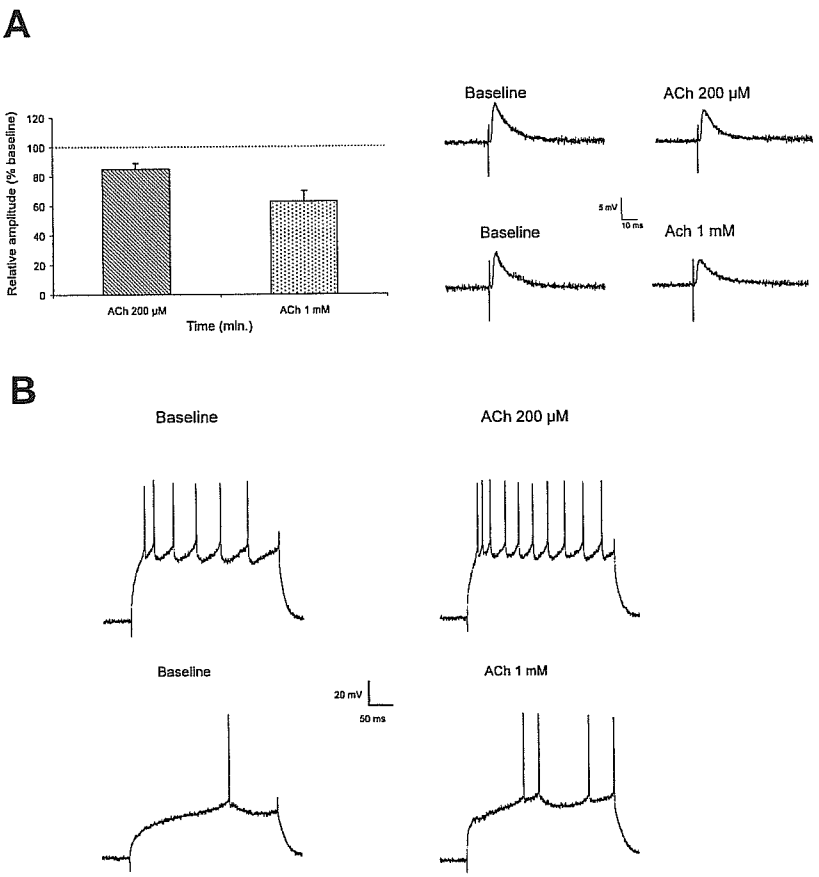

Fig 3.112 Intracellular sharp electrode recording of layer II/III neuron's EPSPs.
(A) Bath application of ACh 200 µM produced a depression of EPSPs that was lower from that produced by 1 mM ACh. Inset representative traces.. Columns represent the mean amplitude of FPs during the last three minutes of ACh application normalized with respect to the mean amplitude of FPs during the last three minutes of baseline. ($\star$ $p < 0.05$) (B) ACh bath application reduced spike frequency adaptation in layer II/III neurons.

3.2 Developmental regulation of ACh modulatory action

The use of the acetylcholinesterase antagonist edrophonium modified the dose-response curve obtained with ACh bath application (see section 3.13), suggesting that AChE activity controls the modulatory action of ACh on FP amplitude. In order to confirm such hypothesis we investigate whether physiological variations in the levels of AChE affect ACh action on FP amplitude. For this mean we take advantage of the postnatal

development in the AChE levels. Indeed it has been reported that, in the mouse cortex, AChE appears on the first postnatal day (P0) increasing progressively until P30 and remaining unchanged thereafter (Hohmann and Ebner 1985)

3.21 Developmental changes in AChE levels in SJL-C57BL/6J mice

We first compared the levels of AChE, in the visual cortex of in SJL-C57BL/6J mice, at different postnatal ages (P13, P16, P23, P30 and Adult). Density of AChE staining in the cortex increased with the animals age. A statistically significant increase was observed between P13 and P16 and between P23 and P30 but not between P16 and P23 or P30 and adult (fig 3.21)

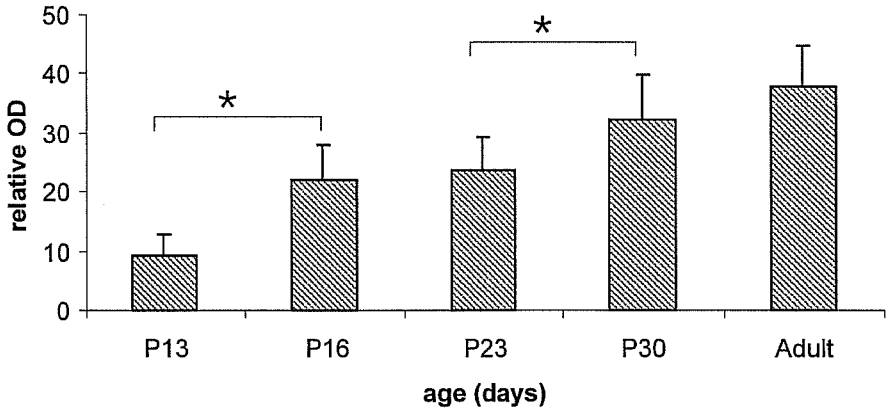

Fig 3.21 density of AChE positive fibers in visual cortex of SJL-C57BL/6J mice during postnatal development. (non parametric test for multiple comparisons * $p < 0.05$.)

3.22 ACh dose response curve varied during postnatal development

We next investigated for any changes in ACh dose-response curve during postnatal development. With this aim FP dose response curves to ACh bath application were obtained from visual cortex slices of mice at three different ages, P13 before eyes opening, P16 after eyes opening, P23 and adult.

Four different concentrations of ACh were applied in this study: 20 μM, 100 μM, 500 μM and 1.5 mM. We found that at P13 all of these concentrations determine a depression

of FPs amplitude (94 ± 4% of baseline for 20 μM; 75 ± 6% of baseline for 100 μM; 69 ± 6 % of baseline for 500 μM; 56 ± 4% of baseline for 1.5 mM ; fig 3.22). Interesting at this age not even transient facilitation was observed suggesting that . At P16 facilitation of FPs amplitude was induced by 20 μM ACh, no variations were seen with 100 μM ACh while depression was induced by 500 μM and 1.5 mM ACh (116± 5% of baseline for 20 μM; 99± 8% of baseline for 10 μM; 63 ± 6% of baseline for 500 μM; 43 ± 5% of baseline for 1.5 mM; fig 3.22). At P23 facilitation of FPs amplitude was induced by both 20 and 100 μM ACh; no variation were observed when 500 μM ACh was applied while depression was induced by 1.5 mM ACh (125 ± 5% of baseline for 20 μM; 130 ± 2 % of baseline for 100 μM; 95 ± 13 % of baseline for 500 μM; 46 + 7 % of baseline for 1.5 mM; fig 3.22). Finally in the adult facilitation of FPs amplitude was induced by 20, 100 and 500 μM while 1.5 mM determine a depression of FPs (128 ± 5 % of baseline for 20 μM; 142 ± 7% for 100 μM; 134 ± 6% for 500 μM; 65 ± 7% for 1.5 mM; fig 3.22). These data showed that during the postnatal development there is a clear shift of ACh dose-response curve towards high concentration of ACh that correlates with the developmental maturation of AChE.

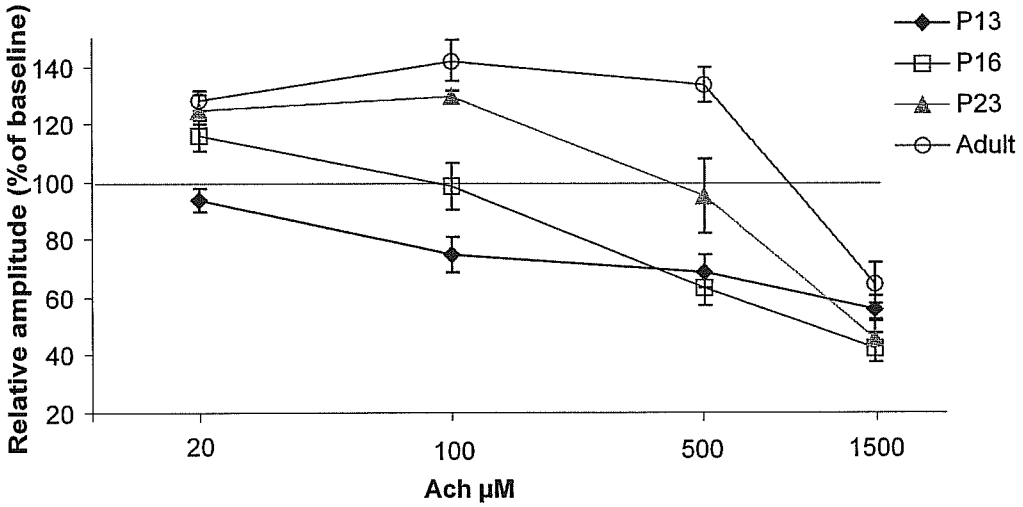

Fig 3.22 Modulatory action of ACh bath application on FPs evoked in visual cortex at different postnatal ages. A shift of dose-response curve towards higher concentration of ACh was observed during postnatal development.

3.23 Muscarinic receptors expression during postnatal development

The differences in the ACh dose-response curve we observed could also be due to changes in the expression of different subtype of muscarinic receptors during postnatal development. Since specific antibody for the different mAChRs are not commercially available we measured mRNA expression level of all five muscarinic receptor subtypes as index of their expression. We found that no one of the mRNA codifying for the different mAChRs changes its levels between P13 and adulthood (fig 3.23).

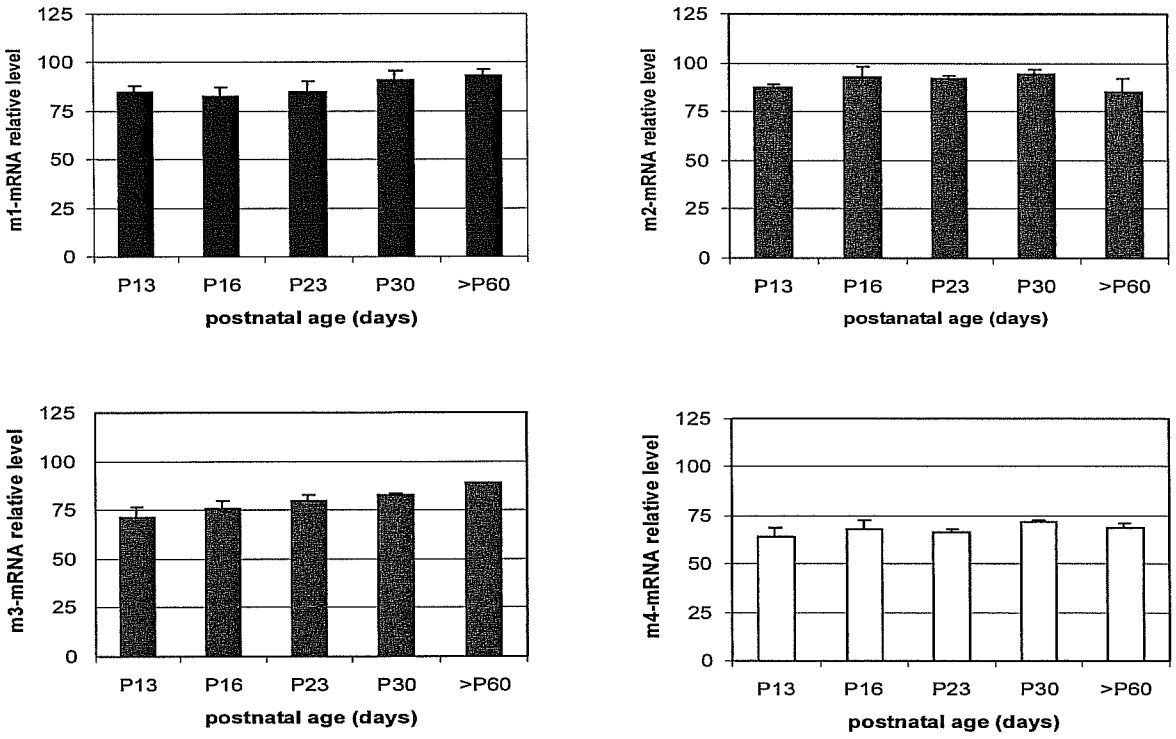

Fig 3.23 Expression of mRNA of M1-M4 mAChRs during postnatal development. No significant different were observed for any of the receptors considered (non parametric test for multiple comparisons).

3.24 Modulatory effects of ACh in dark reared animals

The results obtained at P13, showed that facilitation by ACh was not inducible at a developmental stage when the visual input is absent. However, at P16 just after eyes opening facilitation of synaptic transmission was inducible. These data made us wonder

whether the postnatal development of ACh modulatory action could be affected by visual experience. In order to test this hypothesis we reared mice in the darkness (DR) from P7 until P30 and we compared the dose response curve obtained in these animals with that observed in age-matched controls. We found no differences between the two groups (DR: 116 ± 3 % of basal amplitude, for 20 μM, n=7; 116 ± 4 % of basal amplitude, for 100 μM, n=6; 101 ± 8% of basal amplitude, for 500μM, n=7; 62 ± 3% of basal amplitude, for 1.5 mM, n=5; Control: 124 ± 5 % of basal amplitude, for 20 μM; 120 ± 5 % of basal amplitude, for 100 μM; 104 ± 7 % of basal amplitude for 500 μM; 65 ± 7 % of basal amplitude, for 1.5 mM; fig 3.24)

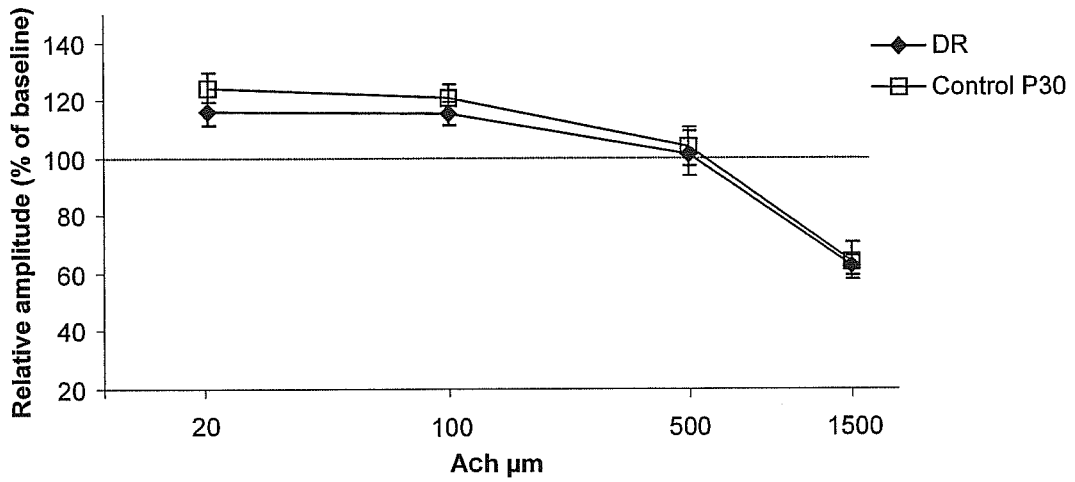

Figure 3.24 Dark rearing do not affect normal development of ACh modulation of FP

Dark rearing did not also affect AChE expression (fig. 3.25). While a significantly reduction in the expression of M_1, M_2 and M_3 mAChRs was observed (fig 3.26).

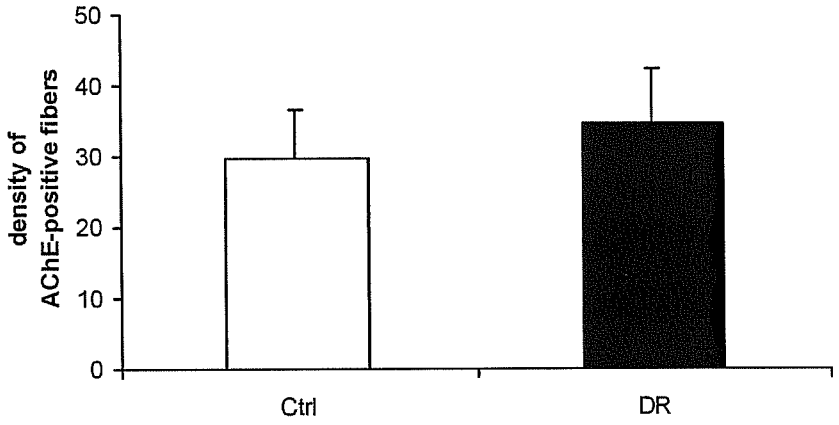

Figure 3.25 Dark rearing did not modified density of AChE positive fibres in visual cortex of SJL-C57BL/6J mice

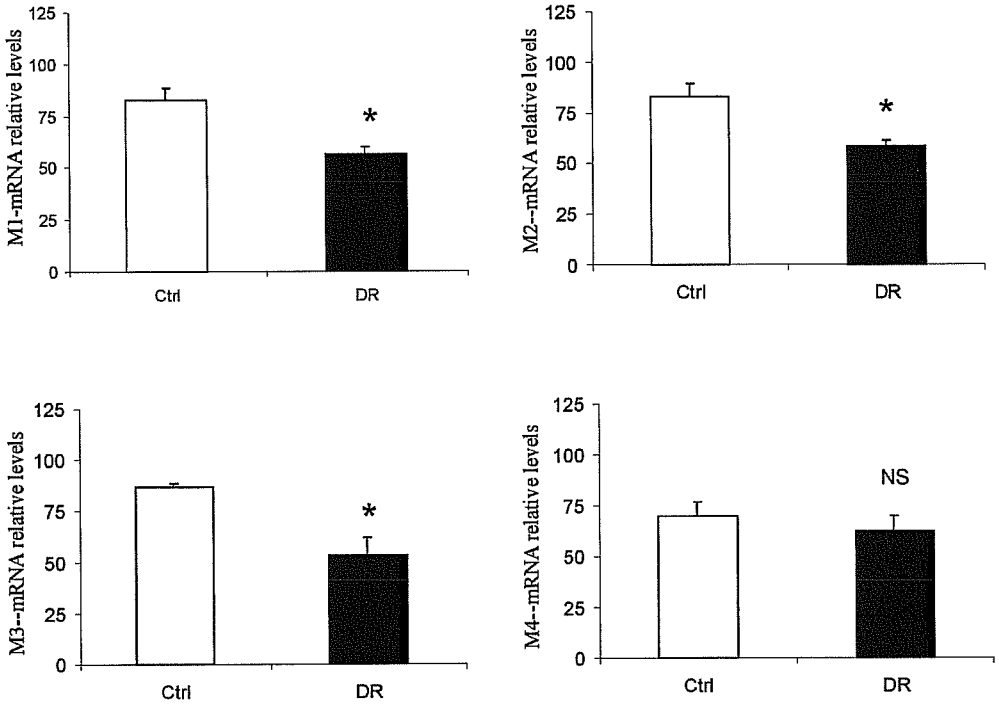

Figure 3.26 Expression of mRNA of M1-M3 mAChRs is reduced in visual cortex of dark reared animals. * $p<0.05$; NS not significant.

These results suggest that, despite dark rearing reduced the expression of three of the mAChRs; the developmental changes in the modulatory action of ACh on FPs are not influenced by visual activity.

3.3 Cholinergic system and synaptic plasticity

Cortical cholinergic innervation has been shown to play an essential role in learning, memory and long term cortical plasticity. Interestingly LTP, which represent the neural substrate of long-term synaptic modifications involving the synthesis of new proteins, appears to be regulated by the cholinergic system as well. In particular, interfering with cholinergic transmission by means of pharmacological tools acting on cholinergic receptors has been shown to affect the capability of cortical synapses to undergo long term plastic modifications (Brocher et al., 1992; Kirkwood et al., 1999; Pesavento et al., 2002). With the aim of directly testing the involvement of cholinergic system in cortical LTP we selectively disrupted cortical cholinergic innervations by using the immunotoxine 192 IgG-saporin selective for cholinergic cells. The first group of experiments was dedicated to know if 192 IgG-saporin was able to selectively lesion BFB cholinergic neurons in mouse. Until recently, no immunotoxin based on saporin method has been reported to work in mouse brain; this lack of action depends on the poor selectivity of antibodies for mouse P75. Indeed, the selectivity of the 192 IgG-saporin toxin conjugate is based on the specific binding of its antibody moiety to the low-affinity nerve growth factor receptor P75, highly expressed on cholinergic neurons of the basal forebrain (Wiley, 1992). Thus, after being recognized and bound by P75-expressing neurons, the receptor-immunotoxin complex is internalized, enabling the cytotoxic component (i.e. saporin) to block protein synthesis, inducing cell death. In our experiments, we used a new 192 IgG-saporin that according with previous results (Berger-Sweeney et al. 2001) was retained to be specific and suitable to produce degeneration of most BFB cholinergic neurons. We used different concentrations of 192

IgG-saporin in adult mouse: the results clearly indicated that 192 IgG-saporin was not able to induce the death of more than 50% of total BFB cholinergic neurons even at the highest doses. For this reason we decided to use the rat as a model for this type of experiments.

3.31 BFB's cholinergic neurons selective immunolesion

Four 4-day old (P4) rat pups, from two different litters were anesthetized by hypothermia and subjected to bilateral injections of 192 IgG-saporin. Two histochemical tissue markers were used to assess the extent and specificity of the cholinergic depletion, namely ChAT and AChE so as to visualize cholinergic neuronal and terminal fibre loss, respectively. The dose and injection volume of the immunotoxine adopted here (0.4 μg in 5 + 5 μl) were chosen on the basis of pilot experiments and previous observations (Leanza et al., 1996a). Accordingly, they proved to be optimal, producing the maximal cholinergic depletion, with no unspecific toxicity or animals' loss. In the same pilot experiments, no difference was detected between uninjected and vehicle-injected rats (see also Leanza et al., 1996a); therefore we consistently used intact animals as controls.

Two-to-three weeks after 0.4 μg 192 IgG-saporin injection, the rats showed a dramatic reduction in the number of ChAT-immunoreactive cells in the nucleus basalis magnocellularis, the medial septum and the diagonal band of Broca. In these nuclei, the few spared cells had a reduced number of processes with little branching (figure 3.31). In addition, an almost complete loss of AChE-positive cholinergic innervation was evident throughout the neocortex and in the various subfields of the hippocampus in saporin-lesioned animals (figure 5.1). In these lesioned animals (n=14), the estimated loss of cholinergic staining density in the hippocampus and fronto-parietal cortex averaged ~80-85% (table 3.31); by contrast, the AChE-positive staining in other brain areas like the thalamus and striatum was unaffected.

Group	n	Par Ctx	CA1	CA3	DG
Control	12	49.60±13.0	72.00±16.7	94.40±19.8	73.20±9.1
Lesioned	14	7.66±4.8*	12.83±3.6*	21.16±7.9*	19.50±3.2*
Lesion (% control)		84.5	82.2	77.6	73.4

3.31 Density of the AChE-positive fibers in different brain regions of uninjected and 192 IgG-saporin lesioned rats.
Values represent standardized relative staining density scores ±S.D. for each of the two groups. (*) Significant difference from the control group at $P<0.001$

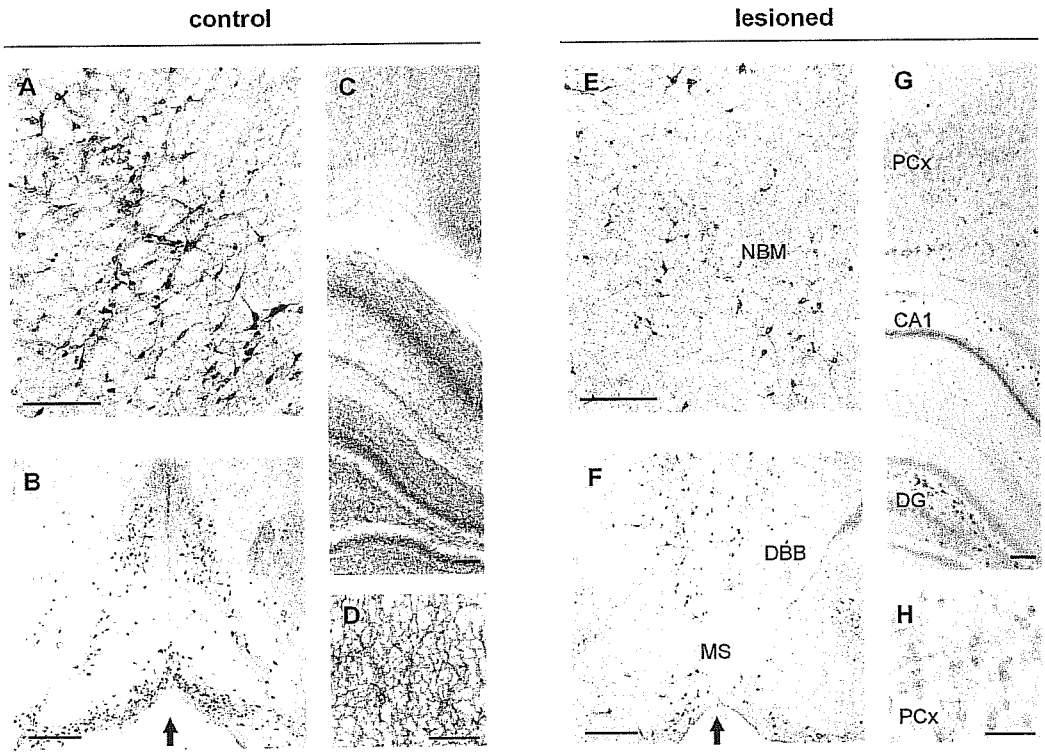

Figure 3.31. Decrease in the number of ChAT-positive cells in the BF and AChE-positive fibers in cortex and hippocampus after immunolesion
Choline acetyltransferase immunoreactivity in the Nucleus Basalis Magnocellularis (A and E) and the medial septum and diagonal band of Broca (B and F) in control and lesioned individuals (insets in A and E show enlargements of cholinergic immunoreactive cells in this brain area). Side pictures (C and G) correspond to the AChE staining in the parietal cortex, CA1 hippocampal subfield and the dentate gyrus of control and saporin-lesioned rats. Lower panels (D and H) show closer views of the stained fibers in the parietal cortex under each treatment. Scale bar in A, B, C, E, F, and G=100 □m; D and H 25 □m. NBM: nucleus basalis magnocellularis; MS: medial septum; DBB: diagonal band of Broca; PCx: parietal cortex; CA1: cornu ammonis 1; DG: dentate gyrus

3.32 LTP is impaired in slices from immunolesioned animals

In order to test if cholinergic deafferentation can affect synaptic plasticity in developing visual cortex we conducted electrophysiological field potential recordings. Recordings were made in layer II/III of occipital slices obtained from control and192 IgG-saporin treated rats while stimulating layer IV, i.e. the intracortical pathway connecting layer IV cells with layer II-III cells. In control rat slices, theta burst stimulation given to layer IV induced a stable LTP (111 ± 5 % of baseline, n=10; figure 3.32a). This value was statistically different ($p < 0.001$) from that obtained using the same stimulation protocol in slices from 192 IgG-saporin-treated rats where an LTD was observed after TBS stimulation (89 ± 2 % of baseline, n=9; figure 3.32a). We then investigated if this shift from LTP to LTD in immunolesioned animals could be due to a lower level of ACh release during TBS stimulation. In order to test this hypothesis, 20 μM ACh was bath applied to slices from 192 IgG-saporin treated rats for 10 minutes, centered around TBS presentation. Under these conditions we observed a complete rescue of LTP whose magnitude (108 ±5 %, n=8; figure 3.32b) was not significantly different from that observed in control animals.

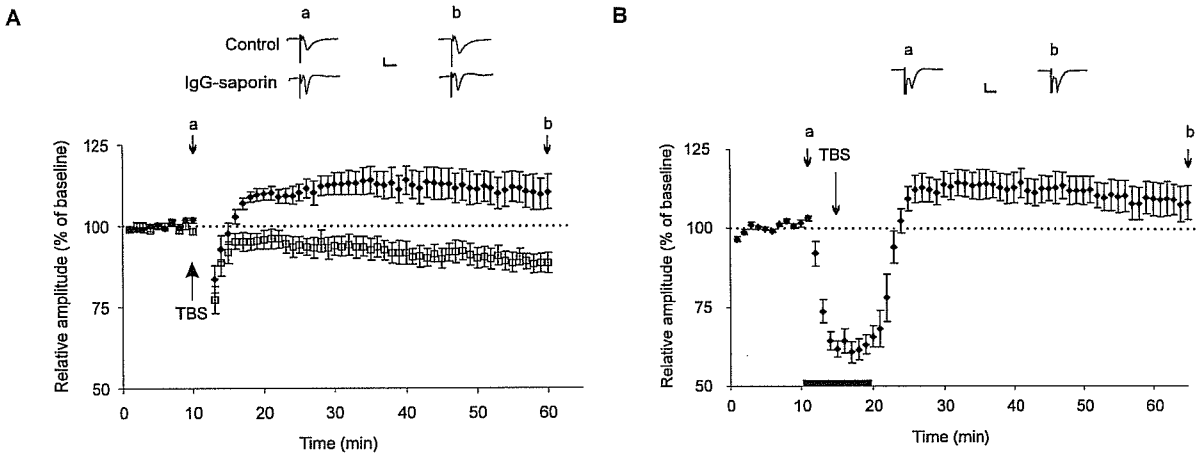

Figure 3.32. LTP is impaired in cortical slices from 192-IGg saporin treated animals.
(A) Theta burst stimulation induced a stable LTP in control slices (filled diamonds) but an LTD in immunolesioned slices (open squares). (B) LTP is recovered in immunolesioned slices when 20 μM ACh was bath applied in concomitance with TBS. Horizontal bar represents the time of ACh application. Inset: representative traces of FPs (a) last minute before TBS (b) last minutes of recording. Scale bar 10 ms/0.5 mV.

From 3.32b, it appears that ACh supplied before TBS induced a decrease in FP's amplitude. In order to investigate if cholinergic immunolesion modifies the ability of cortical neurons to respond to ACh we looked at the effect of bath application of this neurotransmitter on the modulation of cortical synaptic transmission. To this aim, we applied in the bath two different concentrations of ACh, 400 μM and 20 μM while measuring FP amplitude. In control animals, we found that 400 μM ACh induced a statistically significant depression of FP's amplitude (84 ± 4% of baseline, n=6, $p< 0.01$; figure 3.33a) while 20 μM ACh had not effect (99 ± 9 % of baseline, n=5, NS; figure 3.33b). The modulatory effect of ACh was dependent on the activation of muscarinic receptors since it was completely abolished by 10 μM atropine (not shown). In 192 IgG-saporin treated rats, however, 20 μM ACh was already enough to induce a significant depression of FPs amplitude (60 ± 3 %, n=6; $p< 0.001$: figure 3.33c) whose magnitude was even higher than that observed with 400 μM ACh in control animal slices ($p< 0.01$). Thus, the ability of cortical neurons to respond to ACh was increased in 192 IgG-immunolesioned rats. The higher responsiveness to ACh could be due to the reduction in the expression of AChE we observed in the immunolesioned animals. In agreement, with this possibility we found that 20 μM ACh in the presence of acetylcholinesterase inhibitor edrophonium (10 μM) induced a depression of FPs in control slices, similar to that observed in immunolesioned slices (50 ±4 %, n=5; $p<0.001$: figure 3.33d). These results suggest that the decrease of AChE that parallels cortical cholinergic denervation may compensate the reduced release of ACh.

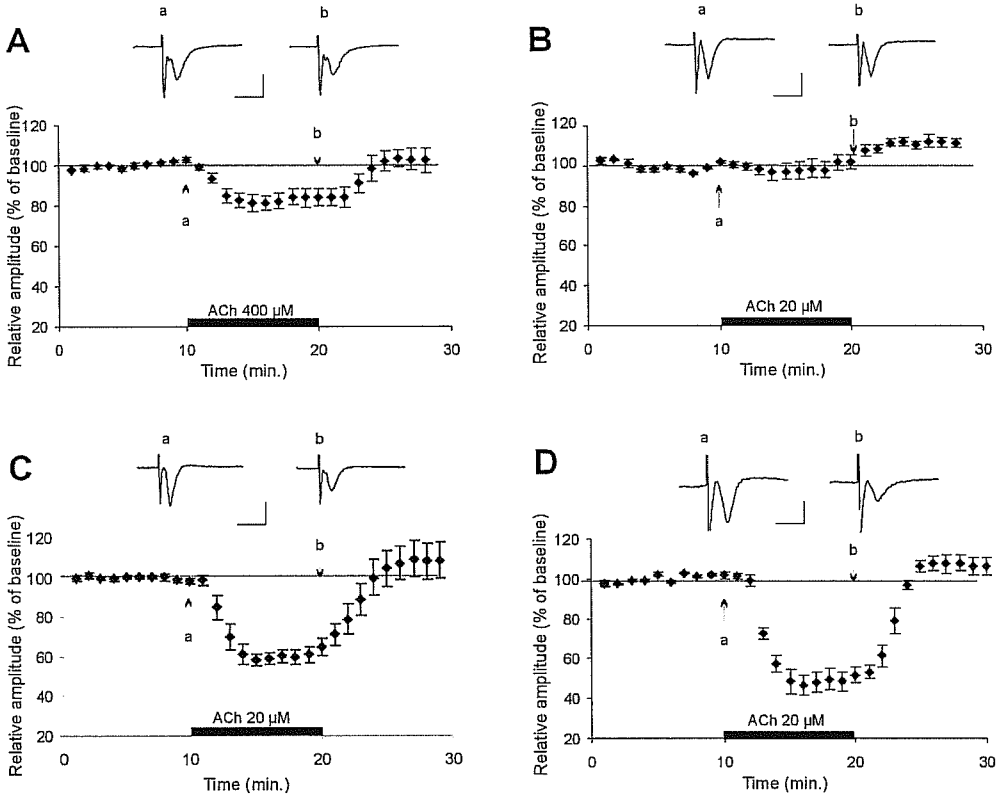

Figure 3.33: Slices from cholinergic lesioned rats show increased response to exogenously applied ACh.
(A) Control slices: bath application of 400 µM ACh reduced evoked FPs amplitude. (B) Bath application 20 µM ACh did not affect FPs amplitude in control slices. (C) Bath application of 20 µM ACh reduced FPs amplitude in slices from saporin treated rats. (D) Depressing effect of 20 µM ACh was reproduced in control slices in the presence of 10 µM edrophonium. Horizontal bars represent the time of ACh application. Inset: representative traces of FPs at the time depicted by the arrows. Scale bar 10 ms/0.5 mV.

The expression of muscarinic receptors (mAChRs) could also be modified in animals chronically deprived of cholinergic input. To investigate this possibility, we measured mRNA expression level of all five muscarinic receptor subtypes in the visual cortex of control (n=3) and saporin-injected (n=3) rats. The specificity of the PCR products was confirmed by restriction endonuclease digestion of each of the gel-purified bands. Reverse transcription of the total RNA obtained from this tissue enabled the amplification of specific PCR products corresponding to the m1, m2, m3, and m4 receptor subtypes, while m5 mRNA was undetectable. As shown in table 3.33, under our experimental conditions, we did not detect changes in the mRNA expression of the m3, or m4 receptor

subtypes after immunolesioning; however, the expression of the m1 and m2 mAChR transcripts was significantly reduced (m1: P=0.006; m2: P=0.045; see also table 3.32). Thus, lesion of basal forebrain cholinergic neurons during postnatal development induces reduction of muscarinic receptors m1 and m2 that could in turn, influence cholinergic modulation of synaptic transmission.

		Receptor subtype				
Group	**n**	**M1**	**M2**	**M3**	**M4**	**M5**
Control	3	77.21±1.07	64.54±3.33	70.96±3.83	68.03±7.15	n.d.
Lesioned	3	65.25±2.27*	48.17±6.21*	66.31±4.28	55.21±9.36	n.d.

Table 3.32. Transcriptional expression of muscarinic acetylcholine receptors in visual cortex. Values represent the mean optical density of the RT-PCR bands of each muscarinic receptor subtype respect to that of ß-actin ± S.D.; (*) indicates significant difference from the control group at P<0.05; n.d = non detected.

3.4 Muscarinic receptors and synaptic plasticity

In order to evaluate the muscarinic contribution to synaptic plasticity we moved again to mice model were we could take advantage of the use of mAChR KO.

3.41 LTP is impaired in M2-M4 mAChR KO

Theta burst stimulation in layer IV was used to induce LTP in visual cortex slices of adult mice. In control mice TBS induce a stable LTP of FP amplitude (126 ± 6 % of baseline, n=9, fig 3.41a). However when atropine 10 μM was bath applied, ten minutes centered on TBS, LTP was impaired (100 ± 3 % of baseline, n=6, p< 0.005 compared to control, fig 3.41b) showing that muscarinic receptors are necessary for LTP induction.

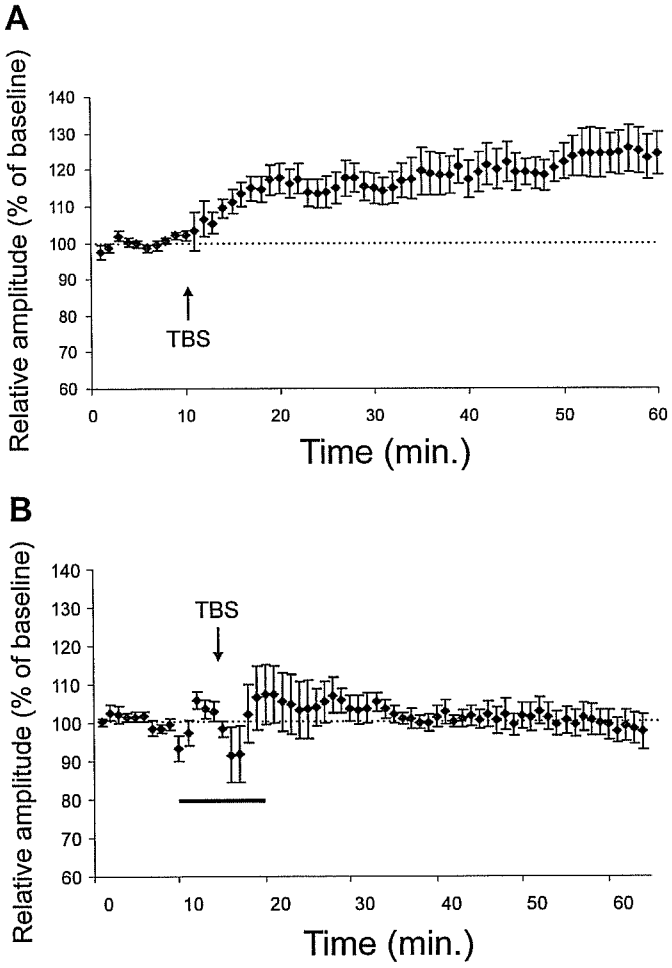

Fig 3.41 LTP required mAChRs activation. (A) TBS induce a stable LTP in visual cortex slices of M1-M3 control mice. (B) Atropine 10 μM delivered during TBS prevented LTP. Horizontal bar indicates the time of atropine bath application .

In slice from M1-M3 double KO mice TBS induced an LTP that was not statistically different from control (124 ± 6 % of baseline, n= 6, fig 3.42a). While in M2-M4 double KO mice TBS failed to induce LTP (102 ± 7 % of baseline, n=8, in M2-M4 KO; 123 ± 6% of baseline, n=7, in control; $p < 0.05$ M2-M4 KO compared to control; fig 3.42b)

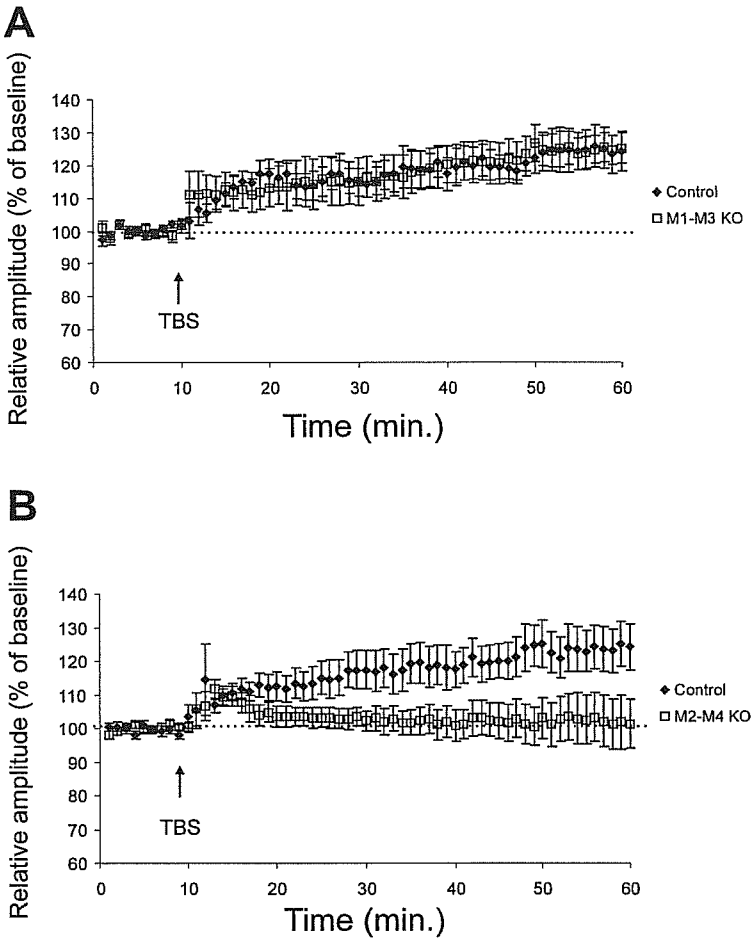

Fig 3.42 LTP is impaired in M2-M4 double KO mice. (A) M1-M3 double KO mice showed an LTP similar to control mice. (B) TBS did not induce LTP in M2-M4 double KO mice

These results show that LTP in mouse visual cortex relay on M2-M4 muscarinic receptors.

4.0 DISCUSSION

The physiological and cognitive states of the brain are influenced by variations in the activity of the modulatory systems such as the cholinergic, serotoninergic and noradrenergic systems. For example, changes in the levels of ACh have been associated with the arousal/sleep cycle, sustained attention and focal attention. A summary of ACh role implies a control of sensory processing in primary sensory cortical areas and higher order cortical areas. Although large amount of data favour this interpretation the link between changes in ACh release and modulation of sensory input is still lacking and a final model is far from being proposed. The absence of a complete model contrasts with the large mass of data obtained in the last twenty years using microdialysis. This technique permits to measure the level of ACh in different brain areas, including cortices, over periods of minutes showing clear changes in extracellular ACh concentration being associated with different brain states.

In order to investigate deeply the role of ACh in cortical synaptic transmission, we have used a quantitative experimental approach (variations in the levels of cholinergic activity) rather than a simply qualitative one (absence or presence of cholinergic activity). Our results for the first time show a concentration dependent effect of ACh on the modulation of synaptic efficacy, with the sign of this modulation depending on the concentration reached by ACh at the level of receptor sites. In particular, variations of extracellular ACh affect in opposite way cortical synaptic efficacy producing either an enhancement or an inhibition of evoked FPs. We found that such effects are influenced by the activity of ChE and relay on specific subtypes of cholinergic receptors, namely muscarinic receptors, thus linking the action of ACh to the activation of particular receptor types. Moreover, we showed that ACh concentration in cortical tissue contributes to modulate long-term changes of synaptic efficacy, such as LTP or LTD induced by specific patterns of afferent neuronal activity. In the absence of muscarinic activation, LTP is not inducible as shown in slices treated with atropine. In addition, the sign of long-term changes, whether LTP or LTD, seems to depend on the endogenous level of ACh; indeed, we showed that burst stimulation of

afferent neurons, in rats with reduced cortical cholinergic innervation, induces an LTD instead of LTP (see Fig 3.32).

4.1 Cholinergic modulation of synaptic efficacy

Cholinergic modulation of synaptic transmission has been widely discussed in the literature; a large number of methodologies have been used, sometimes leading to opposit results. In particular, while there is general agreement that ACh increases intrinsic excitability of cortical neurons (Krnjevic & Phillis, 1963; McCormick & Prince, 1987), some authors report an ACh-mediated suppression of glutamatergic inputs while others report an enhancement (Huerta & Lisman, 1993; Hasselmo &Cekic, 1996; Cox et al., 1994; Gil et al., 1997). However none of such studies focused on possible differences produced by varying the concentrations of ACh applied. Moreover in many of these reports cholinometic drugs resistant to AChE degradation were used to reproduce the action of ACh, in this way neglecting a possible role of AChE in affecting the modulatory activity of the cholinergic system.

In the present study, the effects produced by bath application of different ACh concentrations were evaluated on FPs elicited by the stimulation of three cortical synaptic pathways (WM-layer II/III; layer IV-layer II/III and layer II/III-layer II/III). Previous work has clarified that the amplitude of FPs recorded in different cortical layers by stimulation of WM or layer IV is associated with specific current sinks (current source density analysis, CSD) reflecting the flow of neuronal activity and synaptic activation in the correspondent layer (Mitsdorf 1985). In our experimental conditions we considered the negative field potentials with a latency of 4-7 msec evoked by stimulation of WM or layer IV and recorded in layer II-III. These FPs mirror the excitatory current sink produced by afferent activated fibres making synapses with neurons in layer II-III.

The reported results showed a biphasic action of ACh on evoked FPs. In particular, high concentrations (in the millimolar range) of ACh produced a depression while lower concentrations (ten to hundred micromolar) enhance FP amplitude. Microdialysis studies (performed in the presence of ChE inhibitors) showed that *in vivo*

cortical ACh levels are in the nano-micromolar range (Mitsushima et al., 1996; Acquas et al., 1998; Diez-Ariza et al., 2002), i.e. one or two order of magnitude lower than that necessary to induce depression of synaptic transmission under our experimental conditions. These data suggest that the ACh concentrations used in our study are higher than those present in living animals. However, microdialysis studies use large volumes of extracellular fluid that accumulate over minutes in the extracellular space. Such low temporal and spatial resolution could thus underestimate the actual local ACh concentrations present at a given time in a given place (such as the synaptic cleft). To know if our data have a physiological impact, we checked whether under our experimental conditions was possible to induce an endogenous release of ACh resulting in the modulation of synaptic transmission. Previous reports showed that endogenous ACh release in slices could be obtained by tetanic stimulation of intracortical cholinergic fibres (Bandrowski et al., 2001). In order to evaluate whether the depressing effect observed following bath application of a high concentration of ACh could be reproduced by inducing the release of endogenous ACh, we delivered a train of pulses in layer I, where cholinergic fibres lie horizontally for a few hundred micrometers. Using this approach we were able to demonstrate a mAChR-dependent depression of FPs evoked by stimulation of WM in absence of exogenous applied ACh.

The effects of ACh are due to mAChRs since they are blocked by an antagonist of muscarinic (atropine) but not of nicotinic (mecamylamine) receptors. Facilitation of extracortical input has previously been shown to be induced by ACh through the activation of nicotinic receptors in layer II/III of the barrel cortex (Gil et al., 1997). In the present study, we provide new evidence that ACh is able to enhance or inhibit synaptic transmission in visual cortex though activation of mAChRs and therefore independently of nicotinic receptors. The fact that both, ACh-induced facilitation and depression of evoked FP relay only on muscarinic receptor activation suggests that these two opposite effect could be induced by differential activation of mAChR subtypes. It has been reported that different mAChR subtypes are expressed in the mammalian visual cortex, with characteristic patterns of distribution throughout the cortical layers (Levey et al., 1991; Mrzljak et al., 1993; Aubert et al., 1996; Tigges et al., 1997). Since these studies were conducted mainly in rats, it was mandatory to know

which subtypes of mAChRs were expressed in the mouse cortex. We showed that the transcripts for all five mAChRs (M_1-M_5) are indeed expressed in the adult mouse visual cortex. In order to investigate a possible specificity of mAChR subtypes in controlling ACh-dependent facilitation or depression of synaptic responses, we used a combination of pharmacological tools and M_1-M_5 KO mice (Gomeza et al., 1999a, b; Yamada et al., 2001a, b; Fisahn et al., 2002). We showed that different mAChR subtypes are involved in facilitation/depression of FPs evoked by stimulation of WM. In particular, M_2 and M_4 mAChRs are necessary for the enhancement of synaptic transmission by low ACh concentrations, as shown by the results obtained with M_2 and M_4 KO mice and by using of an M_4 receptor-preferring antagonist. In contrast, the depression of synaptic transmission induced by high ACh concentrations is not modified in M_1-M_5 single KO or M_1-M_3 double KO mice, but is prevented by a high concentration of pirenzepine (2 μM) that blocks most M_1 and M_4 and a considerable portion of M_2, M_3, and M_5 receptors (Caulfield & Birdsall, 1998). In agreement with the results obtained in single and double mAChR KO mice, lower concentrations of pirenzepine, which preferentially block M_1 receptors, did not affect the depression of FPs induced by high concentrations of ACh. These results suggest that ACh-dependent depression involves multiple mAChRs whose precise identity and combined action remains unknown.

When examining the physiological effects induced by the release of ACh, particular attention should be paid to the role of the cholinesterases. In particular AChE is the best-known example of extracellular enzymatic degradation of a neurotransmitter (as opposed to re-uptake). AChE is characterized by one of the fastest catalytic activity known, with a single AChE protein been able to degrade $3\text{-}7 * 10^3$ ACh molecules/sec. Such characteristic is ideal for the rapid stop of ACh activity in the synaptic cleft at the neuromuscular junction. However, the action of AChE appears to be more complex in the CNS where extracellular levels of ACh can be kept high for long periods of time and cholinergic effects are slow and long lasting. Based on AChE distribution and models of neurotransmitter diffusion it as been proposed that AChE in the CNS has a role in regulating the ambient extracellular level of ACh setting the equilibrium between active ACh and ACh hydrolysis (Messamore et al 1993, Descarries et al 1997). According to this hypothesis AChE activity should play an essential role in the

concentration-dependent modulatory action of ACh. We found that reducing the activity of the ChE using the antagonist edrophonium modifies the cortical responses to bath application of different concentrations of ACh. Partial blockade of ChE activity by 1-2 μM edrophonium shifted the dose-response curve of ACh modulatory effects towards lower concentrations of ACh. Increasing the concentration of edrophonium up to 10 μM resulted in a dramatic shift to the left of the ACh dose-response curve with a very low concentration of ACh (5 μM) still inducing depression of synaptic responses. However, even at these low concentrations, a transient facilitation of FPs was observed after application of ACh. The transient facilitation appears to have the same nature of steady state facilitation since it was blocked by atropine and it is probably an effect of the slow diffusion of ACh-containing ACSF in the recording chamber. Following this interpretation, ACh passes through a concentration that is able induce FP facilitation before reaching the steady state concentration responsible of FP depression. The finding that the delay of transient facilitation is inversely related to ACh concentration supports the diffusion hypothesis. Also the variation of the cortical levels of AChE, such as that seen during postnatal development, may affect in a similar way ACh modulatory action. In this case the reduced expression of AChE observed during the first postnatal month correlates with the shift in the ACh dose-response curve towards lower concentrations. Finally, bypassing the cholinesterase activity by the use of cholinometic antagonists that are resistant to ChE degradation, such as charbachol and muscarine, we showed that even at low concentrations these compounds induce depression of FP's similar to that obtained with high concentrations of ACh.

Altogether the reported data prompted us to propose two possible models to explain the ACh effect on synaptic efficacy. Both are based on ChE activity and preferential activation of distinct mAChRs. We showed that low levels of ACh induce facilitation of synaptic efficacy by activating M_4 and M_2 receptors, while multiple mAChRs are required for synaptic depression. The first model relay on affinity differences of ACh for the mAChRs subtypes. Indeed it is reported that the affinity of ACh is higher for M_2 and M_4 than for the other mAChRs (Lazareno & Birdsall, 1995; Page et al., 1995). Thus, due to the high hydrolytic activity of AChE (Rosenberry, 1975; Quinn, 1987; for reviews see Descarries et al., 1997; Massoulie et al., 1999), this

enzyme could maintain ACh levels within a range that leads to preferential activation of M_4 and M_2 receptors, thus facilitating synaptic transmission. However any enzymatic activity is dependent on the concentration of the substrate and AChE posses a saturation threshold beyond which, further ACh increases escape from the catalytic activity (Silver 1974). Thus, when the extracellular levels of ACh exceed the saturation threshold of local AChE, the local ACh concentration could raise leading to activation of multiple mAChRs and depression of synaptic efficacy (see figure 4.1).

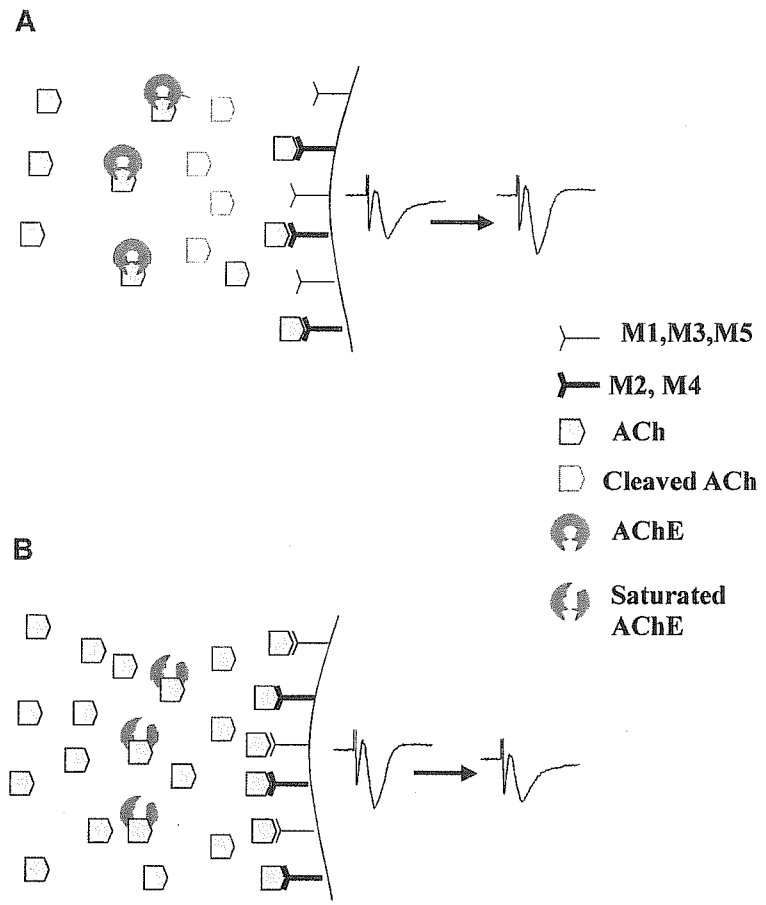

Figure 4.1 Model of ACh modulatory action based on mAChR affinity differences. (A) In the presence of low extracellular ACh levels, AChE activity keeps ACh concentration at the level of mAChRs, in a range that allows the selective activation of the highest affinity mAChR, M2 and M4, thus inducing FP facilitation (B) When the extracellular concentration of ACh exceed the saturation threshold of AChE the local ACh concentration can rise, activating in this way also the other subtypes of mAChRs and inducing FP depression.

The second model takes into consideration possible spatial differences in the distribution of AChE and the mAChRs subtype (Rotundo and Carbonetto 1987, Hersch et al 1994, Van der Zee et al 1989). Here the M2 and M4 receptors controlling FP facilitation are located relatively far away from AChE clusters and hence are not influenced by ACh degradation. On the other hand AChE activity prevents ACh to activate the cluster of mAChRs that control FP depression. Again, once the ambient level of ACh exceeds the saturation threshold of AChE also these receptors can be activated, determining in this way a FP depression (Fig. 4.2).

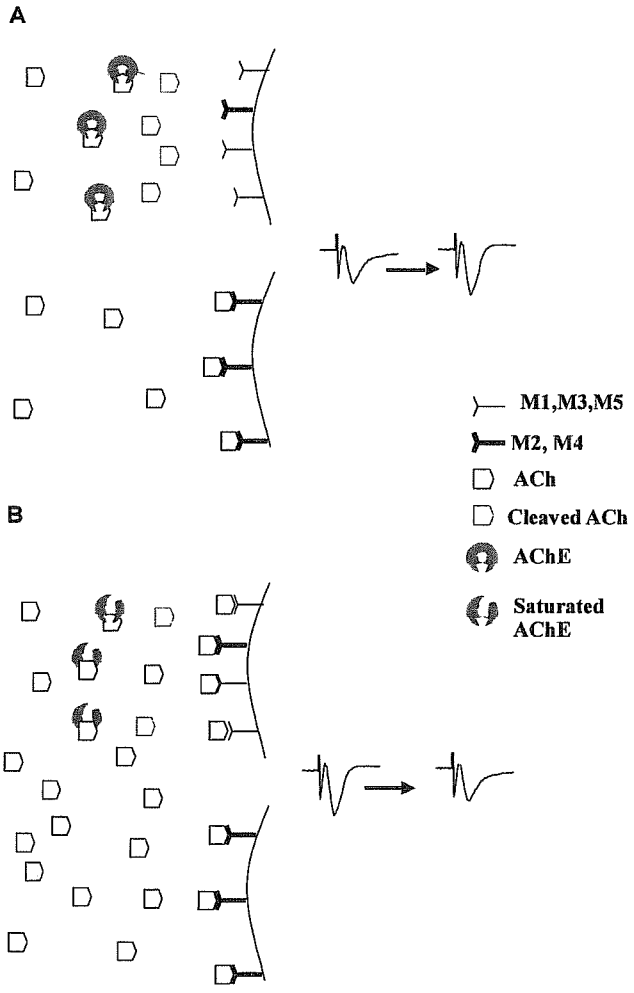

Figure 4.2 Model of ACh modulatory actions based on differences in mAChR subtypes spatial distribution. (A) In the presence of low extracellular ACh, ACh activity prevents the activation of mAChR cluster controlling FP depression while M2-M4 mAChR, located further from AChE, are activated inducing FP's facilitation. (B) In the presence of high ACh extracellular concentration, AChE saturation allows the activation of mAChR that produce FP depression.

This model does not rule out the possibility that the clusters of mAChRs controlling facilitation and those controlling depression could be located in different cellular compartments (for example presynaptic axons and postsynaptic soma).

Following these models the clusters of ChE proteins would exert a buffering action at the level of mAChRs, keeping in this way constant the local concentration of ACh, regardless of the ambient concentration. As a consequence, FP depression would be prevented for a broad range of exogenously applied ACh concentrations. When ChE activity is reduced, the saturation threshold would shift to lower ACh concentration and likewise the dose-response curve. In the extreme case of the absence of ChE activity, facilitation or depression of synaptic transmission would merely depend on different properties of mAChRs subtypes such as ligand affinity and receptor relative abundance.

By stimulating different cortical pathways, we showed that the modulatory action of ACh on synaptic transmission is input-specific. We found that the ACh dose-response curves obtained by stimulation of layer II/III and layer IV intracortical pathways were shifted towards lower ACh concentrations with respect to those obtained by WM stimulation. In particular, different ranges of ACh concentrations were able to modulate synaptic transmission in our experimental system. High ACh concentrations, in the mM range, inhibit synaptic transmission of both intracortical pathways and WM. Since WM contains extracortical connections, we suggest that at high ACh concentrations, there is a suppression of both intracortical and extracortical inputs. Intermediate ACh concentrations (500 μM) facilitate responses from extracortical input while inhibiting intracortical inputs. Considering that about 80% of the excitatory synapses in the cortex are of intracortical origin (Douglas & Martin, 1991), the specific cholinergic enhancement of extracortical connections appears to be a suitable mechanism to increase the influence of extracortical inputs on the cortical network. ACh in the range of 20-50 μM facilitated both extracortical and intracortical inputs and, finally, concentrations lower than 20 μM favored only intracortical connections. All the possible enhancement/ depression combinations between the two types of inputs can thus be obtained by controlling the extracellular levels of ACh.

Then, changes in local ACh levels appear to be responsible for the fine adjustment of cortical responsiveness to different inputs in primary visual cortex. Since the activity of basal forebrain cholinergic nuclei regulates cortical efflux of ACh (for reviews see Hasselmo & McGaughy , 2004; Pepeu & Giovannini, 2004), we propose that local ACh concentrations acting through specific mAChR subtypes could modulate the cortical flow of information. This mechanism may play a key role in switching the cortex through different cognitive states associated with high or low activity of the cholinergic system (Sarter et al., 1996; Passetti et al., 2000; Dalley et al., 2001; Pepeu & Giovannini, 2004). Consistent with this concept, Hasselmo and McGaughy (2004) proposed a model were high levels of ACh favor encoding of sensory information by enhancing extracortical over intracortical inputs while low levels of ACh promote memory consolidation inducing enhancement of intracortical versus extracortical inputs.

4.11 Postnatal changes in ACh modulatory action

We showed that ACh dependent facilitation of synaptic transmission is absent before eye opening, while ACh dependent depression is normally expressed even at this early developmental stage. Thus, facilitation of extra-cortical inputs contained in the white matter occurs in concomitance with efficacy of patterned vision.

What are the mechanisms controlling the maturation of ACh modulatory action?

The fact that ACh-dependent facilitation appears after eye opening suggests the possibility that its development could be under the control of visual experience. To test this hypothesis we reared mice in darkness for a brief period starting before eye opening (P7-P30). The results clearly showed that dark reared animals are characterized by normal ACh dose-response curve, thus excluding that visual experience could control the maturation of ACh-dependent facilitation. In addition, these results indicate that neuronal activity triggered by vision does not interact with the balance between ACh-dependent facilitation and depression. It interesting to note that AChE expression was not affected by dark rearing. Thus, other factors, possibly linked to maturation of

cortical cholinergic system, including AChE and cholinergic receptors, are involved in mechanisms underlying maturation ACh modulatory action.

One possibility is that maturation of different subtypes of mAChRs is involved in mechanisms underlying ACh modulatory action. For example, maturation of M_2 and/or M_4 receptors which control ACh-dependent facilitation could be delayed respect to other mAChRs in this way accounting for absence of ACh-dependent facilitation. Considering the ontogenetic expression of the different mAChRs subtypes in rodents, the even receptors appear to reach the maximal adult-like levels earlier than the odd ones (around the second postnatal week for M_2, M_4 and the fourth postnatal week for M_1M_3) (Lee et al 1990, Wall et al 1992). However, the second messenger cascade activation, i.e. PI turnover for odd mAChRs and adenyl cyclase suppression for the even receptors, follow the opposite pattern of development with the former being already present at birth while the latter starting from the second postnatal week (Lee et al 1990). This suggests that the transduction system linked to the activation of M_2 and M_4 mAChRs develops later than that linked to the activation of M_1 and M_3 and explains the absence of ACh dependent FP facilitation before two weeks of age.

The variations in the ACh dose-response curve observed during postnatal development, after eye opening (P16- adult), appear to reflect the developmental changes in the expression of AChE (Hohmann and Ebner 1985). Indeed, we found a positive correlation between the increase of cortical AChE staining and the development of the ACh modulatory response towards the adult state. On the other hand the expression of mAChRs, that could also affect ACh influence on FPs, does not change in the postnatal period considered. The fact that in some case the increase in AChE staining between two consecutive ages is not statistically significant (i.e. Between P16 and P23 or P30 and adult), while are evident the physiological variations of the response to ACh, is probably due to a limitation in the sensitivity of the detection technique, which is not able to discriminate small variations of AChE.

Several reports suggested that neuronal activity regulates the expression of mAChRs during postnatal development. In particular, the work produced by the group of Schliebs in the visual cortex of monocularly deprived animals (Kumar and Schliebs, 1992; Rossner et al., 1993) showed a transient alteration of mAChRs following visual

deprivation. More recently, Gu et al. (1998) reported that blocking the neuronal activity in the lateral geniculate nucleus by injection of tetradotoxin (TTX) retarded the developmental expression of mAChRs in visual cortex. However, using TTX was not possible to discriminate between evoked and spontaneous activity. In the present study, using RT-PCR, we demonstrate that absence of visual experience in dark reared mice induced developmental changes of mAChRs transcripts. It is interesting to note that modulatory action of ACh did not change in dark reared animals in spite of clear-cut changes in mAChRs transcripts.

Thus, the cortical response to exogenously applied ACh change during postnatal development. The distribution of cortical cholinergic fibres reaches the adult pattern already by P11 (Calarco and Robinson 1995). Additionally the electrically induced release of ACh in cortical slices at P20 is similar to that in adulthood (Pedata et al 1983), suggesting that cortical ACh efflux could be adult-like starting from the second-third postnatal week In this way the reduced AChE expression during postnatal development appears to have a functional meaning which is more complex than a simple compensation of the reduced cholinergic release. The delayed cortical development of both the second messenger pathway coupled to activation of M_2 / M_4 receptors and AChE favours the early development of ACh-dependent depression. The physiological implication of this result is that local ACh release reduces the flow of extracortical inputs to cortical cells during postnatal development. Whether similar regulation occurs also for the intracortical synaptic pathways remains to be investigated.

4.12 Single cell recordings

When considering the effects of ACh at the level of single cell recording, we found a mismatch between FPs and postsynaptic EPSPs. Indeed, whereas high concentrations of ACh depressed both FPs and EPSPs, low ACh concentrations facilitate the former while depressing the latter. Why we observed this discrepancy and what are the underlying process and functional meaning?

We define synaptic efficacy as the probability that an input produces a firing response in the postsynaptic cell. Following this definition synaptic efficacy should depend on the evoked post-synaptic potential and the cell excitability. It has previously been reported that application of ACh on cortical neurons produced an increase cellular excitability (Krnjevic & Phillis, 1963; McCormick & Prince, 1987). In our experimental condition, we showed that both, high and low concentrations produced an increase in the firing frequency and a similar membrane depolarization in cortical neurons. However, depression of EPSPs was significantly higher in the presence of high ACh concentrations. These results suggest the hypothesis that different concentrations of ACh could affect synaptic efficacy by regulating the balance between postsynaptic excitability and synaptic depression. On this ground low concentration of ACh would act by increasing membrane excitability while high concentration would mainly decreasing synaptic transmission, thus resulting in opposite effects on synaptic efficacy. Following this hypothesis, FPs should reflect changes of cellular firing as well as variation of post-synaptic potentials.

Alternatively the differences observed between FP and single cell recording could be due to heterogeneity in the response to ACh application among different neuronal subtypes (for example glutamatergic versus GABAergic neurons). The resultant of such differences on the whole cortical activity, detected by FP recording, could thus be discordant from the behaviour observed in a single cellular subtype.

However, further investigations are needed to definitively clarify the process accounting for discrepancy between single cell response and mass response to ACh.

4.20 Cholinergic modulation of synaptic plasticity

Cortical cholinergic innervation has been shown to play an essential role in learning, memory and cortical plasticity. Interestingly LTP, which is believed to represent the neural substrate of synaptic modification and memory, appears to be regulated by the cholinergic system as well. In particular, interfering with cholinergic transmission by means of pharmacological tools acting on ACh receptors has been shown to affect the ability of cortical synapses to undergo plastic modifications (Brocher et al., 1992;

Kirkwood et al., 1999; Pesavento et al., 2000). These studies used pharmacological tools to investigate the action of ACh; however, whether endogenous ACh was necessary to maintain a normal expression of LTP in the visual cortex remained question that still needs to be answered. The use of the immunotoxine 192 IgG-saporine, which selectively induces the death of cholinergic neurons of the BFB allowed us to directly test the effect of reduced cholinergic innervation on LTP expression. Finally, we showed that specific subtypes of mAChRs mediate ACh control of LTP expression. This type of investigation was possible by using of different mAChR KO mice.

4.21 Synaptic plasticity in cholinergic deafferented cortex

Cortical neurons receive cholinergic inputs mainly from the nucleus basalis magnocellularis (Mesulam et al., 1983; Eckenstein et al., 1988). At P4, when the immunotoxin was injected the cholinergic fibres from BFB have already reached the occipital cortex with the vast majority of axons confined in layers V and VI (Calarco and Robertson, 1995). Our results showed that injection of the immunotoxin 192 IgG-saporin in the lateral ventricles of the developing rat determined a massive reduction of cholinergic neurons in the nucleus basalis magnocellularis and the medial septum which was paralleled by a dramatic reduction of cortical AChE staining, a marker for cholinergic innervation.

In the developing visual cortex, these lesion-induced changes resulted in a clear-cut impairment of LTP. In fact, in lesioned animals, TBS was seen to evoke a stable LTD, instead of LTP. The shift from LTP to LTD in these slices is likely to be due to the lack and/or reduction of cholinergic input, as demonstrated by the prompt LTP rescue when ACh was exogenously supplied to immunolesioned slices. It is interesting to note that application of ACh restricted to the time of TBS stimulation was sufficient for a partial rescue of LTP. This suggests that local presence of ACh is necessary during the induction phase but is not required for the maintenance of LTP; it is tempting to speculate that, at least in the conditions used here, the same TBS stimulation might induce the increased ACh output required for LTP induction in normal animals.

Thus, for the first time, our results suggest that the entity of cortical cholinergic release could be a determinant factor in controlling the direction of plastic modifications.

Previous studies on the adult rat reported that selective lesion of central cholinergic neurons using this immunotoxin provokes behavioral deficits in cognitive tasks (Waite et al., 1994; Leanza et al., 1995; 1996b). However, the effects of selective cholinergic loss appear to be much milder when the lesion is performed early after birth. In fact, no cognitive deficits have been detected several months after an early postnatal immunotoxin lesion, in spite of massive cholinergic loss, (Leanza et al., 1996a). Likewise, hippocampal LTP has been observed unchanged shortly after a neonatal but not an adult 192 IgG-saporin lesion (Motooka et al., 2001). In contrast with the results obtained in hippocampus, the lesion of cholinergic system in developing visual cortex affects synaptic plasticity when investigated shortly after lesion. Discrepancy between hippocampus and cortex may arise from regional differences: the lack of functional effects in the hippocampus might probably be due to compensatory mechanisms taking place in this region but not in the visual cortex and acting on either spared cholinergic or non-cholinergic systems. However, we can not exclude that spared cholinergic fibres can compensate for the partial loss of cholinergic imputs (Leanza et al., 1996), thus attenuating synaptic plasticity deficits in the visual cortex, when long survival time after immunolesion is employed. When we checked if chronic deprivation of cholinergic input could have modified the modulatory action of ACh on synaptic transmission we found that immunolesioned slices showed an increased sensitivity to exogenously applied ACh. A possible explanation for this phenomenon could be the decrease of AChE in cholinergic deafferented animals with a consequent increase of the availability of ACh. Indeed, we showed that reducing AChE activity in control slices by edrophonium mimicked the increased response to ACh observed in 192 IgG-saporin treated animals. Alternatively, but not necessarily in contrast, the augmented response to ACh in lesioned rats could be due to a change in the expression of acetylcholine muscarinic receptors. Using RT-PCR we have observed a decrease in m1 and m2 receptor mRNAs, but no changes in the transcriptional expression of the m3, and m4 mAChRs subtypes. The m2 mAChR is the most abundant mAChR subtype in the basal forebrain where it is highly expressed by cholinergic neurons, among others. This receptor is mainly targeted to the

presynaptic membrane (Levey et al., 1996) then, the small but statistically significant decrease in the level of m2 message that we observed in the visual cortex could be explained by its cholinergic deafferentation, which consequently would reduce the amount of message for this receptor. Attention should be paid however, to the fact that transcript variations may not necessarily reflect changes in the translational profile of these receptors.

These results are apparently in contrast with those proposed by Rossner and co-workers (1995) who found that immunolesioning adult rats resulted in significant increases in the mRNA expression level of the m1, m3, and m4 receptor subtypes and no changes for m2 in occipital cortex. However some methodological differences between this and our work, such as the older animals and/or the shorter time post-injection before processing the animals used by Rossner, could account for the differences in the results. Indeed, from previous reports (Waite et al., 1994) it is known that 192 IgG-saporin lesion is not complete before 2 weeks post-injection (i.e. the time used here), while in Rossner et al (1995) the animals were sacrificed one weak after immunotoxine injection.

In conclusion, our results show that selective cholinergic deafferentation early after birth determines an increased responsiveness to ACh in cortical slices, possibly as a consequence of a reduction in AChE levels and/or different mAChRs subtypes, and an alteration of synaptic plasticity.

4.22 ACh action on LTP is mediated by subtypes of muscarinic receptors.

We showed that, in visual cortex, LTP induced by theta-burst stimulation of layer IV required the activation of muscarinic receptors. Using muscarinic receptor KO mice, we aimed to insulate the subtypes of mAChRs involved in LTP regulation by ACh. We showed that LTP was impaired in M2-M4 double KO mice whereas normal potentiation could be induced in M1-M3 double KO. These data confirmed the involvement of cholinergic system in synaptic plasticity and, for the first time, revealed specific subtypes

of mAChRs subtypes control such phenomenon. However, whether a single specific subtype of M2 or M4 is sufficient to regulate LTP is still under investigation.

It is conceivable that different mAChRs could control different forms of long term synaptic plasticity, for example M2-M4 receptors acting on LTP and M1-M3 receptors controlling LTD. Future experiments on single receptors KO mice are necessary to clarify this issue. However, the data obtained until now open the possibility that cortical bidirectional plasticity (Bear 2003) could depend on the activation of different subtypes of mAChRs and, consequentially, be under the control of cortical cholinergic release. Recently, it has been shown that, both in hippocampus and cortex, the selective inhibition of the NMDA receptors subunit NR2A prevents the induction of LTP but not LTD while the inhibition of NR2B results in the opposite action. Since ACh facilitates NMDA response (Markram and Segal 1990, Calabresi et al 1998a), cholinergic release could then control bidirectional plasticity through the selective activation of different NMDAr subunits (i.e. NR2A, NR2B).

5.0 CONCLUSIONS

In the present study we showed that ACh has multiple and opposite actions on cortical activity and plasticity, and that the nature of these phenomena depend at least on three factors:

i) the concentration of ACh;

ii) the cortical pathway (intracortical and extracortical pathways);

iii) the developmental stage of the animal.

The combined action of these factors allows the cholinergic system to exert a complex cortical modulatory activity. In this way larger number of degrees of freedom characterize the action exerted by the cholinergic system than that simply due to the presence or absence of ACh release. Such complex interactions appear to control the dynamic flow of cortical information that is associated with the different physiological states of the brain. We found that ChE activity and the specificity of action of the different subtypes of muscarinic receptors represent the key elements of the cholinergic modulation. By controlling the local ACh concentration, AChE (and maybe BuChE) appears to constitute the bridge between the above-mentioned factors (i, ii, iii). Indeed, the differences in the sensitivity to ACh showed by the different cortical pathways (intracortical *versus* extracortical pathways) conferring input specificity to ACh action could be simply obtained by local variations in ChE activity at the level of cholinergic sites receiving the specific inputs. Moreover the variations of AChE levels during the postnatal developmental, at least after eye opening, are sufficient to explain the observed shift in the response to ACh. Considering the heterogeneity of AChE cortical distribution (Descarries et al 1997), this enzyme introduces a mechanism by which the diffuse, and apparently non-selective, cholinergic release could have a heterogeneous, local-specific, modulatory action. Following this view, changes of the levels and distribution of AChE (Baregggi and Giacobini 1978, Skau and Brimijoin 1978, Greenfield and Smith 1979, De Sarno et al 1987) would be able to change the pattern of cortical responses produced by the cholinergic release.

Our data also showed that variations in the cholinergic release are able to affect cortical synaptic plasticity (in the form of LTP/LTD) and suggest that the versus of the modification (e.i strengthening or weakening of synaptic contacts) could be determined by the levels of ACh that are present in the cortex when a specific, plasticity-inducing, pattern of neural activity is produced. In this way the activity of the cholinergic system could have an essential role in shaping cortical connections; this could be particularly important during the developmental stage of the animal when the fine wiring of neuronal connections in the brain is reached through a process of stabilization/elimination of synaptic contacts. Different subtypes of mAChRs control LTP and possibly LTD conferring specificity to ACh action on long term synaptic plasticity. Also in this case appears to be present a specificity of action of the different subtype of mAChRs.

Cholinergic impairment is associated with age-related cognitive deficits such as those occurring in sporadic Alzheimer's disease (AD) (Auld et al., 2002). AChE inhibitors, which increase the level and action of ACh in the brain, are used for the treatment of AD. However, the present data suggest that AChE has a more complex physiological meaning than simply reducing the extracellular level of ACh after synaptic release. A better understanding of AChE role in cortical activity could thus allow a more targeted therapeutic action. The physiological activity of the cholinergic system depends on the selective activation of specific mAChR subtypes. Therefore, it would be interesting to study new pharmacological tools targeted to specific mAChRs, for example allosteric ligands that can change the binding affinity of ACh for distinct mAChR subtypes (Tucek & Proska, 1995; Jakubik et al., 1997). This strategy, together with the use to therapeutic agents that increase the bio-availability of ACh, might be more suitable to halt the cognitive impairment in neurodegenerative diseases affecting the cholinergic system.

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