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Tesi di Dottorato

Role of nitric oxide and endocannabinoids in synaptic plasticity in the perirhinal cortex and in visual recognition memory

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ABSTRACT

Introduction and aims of the research Nitric oxide (NO) and endocannabinoids (eCBs) are major retrograde messengers, involved in synaptic plasticity (long-term potentiation, LTP, and long-term depression, LTD) in many brain areas (including hippocampus and neocortex), as well as in learning and memory processes. NO is synthesized by NO synthase (NOS) in response to increased cytosolic Ca^{2+} and mainly exerts its functions through soluble guanylate cyclase (sGC) and cGMP production. The main target of cGMP is the cGMP-dependent protein kinase (PKG). Activity-dependent release of eCBs in the CNS leads to the activation of the $G\alpha_{i/o}$-coupled cannabinoid receptor 1 (CB1) at both glutamatergic and inhibitory synapses. The perirhinal cortex (Prh) is a multimodal associative cortex of the temporal lobe, critically involved in visual recognition memory. LTD is proposed to be the cellular correlate underlying this form of memory. Cholinergic neurotransmission has been shown to play a critical role in both visual recognition memory and LTD in Prh. Moreover, visual recognition memory is one of the main cognitive functions impaired in the early stages of Alzheimer's disease. The main aim of my research was to investigate the role of NO and ECBs in synaptic plasticity in rat Prh and in visual recognition memory. Part of this research was dedicated to the study of synaptic transmission and plasticity in a murine model (Tg2576) of Alzheimer's disease.

Methods *Field potential recordings.* Extracellular field potential recordings were carried out in horizontal Prh slices from Sprague-Dawley or Dark Agouti juvenile (p21-35) rats. LTD was induced with a single train of 3000 pulses delivered at 5 Hz (10 min), or via bath application of carbachol (Cch; 50 μM) for 10 min. LTP was induced by theta-burst stimulation (TBS). In addition, input/output curves and 5Hz-LTD were carried out in Prh slices from 3 month-old Tg2576 mice and littermate controls.

Behavioural experiments. The spontaneous novel object exploration task was performed in intra-Prh bilaterally cannulated adult Dark Agouti rats. Drugs or vehicle (saline) were directly infused into the Prh 15 min before training to verify the role of nNOS and CB1 in visual recognition memory acquisition. Object recognition memory was tested at 20 min and 24h after the end of the training phase.

Results Electrophysiological experiments in Prh slices from juvenile rats showed that 5Hz-LTD is due to the activation of the NOS/sGC/PKG pathway, whereas Cch-LTD relies on NOS/sGC but not PKG activation. By contrast, NO does not appear to be involved in LTP in this preparation. Furthermore, I found that eCBs are involved in LTP induction, but not in basal synaptic transmission, 5Hz-LTD and Cch-LTD. Behavioural experiments demonstrated that the blockade of nNOS impairs rat visual recognition memory tested at 24 hours, but not at 20 min; however, the blockade of CB1 did not affect visual recognition memory acquisition tested at both time points specified.

In three month-old Tg2576 mice, deficits in basal synaptic transmission and 5Hz-LTD were observed compared to littermate controls.

Conclusions The results obtained in Prh slices from juvenile rats indicate that NO and CB1 play a role in the induction of LTD and LTP, respectively. These results are confirmed by the observation that nNOS, but not CB1, is involved in visual recognition memory acquisition. The preliminary results obtained in the murine model of Alzheimer's disease indicate that deficits in synaptic transmission and plasticity occur very early in Prh; further investigations are required to characterize the molecular mechanisms underlying these deficits.

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All my love and gratitude to my mother and father, for being a constant source of inspiration, affection and support.

This thesis is dedicated to you Lydia.

LIST OF MAIN ABBREVIATIONS

2-AG: 2- arachidonyl glycerol
$5HT_2$: Metabotropic receptor for serotonin 2
5Hz-LFS: LFS consisting of 3000 pulses delivered at 5 Hz
5Hz-LTD: LTD induced with 5Hz-LFS application
7-NI: 7-nitroindazolo
AA: Arachidonic acid
ACEA: *N*-(2-Chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide
aCSF: Artificial cerebrospinal fluid
AD: Alzheimer's disease
ADAM: Alpha disintegrin metalloproteinase
AEA: Anandamide
ALS: Amyotrophic lateral schlerosis
AM251: 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide
AM404
AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPAR: AMPA receptor
AP2: Adaptor protein 2
AP-5: D-(-)-2-amino-5-phosphonovaleric acid
ApoE: Apolypoprotein E
APP: Amyloid precursor protein
ATP: Adenosine-5'-triphosphate
Aβ: Beta amyloid peptide
BACE-1: Beta amyloid cleaving enzyme-1
BDNF: Brain derived neurotrophic factor
BH4: Tetrahydrobiopterin
CA1: Cornus ammonis field 1
CaMKII/IV: Calcium-calmodulin protein kinase II/IV
CaMKK: Calcium/calmodulin protein kinase kinase
cAMP: 3'-5'-cyclic adenosine monophosphate
CAPON: Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein
CB1: Cannabinoid receptor type 1
CB2: Cannabinoid receptor type 2
Cch: Carbachol
Cch-LTD: Charbachol bath application mediated LTD
cGMP: 3'-5'-cyclic guanosine monophosphate
CNG: Cyclic nucleotide gated channels
CNQX: 6-Cyano-7-nitroquinoxaline-2,3-dione
CNS: Central Nervous System
CREB: cAMP responsive element binding protein
C-terminal: Carboxyl-terminal
D_2: Metabotropic receptor for dopamine 2
DAG: Diacylglycerol
DAG: Diacylglycerol
DEA/NO: diethylamine NONOate
DEA/NO: Diethylamine NONOate
DG: Dentate gyrus
DGL: Diacylglycerol lipase
DGL: Diacylglycerol lipase
DHPG: 3,5-Dihydroxyphenylglycine
DHPG-LTD: DHPG application-evoked LTD
DSE: Depolarization-dependent suppression of excitation
DSI: Depolarization-dependent suppression of inhibition
EC: Entorhinal cortex
eCB-LTD: eCB mediated LTD
eCBs: Endocannabinoids
EMT: Endocannabinoid membrane transporter
eNOS: endothelial NOS
EPSP: Excitatory postsynaptic potential
ERK: Extracellular signal related kinase
FAAH: Fatty acid amide hydrolase
fEPSP: Field excitatory postsynaptic potential
GABA: γ-amino-butyric acid
GluR: AMPAR subunit
Glutamate: L-glutamate
GPCR: G-protein coupled receptor
GRAB-1:
GTP: Guanosine-5'-triphosphate

HCN: Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel
HD: Huntington disease
HFS: High frequency stimulation
Hsp 90: Heat shock protein 90
IEG: Immediat e early gene
I-LTD: LTD of the inhibitory synapses
iNOS: inducible NOS
IP3: Inositol 1,4,5-triphosphate
IP_3: Inositol 1,4,5 trisphosphate
KAR: Kainate receptor
KO mouse: Knockout mouse
KT5823: ,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S, 12R-epoxy-1H-diindolo[1,2,3-fg:3',2', 1'-kl]pyrrolo[3,4-i][1, 6]benzodiazocine-10-carboxylic acid, methyl ester
KT5823: 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S, 12R-epoxy-1H-diindolo[1,2,3-fg:3',2', 1'-kl]pyrrolo[3,4-i][1, 6]benzodiazocine-10-carboxylic acid, methyl ester
Kv1: Voltage dependent potassium channels 1
Kv3: Voltage dependent potassium channels 3
LFS: Low-frequency stimulation
L-NAME: L-NG-Nitroarginine methyl ester
L-NMMA: NG-methyl-L-arginine
LTD: Long-term depression
LTP: Long-term potentiation
M1: Muscarinic receptor for acetylcholine type 1
M3: Muscarinic receptor for acetylcholine type 3
MAPK: Mitogen activate protein kinase
MAPT: Microtubule associated protein tau
MCI: Mild cognitive impairment
MEK: MAPK/ERK kinase
MGL: Monoacylglycerol lipase
mGlu Receptor: Metabotropic glutamate receptor
MK-801: Dizocilpine
MLA: Methyllycaconitine
MS: Multiple schlerosis
NCS1: Neuronal calcium sensor protein 1
NCX2057: 3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid
NMDA: N-methyl-D-aspartate
NMDAR: NMDA receptor
nNOS: neuronal NOS
NO: Nitric oxide
NOS: Nitric oxide synthase
NPA: N-ω-propyl-L-arginine
NR (1,2A/B,3): NMDAR subunit (1,2A/B,3)
NS2028: 4H-8-Bromo-1,2,4 - oxadiazolo [3,4-d]benz[b][1,4]oxazin-1-one
NS2828: 8-bromo-4H-2,5-dioxa-3,9b-diaza-cyclopenta[a]naphthalen-1-one
N-terminal: Amino-terminal
NVP: (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]phosphonic acid
ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
p75NTR: pan neurotrophin receptor 75
PC: Purkinje cells
pCREB: phosphorylated CREB
PD: Parkinson's disease
PDE (1-11): Phosphodiesterase (1-11)
PDZ: PSD-95, discs large, zona occludens-1
PGE2: Prostaglandin E2
PGF2: Prostaglandin F2
PICK 1: Protein interacting with C kinase
PIP_2: Phosphatidylinositol 4,5 bisphosphate
PKA: Protein kinase A
PKC: Protein kinase C
PKG: Protein kinase G
PLC: Phospholipase C
POR Postrhinal cortex
PP: Protein phosphatase
Prh: Perirhinal cortex
PS1/2: Presenilin ½
PSD: Postsynaptic density
PSD-95: Postsynaptic density protein 95
RNS: Reactive nitrogen species

ROS: Reactive oxygen species
SC: Shaffer's collaterals
sGC: Soluble guanylate cyclase
SNP: Sodium nitroprusside
SOD: Superoxyde dismutase
STDP: Spike-timing dependent plasticity
TBS: Theta-burst stimulation
t-LTD: Spike-timing dependent depression
t-LTP: Spike-timing dependent potentiation
tPA: tissue plasminogen activator
TrKB: Tyrosine kinase receptor B
VCAM-1: Vascular cell adhesion molecule-1
VEGF: Vascular endothelial growth factor
VGCC: Voltage gated calcium channels
VGNC: Voltege gated sodium channels
Weak 5Hz-LFS: 5Hz-LFS consisting of 1350 pulses
WIN55,212-2: (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone
YC1: 5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol

1. INTRODUCTION

1.1. Memory and learning: classification and short historical review

Memory and learning have been studied since ancient times, but only in the last few decades the cerebral structures involved have been identified and the physiological and molecular mechanisms underlying these processes have begun to be clarified.

Memory has been classified as explicit (facts and events) and implicit (e.g. classical and operant conditioning and procedural memory; Fig 1.1.). Cerebral areas associated with these mnemonic processes are extensively studied by psychologists, neurophysiologists and psychiatrists. In a situation of learning, many of these brain areas may be working simultaneously, each one processing different streams of information about the perceived event (visual, location, sound, emotional content, etc.). The associative learning is believed to emerge from the coordinated activity of different brain areas. This is how organisms, including humans, encode causality in the perceived world.

Memory can also be classified as short- and long-term. Short-term memory has a limited temporal window (minutes) and needs to be continuously renewed to be maintained; long-term memory lasts longer (from hours to years).

Different functions are associated to specific brain regions, and the same applies for implicit and explicit memory processes (reviewed by Squire, 2004). In the 1940's, Penfield's electrophysiological experiments on epileptic patients and studies of hippocampal lesions in primates and humans were some of the first experimental evidences supporting the idea of structure - function relations for mnemonic processes (reviewed by Feindel, 1982). Before surgical ablation of the temporal lobe, Penfield's patients, who were conscious throughout the procedure

(local anaesthesia), were electrically stimulated in different areas of the temporal cortex. During the stimulation, Penfield's patients verbally referred back to past experiences.

Another famous example comes from the case of patient H.M., who suffered from an aggressive form of epilepsy. Bilateral partial ablation of the temporal lobes, including both hippocampi, was performed as a treatment. After the surgery, H.M. suffered from a severe form of anterograde amnesia: he could recollect everything that happened until the day of the surgery, but from that moment onwards his long-term memory was severely impaired although procedural memory processing remained intact.

In the coming years, lesion studies on primates and rodents demonstrated that the hippocampus and associated structures (perirhinal, entorhinal and parahippocampal cortices) are selectively involved in acquisition and consolidation of declarative but not implicit memory processes (reviewed by Squire, 2004).

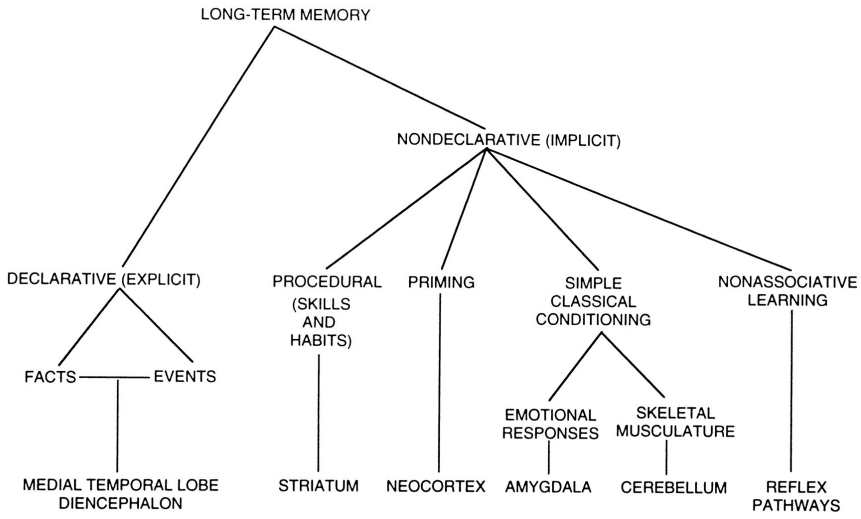


Fig 1.1. Schematic representation and functional classification of long-term memory and associated brain structures (modified from Squire, 2004).

1.2. Cellular correlates of learning and memory: synaptic plasticity

What is memory from a physical point of view? In pure physical terms, memory is the ability of a system to acquire, store and recollect information. In a biological system, a complex phenomenon like memory is characterized by cellular and molecular mechanisms that have been the subject of extensive investigation in the last 40 years. Considering that most of the stored information is acquired from sensorial experience, the brain has to undergo many long-term functional and structural modifications corresponding to the mnemonic tracks left by perceived experiences. In his Croonian Lecture to the Royal Society in 1894, Ramòn y Cayal proposed that memory formation relies on reinforcement of the signalling between neurons in the involved areas:

[…] it can be accepted that the mental exercise brings to a major development of the dendritic apparatus and of the axonic collaterals system in the mostly used brain areas […].

In this concept, the notion of synaptic plasticity is already maturing: the ability of chemical synapses to increase or decrease the efficiency of transmission between neurons according to the frequency of the stimulation and to the previous history.

The idea that memory and learning result from the alteration of activity of specific synapses was further highlighted by the Canadian psychologist Donald Hebb in 1949 in his book titled “Organization of the behaviour”:

“When an axon of cell A is near enough to excite B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.”

This Hebbian rule has been formulated on pure theoretical basis, hypothesizing that such a mechanism could stabilize specific patterns of neuronal activity: if a

neuronal activity pattern corresponds to a specific behaviour, stabilizing that pattern means memorizing that behaviour (Hebb, 1949).

Most part of both excitatory and inhibitory synapses show a rich repertoire of plastic modalities that work on time scales comprised between milliseconds and weeks (e.g. paired-pulse facilitation, paired-pulse depression, long-term potentiation, long term depression, suppression of inhibition etc.). With regards to the cellular and molecular basis of memory and learning, long-term potentiation (LTP) and long-term depression (LTD) have represented the main experimental model in the last 30 years. Both LTP and LTD have been observed in many brain structures such as hippocampus, neocortex and subcortical structures. In 1973, Bliss and Lømo were the first to observe that high frequency stimulation (HFS) of the perforant path (PP) of the hippocampus of anesthetized rabbits *in vivo* determined a significant and prolonged potentiation of synaptic transmission in the dentate gyrus and termed this phenomenon LTP. It has been mainly explored in hippocampus, but it can also be induced in other brain structures, such as the perirhinal cortex (Prh). LTP is characterized by a long-term (more than 3 h) increment of synaptic strength following a short period of coordinated neuronal activity, such high frequency stimulation of afferent fibres. Even if LTP is persistent, it's not irreversible: the synaptic strength can be returned to basal levels through low frequency stimulation (LFS) of the afferent fibres (depotentiation, DP; Barrionuevo et al., 1980). In addition, when LFS is applied to a non-potentiated pathway it leads to LTD (Bear and Dudek, 1992). LTD can be returned to basal levels after HFS through the process termed de-depression. Thus, the strength of synaptic transmission can be altered in a bidirectional and reversible way: the dynamic storage of large amounts of information at neuronal level may be constantly redefined. Furthermore, these forms of Hebbian plasticity

act through positive feedback processes, that if left without control measures may destabilize the neuronal networks by driving neurons into maximal and/or minimal firing frequency averages: by degrading the signals that propagate through the network, this ultimately disrupts the ability of the neurons to encode further plastic changes. Homeostatic forms of synaptic plasticity should integrate negative feedback systems in order to keep the synaptic transmission and plasticity within a dynamic functional range, either increasing or decreasing the strength of all the synaptic inputs: this can be achieved by either keeping at the same time their relative weight (synaptic scaling) or by modifying the ability of the synapses to undergo further plastic changes (metaplasticity; reviewed by Pérez-Otaño and Ehlers, 2005)

1.2.1. Long term potentiation (LTP): induction and expression mechanisms

LTP is defined as the long-term increase of synaptic strength subsequent to the application of a HFS (usually 100 Hz) on the presynaptic fibres. A lot of what we know about LTP arises from experiments conducted at Schaffer Collaterals (SC) / CA1 glutamatergic synapses. The induction of LTP at these synapses involves the activation of N-methyl-D-aspartate receptors (NMDAR), a class of ionotropic receptors for glutamate, permeable to calcium (Ca^{2+}) (reviewed by Collingridge and Bliss, 1995). HFS or presynaptic stimulation coupled to post-synaptic depolarization removes the voltage-dependent block of the NMDAR by the displacement of the magnesium (Mg^{2+}) ion placed on the extracellular side of the channel pore (Mayer et al., 1984). NMDAR are referred to as coincidence detectors, as the contemporary presence of glutamate in the synaptic cleft in combination with postsynaptic depolarization, determines the opening of NMDAR resulting in the influx of Ca^{2+}. NMDAR are formed by hetero-oligomeric assemblies

of NR1 subunits with NR2 (A-D) and NR3A (Monyer et al, 1994). According to Liu et al., 2004, HFS applied to a glutamatergic pathway activates NR2A subunit containing NMDAR leading to the activation of Ca^{2+}/calmodulin dependent Kinase II (CaMKII). On the other hand, if in the subunit NMDAR is comprised the NR2B subunit, the Ca^{2+} influx will result in the activation of the Ca^{2+}/calmodulin dependent phosphatase calcineurin that is responsible for LTD induction (Mulkey et al., 1993, 1994).

The LTP expression mechanisms are not yet fully understood. The activation of CaMKII triggers the insertion of further GluR1 containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) on postsynaptic dendritic processes (Malinow and Malenka, 2002). Recent studies highlighted two other main signal transduction pathways responsible for the long-term modifications needed for the consolidation of LTP: the cAMP-dependent protein kinase (PKA) and the mitogen activated protein kinase (MAPK). Intracellular Ca^{2+} influx induces the activation of adenylate cyclase (AC) leading to the subsequent increase of intracellular cAMP which activates PKA. The expression in transgenic mice of the inhibitory form of the regulatory subunit of PKA R(AB) does not affect the early phase of LTP but disrupts its consolidation. MAPK is also termed extracellular signal-related kinase (ERK). In the dentate gyrus, the application of a LTP-inducing stimulation determined rapid phosphorylation and nuclear translocation of MAPK (English and Sweatt, 1996; Davis et al., 2000 a). Both PKA and MAPK can phosphorylate and activate the transcription factor cAMP-responsive element binding protein (CREB) (Yin and Tully, 1996; Silva et al., 1998). CREB triggers the expression of genes responsible for long-term modifications (functional and structural) underlying the late phase of LTP. Finally, many lines of evidence have

consistently shown that protein synthesis blockade prevents the long-term expression of LTP, without affecting induction (reviewed by Blitzer et al., 2005).

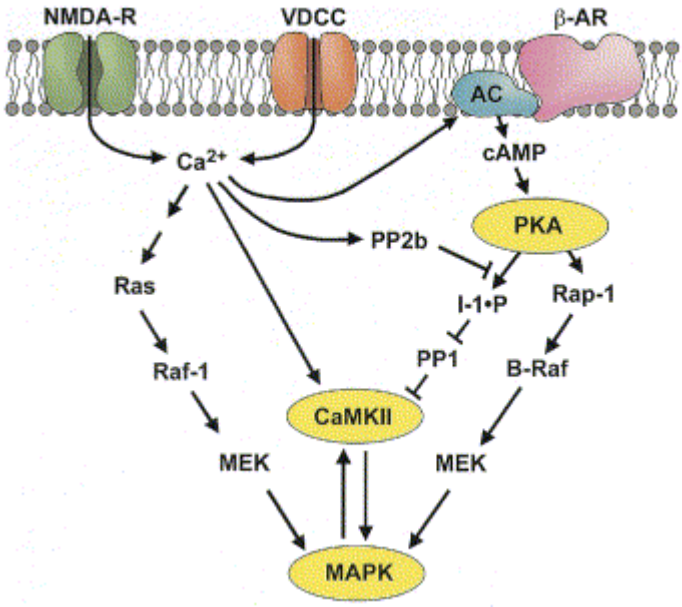


Fig 1.2. Schematic representation of LTP induction and expression mechanisms. The three most important downstream signalling pathways are CaMKII, MAPK and PKA. Ca^{2+} influx occurs through NMDAR and voltage-gated Ca^{2+} channels (VGCC). AC can also be activated by stimulation of G_s coupled receptors such as β-adrenergic receptors (modified from Blitzer et al., 2005).

Many recent studies have highlighted the key role of brain-derived neurotrophic factor (BDNF) in both induction and expression of LTP (Lu and Gottschalk, 2000; Aicardi et al., 2004; Santi et al., 2006; Minichiello et al., 2009). In particular, it has been suggested that activity-dependent release of BDNF from the presynaptic site is necessary for the induction of LTP, while the activity-dependent sustained production and secretion from the post-synaptic site is necessary for the expression of LTP (Aicardi et al, 2004; Reviewed by Lu et al., 2008). Pro-BDNF is cleaved to mature BDNF (mBDNF) via proteolytic cleavage by the tissue plasminogen activator (tPA). mBDNF binds the tyrosine-kinase coupled receptor B (TrkB) which in turn phosphorylates various substrates, including MAPK/ERK resulting in the activation of MAPK/ERK kinase (MEK). TrkB activation also leads to phospholipase C (PLC) activation, which in turn activates both PKC and calcium calmodulin kinases kinase (CaMKK), subsequently resulting in CaMKIV and CREB activation (Reviewed by Minichiello, 2009; Fig 1.3).

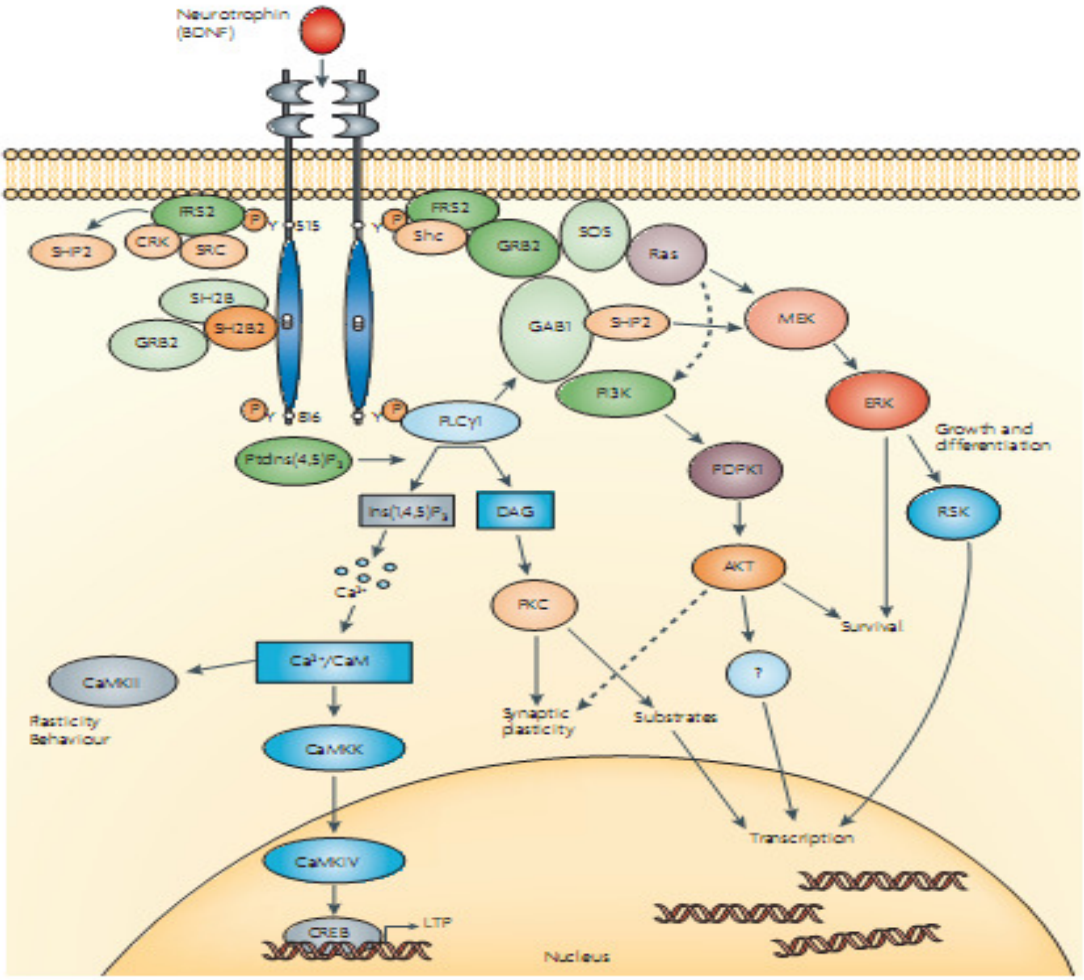


Fig 1.3. Activation of TrkB triggers three main intracellular signalling pathways. i) Ras–MAPK, which promotes neuronal differentiation and growth through MAPK/ERK kinase (MEK); ii) phosphatidylinositol 3-kinase (PI3K) cascade, which promotes survival and growth of neurons and other cells through Ras or GRB-associated binder 1 (GAB1); iii) phospholipase Cγ1, which mediates synaptic plasticity through CaMKII and CaMKK/CaMKIV signalling cascade (modified from Minichiello et al., 2009).

1.2.2. Long term depression (LTD): induction and expression mechanisms

The first evidence for LTD came from the observation that LTP inducing protocols in hippocampal SC/CA1 synapses generated a reversible depression in the non tetanized pathway (Lynch et al., 1977); this phenomenon is called heterosynaptic plasticity. Homosynaptic depression was later shown in hippocampus: the application of LFS reversed LTP in a pathway previously tetanised; this phenomenon is now known as depotentiation (Barrionuevo et al., 1980). Later, it was demonstrated that LFS can induce LTD in CA1 even without previous LTP induction (Dudek and Bear, 1992; Mulkey and Malenka, 1992).

LTD can be induced in many brain areas other than the hippocampus including the visual cortex (Artola et al., 1990), striatum (Calabresi et al., 1994), perirhinal cortex (Ziakopoulos et al., 1999; Cho et al., 2000), amygdala (Wang and Gean, 1999), posterior cingulus (Hedberg and Stanton, 1995) and prefrontal cortex (Hirsch and Crepel, 1991).

The first studies on homosynaptic LTD showed that application of LFS consisting of 900 pulses delivered at 1 Hz, induces LTD that relies on the activation of NMDAR (Dudek and Bear, 1992; Mulkey and Malenka, 1992). NMDAR-dependency of LTD is often age-related (Kemp et al., 2000, Jo et al., 2006), consistently with the developmental change in subunit composition of NMDAR (Monyer et al., 1994).

It is of interest to note that LTD induction can be mediated by other receptors like kainate receptors (KAR), metabotropic glutamate receptors (mGluRs) and type I muscarinic acetylcholine receptors (M1) (reviewed by Kemp and Bashir, 2001). Group I and II mGluRs are involved in depotentiation in CA1 (Bashir and Collingridge, 1994); mGluRs agonists have been shown to induce LTD in CA1

(Fitzjohn et al., 2001), in the dentate gyrus (O'Mara et al.,1995a; Huang et al., 1999) and in Prh (McCaffery et al., 1999).

By far, the majority of AMPAR are impermeable to Ca^{2+}, due to the editing of the mRNA of the glutamate receptor subunit 2 (GluR2). However, LTD in CA3 has been shown to require co-activation of mGluRs and Ca^{2+} permeable AMPA (Laezza et al., 1999). Increasing evidence suggests the pivotal role of AMPA trafficking in LTD. Activity-dependent internalization of AMPA receptors is central for recognition memory and LTD induction in Prh (Griffiths et al., 2008).

KAR involvement in LTD is not yet entirely demonstrated, but it's suggested by the observation that the co-application of the mGluR antagonist MCPG and the AMPA/KA receptor antagonist CNQX inhibits LTD induction in the hippocampus (reviewed by Kemp and Bashir, 2001).

The application of LFS consisting of 3000 pulses delivered at a frequency of 5 Hz in Prh slices of juvenile rats resulted in M1-dependent LTD (Jo et al., 2006); in addition, the bath application of the acetylcholine analogue carbachol (Cch) was shown to induce chemical M1-dependent LTD in Prh slices of adult rats (Massey et al., 2001).

The central intracellular event for LTD induction is the cytosolic Ca^{2+} concentration increase, as observed for LTP (Lynch et al., 1983; Bliss and Collingridge, 1993): LTP-inducing stimuli determine rapid and high increases in intracellular Ca^{2+}, while for LTD the increase is low and slow (Lisman, 1989). Presynaptic Ca^{2+} increase is also necessary for LTD induction (Kobayashi et al., 1996, 1999). The source for the increase of post-synaptic Ca^{2+} can be both extracellular and intracellular. In the first case, it relies on the activation of NMDAR, Ca^{2+} permeable AMPAR or voltage-gated Ca^{2+} channels (VGCC; Cummings et al., 1996; Christie et al., 1997; Wang et al., 1997 a; Norris et al.,

1998; Otani and Condor, 1998). In the second case, Ca^{2+} comes from intracellular stores as a consequence of the activation of G_q-coupled receptors like group I mGluRs or M1.

The increase in intracellular Ca^{2+} determines the formation of the Ca^{2+}/calmodulin (CaM) complex activating Ca^{2+}/CaM-dependent phosphatase calcineurin. This happens if the Ca^{2+} increase is low and slow. LFS-dependent calcineurin activation inactivates inhibitor 1, by dephosphorylation. This results in the activation of phosphatase 1 and 2 (PP1/2) which results in LTD via dephosphorylation of various targets such as AMPAR and CaMKII (reviewed by Kemp and Bashir, 2001; Fig 1.4.).

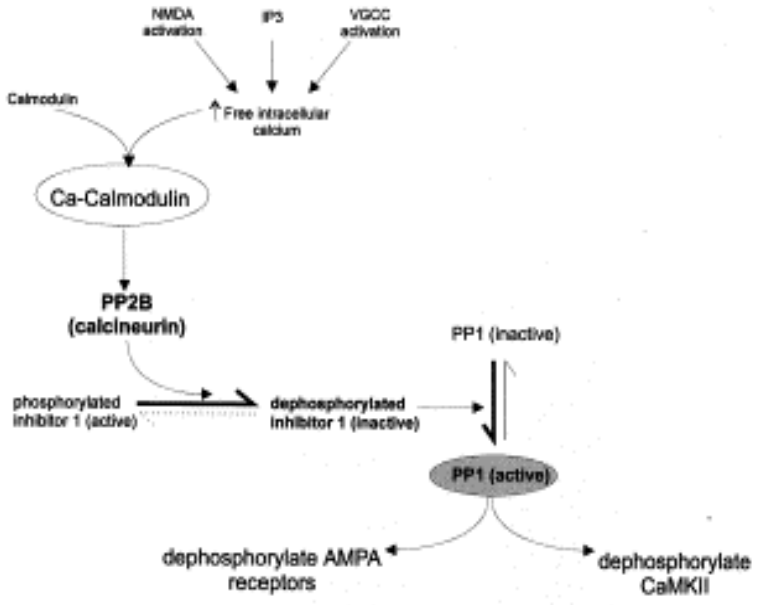


Fig 1.4. LTD induction requires increased intracellular calcium concentration from the extracellular space via NMDAR or VGCC activation or IP_3-mediated opening of intracellular stores. The Ca^{2+}-calmodulin complex activates the protein phosphatase calcineurin that dephosphorylates the phosphatase inhibitor 1 inactivating it. This enables the activation of protein phosphatase 1 (PP1) that dephosphorylates targets like CaMKII and AMPA receptors (Modified from Kemp and Bashir, 2001).

In the last three decades, LTD expression mechanisms have been deeply investigated in various brain regions. It was concluded that different induction mechanisms determine different expression mechanisms. Hippocampal NMDAR-LTD in CA1 relies on the increased internalization of AMPAR via

dephosphorylation of Ser-845 (target of PKA) and Ser-831 (target of CaMKII) of the GluR1 subunit, altering the conductance and the probability of opening of AMPARs. In addition, LFS determines increased internalization of postsynaptic AMPARs through a dynamin-dependent, clathrin-mediated process (Lüscher et al., 1999; Lüthi et al., 1999; Carroll et al., 1999; Man et al., 2000; Beattie et al., 2000; Wang and Linden, 2000; Henley, 2003; Collingridge et al., 2004; Griffiths et al., 2008). In specific, it has been shown that AP2, a clathrin adaptor protein, is important for the internalization of surface AMPARs and for the expression of NMDAR-dependent LTD (Lee et al., 2002).

The last event that temporally characterizes LTD is a change in protein synthesis, which is essential for mGluR-dependent LTD in CA1 (Huber et al., 2000) and for Cch-induced LTD in Prh (Massey et al., 2001): LTD induction has been shown to correspond to long-term changes in spine morphology, specifically in a reduction in spine density (Halpain et al., 1998). Fig 1.5. illustrates the main LTD induction pathways and related post-synaptic changes.

It is of interest to note that the immature form of BDNF, proBDNF, has been demonstrated to be involved in LTD induction via activation of the pan-neurotrophin receptor 75 (p75NTR) (Woo et al., 2005; Rösch et al., 2005).

1.2.3. Biological relevance of LTP and LTD

Many studies showed that both LTP and LTD can be induced in the same pathway depending on the frequency of firing of the presynaptic fibres (Bliss and Collingridge, 1993; Bear and Malenka, 1994).

Certain properties of LTP and LTD provide support to the hypothesis that they underlie complex mnemonic processes. Firstly, synapses can be independently modified: only co-active synapses participate in the plastic change, i.e. stimulation does not affect surrounding synapses, thus is input-specific. This evidence

suggests that every single synapse can be individually used to store information. Since every neuron is surrounded by about 10,000 synapses and brain areas involved in learning and memory contain billions of neurons, the mnemonic capacity of the brain is extraordinary. Secondly, LTP and LTD are associative phenomena: this property allows for small changes in certain synapses in order to produce a distributed storage in a complex and organic memory within a neuronal network. This implies that LTP and LTD cannot be triggered by the activity of a single neuron, but only as a consequence of associate activation of many inputs. This functional need comes from the fact that both LTP and LTD induction require a sufficient degree of depolarization of the postsynaptic membrane (Bilkey, 1996; reviewed by Diamond and Rose, 1994). LTP and LTD have a reciprocal connection in the generation of stored memories. LTP is associated with the storage of new information, but, since the brain represents a system with limited capacity, soon the newly stored information would reach a saturation point, destabilizing the system and making the recollection more challenging. LTD has the role to improve the signal/noise ratio associated to the acquisition and recollection of newly stored information (reviewed by Rosenzweig and Barnes, 2003; Fig 1.6).

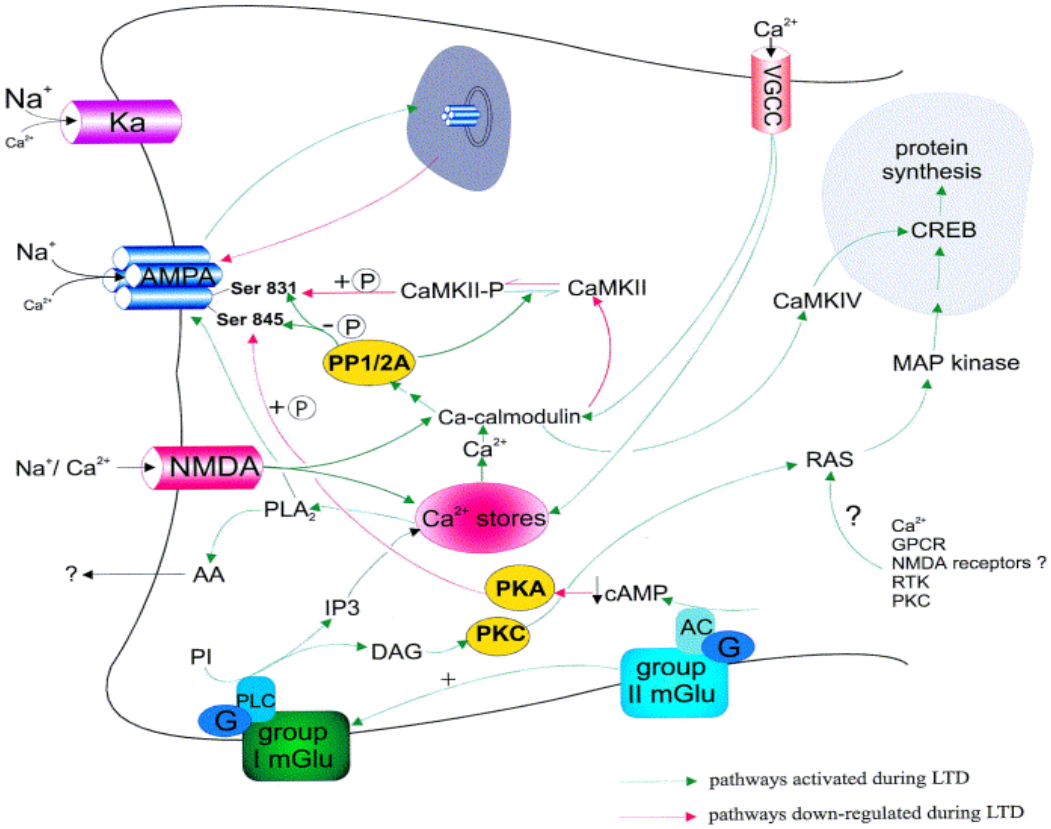


Fig 1.5. Schematic representation of the main LTD induction pathways. Abbreviations: AC, adenylate cyclase; AA, arachidonic acid; CaMKII, calcium/calmodulin-dependent kinase II; CREB, cAMP responsive element binding protein; IP_3, inositol trisphosphate; Ka, kainate receptor; mGluR, metabotropic glutamate receptor; MAPK, mitogen activated protein kinase; PI, phosphatidil inositol; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PP1/2 A, protein phosphatase 1/2 A; TKR, tyrosine kinase receptor (Modified from Blitzer et al., 2004).

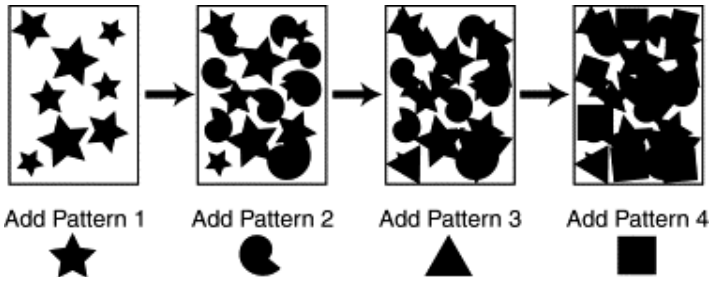


Fig 1.6. Schematic representation of an information storage system with limited capacity. If we assume that a neural network coincides with a sheet of white paper, we can draw on it different shapes, coincident to new encoded patterns of neural activity. If the network contains too much information it becomes useless. Acquisition of new shapes/patterns is mediated by LTP. If the system keeps accumulating patterns one on another, soon every single shape becomes indistinguishable from others: recollection is therefore impossible and most of the information will be lost. The role of the LTD is to organize the patterns and select the stronger ones to increase memory capacity and recollection (Rosenzweig and Barnes, 2003).

1.3. Perirhinal cortex (Prh): anatomy and functions

At the beginning of the 1980's, the systematic exploration of amnesia in animal models lead to the identification of cortical structures involved in declarative memory formation; these were primarily located in the medial temporal lobe, comprising the hippocampus, dentate gyrus, subiculum, entorhinal cortex (EC), parahippocampal cortex (Prp) and Prh (Zola-Morgan et al., 1986; reviewed by Squire and Zola-Morgan, 1991). Prh is a periallocortex, located on the ventral surface of the temporal lobe. In both primates and rodents, Prh comprises two cytoarchitectionally different regions: 35 and 36 Brodman areas (Fig. 1.8.). Area 35 is thin and agranular (IV layer is missing) and it is located ventrally to the rhinal sulcus; area 36 contains a thin IV layer, in which granular cells are mixed with pyramidal cells from III and V layer (Burwell, 2001).

Prh has strong reciprocal connections with the hippocampus and the subiculum. These connections are both direct through the lateral perforant path, and indirect through EC (Deacon et al., 1983; Burwell et al., 1995; Liu and Bilkey, 1996; Naber et al., 1999). Furthermore, Prh receives both unimodal inputs from associative unimodal cortices (i.e. visual, somatosensory, auditory and olfactive) and multimodal inputs from associative multimodal cortices (i.e. medial and ventro-lateral prefrontal cortex, cingulate anterior area, retrosplenial cortex; Fig 1.7). Visual information, transmitted from unimodal associative visual cortex, is then transmitted to the Prh (Meunier et al., 1993; Wiig and Bilkey, 1995; Ennaceur and Aggleton, 1997). Since a few years ago, Prh was merely considered a structure where sensory information was transmitted from sensory related cortices to the hippocampal formation, without ascribing any role in memory formation. Since the early 1990's many studies highlighted that Prh interacts, directly or via integration with other brain areas, for various mnemonic functions, with a crucial role in visual

recognition memory (Buffalo et al., 1998; reviewed by Brown and Aggleton, 2001; Massey et al., 2001; Warburton et al., 2003; Massey et al., 2008) and fear conditioning (Corodimas and LeDoux, 1995). In the last decade many studies clarified the cellular and molecular mechanisms underlying Prh-dependent visual recognition memory.

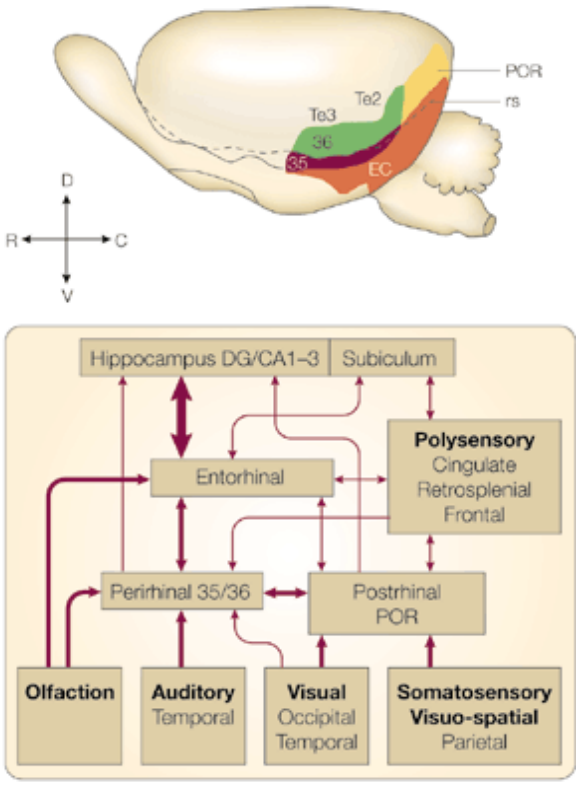


Fig 1.7. Location of the Prh in the rat brain and schematic representation of connections to and from other brain areas. Rat Prh network is characterized by 3 main connections. The first one is a strong reciprocal connection with the hippocampal formation through lateral EC (Suzuki and Amaral, 1994). The second one is characterized by afferent projections from unimodal and multimodal associative cortices, including visual, somatosensory, auditory and olfactory associative areas, medial and ventro-lateral prefrontal cortex, anterior cingulus and retrosplenial and Prp cortices (Burwell et al., 1995). The third one is characterized by the reciprocal connection with the amigdaloid complex. The thickness of the arrows indicates the connection density (Modified from Brown and Aggleton, 2001).

1.3.1.Perirhinal cortex and visual recognition memory

Recognition memory is a primary aspect of our ability to remember. It is based on the capability of both identifying and judging the prior occurrence of a visually perceived experience (Mandler et al., 1980).

Recognition memory is a main component of the sort of memory lost in anterograde amnesia. However, it has been proposed that within the two main components of recognition memory, identification and judgement of prior occurence, only the latter is directly compromised in anterograde amnesia (Mandler et al., 1980; Jacoby et al., 1981; Gardiner et al., 1990; Aggleton and Brown, 1999).

The visual component of recognition memory corresponds to the sensation of familiarity of a visual stimulus without active recollection of the attributes of that vision. This discrimination of familiarity is defined as 'knowing' (I know I've seen this thing before) whereas the active recollection of the attributes is defined as 'remembering' (I actively remember the name of the object, what the material is made of, where I've seen it before etc.). There are two main models describing recognition memory. The first model is the single process model. In this model 'knowing' and 'remembering' are considered a single process that differ only quantitatively: knowing corresponds to a weaker mnemonic trace whereas remembering is related to recollection and hence is related to a stronger mnemonic pattern. The second model is the dual process model, according to which 'knowing' and 'remembering' are two qualitatively distinct processes mediated by two distinct structures. In the dual model the hippocampus is usually related to 'remembering' because of its structural complexity and the many lines of evidence showing its role in recollection; on the other hand Prh has been shown to

be selectively involved in recognition memory. So far, the dual model is the most accepted (reviewed by Brown and Aggleton, 2001).

Prh is critically involved in recognition memory as shown by several lesion studies in both primates and rodents (Zola-Morgan et al., 1989; Meunier et al., 1993; Mumby et al., 1994; Meunier et al., 1996; Winters et al., 2004). Other evidence highlighting the pivotal role of Prh in visual recognition memory comes from electrophysiological recordings from the medial temporal lobe of monkeys performing recognition memory tasks (Brown and Wilson, 1987; Fahi et al., 1993; Li et al., 1993; Miller et al., 1993; Xiang et al., 1998). These studies consistently showed a decrease in neuronal responsiveness subsequent to the presentation of a previously encountered visual stimulus (Brown, 1996; Desimone, 1996; Eichenbaum et al., 1996; Ringo, 1996; Brown and Xiang, 1998; Suzuki and Eichenbaum, 2000; Eichenbaum, 2000). The decrease in the overall neuronal responses brings into account information about prior occurrence of a stimulus mediating the discrimination between familiarity or recency of that stimulus (Miller et al., 1993; Sobotka and Ringo, 1993; Xiang and Brown, 1998). These response reductions are observed in Prh (in ~25% of the recorded neurons) and rarely in the hippocampus (<~1%) (Brown and Wilson, 1987; Miller et al., 1993; Sobotka and Ringo, 1993; Xiang and Brown, 1998). In the last ten years many studies have aided in the clarification of the molecular mechanisms underlying the acquisition, consolidation and recollection of visual recognition memory. Three main approaches have been followed, primarily conducted in rats: 1) behavioural measures in animals with bilateral cannula into Prh: the local infusion of drugs or transfecting viruses in the Prh and the consequent deficit or enhanced performance in an object recognition task has teased about the roles of various membrane receptors and associated cellular signalling pathways involved in

recognition memory processes. The most applied behavioural protocol used to investigate visual recognition memory in rats is the spontaneous novel object exploration task, described in section 1.3.1.1; 2) Electrophysiological recordings on acute brain slices: the measure of basal synaptic transmission and synaptic plasticity in Prh slices has helped to define which are the cellular correlates of recognition memory. This *in vitro* approach can be carried out on: i) naive slices treated with various drugs ii) slices from transgenic animals iii) slices from animals locally transfected in Prh with viruses inducing a genetical modification, and finally iv) slices from animals that underwent the paired-viewing protocol (described in Brown and Aggleton 2001). This protocol consists in keeping the animal's head in a window facing a screen showing two different images. The two images are projected in a manner as to occupy the monocular visual field of each eye independently. Sets of images are then repetitively projected. At the end of a period of training, a set of novel images are projected towards only one eye, in order to encode novelty in one hemisphere and familiarity in the other. Note that the visual information perceived in one eye is processed in the contralateral hemisphere of the brain. This treatment results in one of the hemispheres being conditioned with 'familiar' and the other hemisphere with 'novel' images respectively (Fig 1.8.). Upon surgical removal of these hemispheres, electrophysiological experiments (Warburton et al., 2003; Massey et al., 2008) and immunohistochemical studies can be performed in order to evaluate how the encoding of familiar or novel information influences neuronal activity. 3) Immunohistochemical imaging has been used for the evaluation of molecular changes in the Prh of rats that underwent the paired-viewing protocol; it can be examined in a quantitative manner by analysing the differential expression of

genes, e.g. the immediate early gene (IEG) c-fos levels in the 'novel' versus the 'familiar' hemisphere.

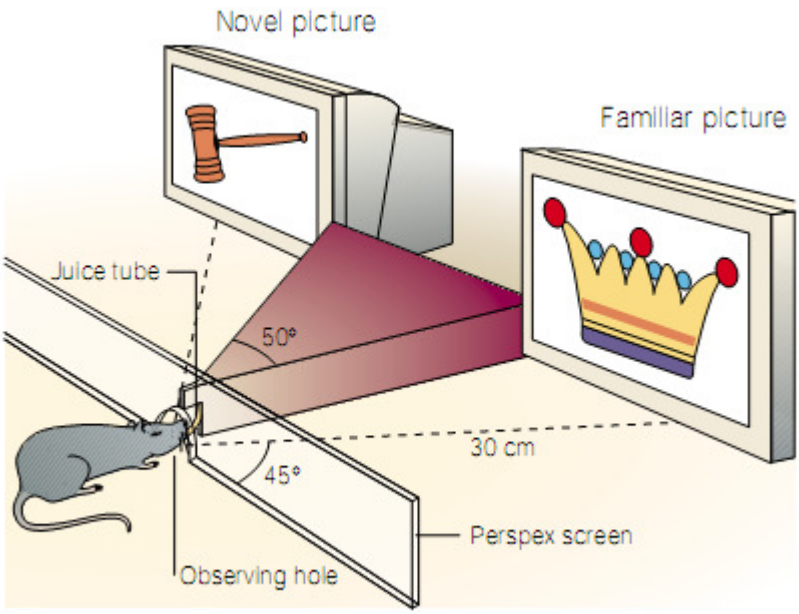


Fig 1.8. In the paired-viewing protocol, a rat is exposed to two pictures simultaneously, one novel and one familiar. Each figure is projected on a screen in order to occupy the visual field of only one eye and it is therefore selectively processed in the contralateral hemisphere (Modified from Brown and Aggleton, 2001).

1.3.1.1. Perirhinal cortex and visual recognition memory: behavioural studies

In order to better understand the cellular correlates underlying visual recognition memory formation, many studies evaluated the visual recognition performance with the spontaneous novel object exploration task on intra-Prh cannulated rats. This test consists of a training phase where the animal is placed in an arena with two identical objects where it is then left to explore. In a second phase of the test, a delay is introduced that varies between minutes to hours (2 min to 24 h). During the test phase, the animal is placed back into the same arena with one familiar and one novel object: if the animal remembers the familiar object, it would preferentially explore the novel one due to innate behavioural patterns; if not, it would explore the novel and familiar objects equally (Warburton et al.,

2003). The infusion technique can be used to analyse different stages of memory formation according to the different stage of the experiment when the drug is applied: i) acquisition: if the drug is infused before the training phase ii) consolidation: after the training phase iii) retrieval before the test phase (Ennaceur and Delacour, 1988; reviewed by Brown et al., 2010; Warburton et al., 2003).

The reversible inactivation of Prh with the glutamatergic AMPAR antagonist CNQX has been shown to impair both acquisition and retrieval of visual recognition memory in the spontaneous novel object preference task (Winters and Bussey, 2005). In addition, the selective blockade of NMDAR or group I and II mGluRs produced an impairment at longer (24 h) but not at shorter (20 min) delays (Barker et al., 2006a,b), when the drug is applied before the training phase. This treatment however did not affect retrieval. The blockade of glutamatergic KAR produced deficits after a 20 min delay but not after 24 hours (Barker et al., 2006b). This unusual temporal pattern (amnesia and then remembering) can be perhaps explained because some neurons (called 'familiarity' or 'slow change neurons') in the Prh of monkeys responded less strongly if a novel stimulus was repeated after a certain amount of times (min to h); if the delay was shorter they kept responding strongly (Xiang and Brown, 1998). Other neurons have reduced responses in monkey even when stimuli are repeated after very short delays (even <1 s; Miller et al., 1993). Thus, two different populations of neurons within Prh provide the information of prior occurence of the stimulus at different time delays (20 min and 24 h). In both cases AMPAR-dependent transmission seems to be essential for recognition memory, while NMDAR blockade induces a deficit in acquisition at longer delays and KAR at shorter delays.

A similar pharmacological dissociation was also observed as a consequence of the antagonism of muscarinic or nicotinic receptors. The muscarinic antagonist

scopolamine was shown to impair visual recognition memory at 20 minutes but not at 24 hours; the opposite pattern was observed with the α 7 nicotinic antagonist MLA (Tinsley et al., submitted). Therefore, the muscarinic receptor and the KAR appear to be involved in a form of acquisition that takes place in the first 20 min, without however affecting the performance at longer delays.

The role of cholinergic transmission in visual recognition has been deeply investigated and many studies highlight its pivotal role in this form of memory. It has been demonstrated that scopolamine partially impairs object recognition memory at 24 hours (Winters and Bussey, 2005). The selective targeting of basal forebrain cholinergic fibres with the immunotoxin 192 IgG-saporin caused a permanent cholinergic denervation of fibres targeting the Prh, impairing spontaneous novel object exploration in rats (Winters and Bussey, 2005). Also, it has been shown that scopolamine affects both visual recognition memory and synaptic plasticity in Prh: systemic injections or intra-Prh infusions of scopolamine before the test phase in the spontaneous object recognition memory task significantly impaired object recognition memory at 15-20 min delays, and disruptions in the decremental responses to familiar versus novel pictures in Prh neurons as measured with Fos expression were observed. Furthermore, bath application of scopolamine prevented *in vitro* LTD but not LTP in Prh slices (Warburton et al., 2003; Massey et al., 2001). Another study showed that intra-Prh infusion of scopolamine disrupted recognition memory at 24 h whereas infusion before the retrieval phase improved the performance, an effect probably due to the elimination of interferences coming from cholinergic fibres in the retrieval phase (Winters et al., 2006; Winters et al., 2007). To sum up, if AMPARs mediate several aspects of acquisition, consolidation and retrieval, NMDARs are considered crucial for consolidation and acquisition, whereas muscarinic cholinergic transmission in

Prh seems to be selectively involved in acquisiton (reviewed by Winters et al., 2008).

Acquisition is also influenced by the intra-Prh infusion of lorazepam (Brown and Brown, 1990; Wan et al., 2004) and L-type voltage-dependent calcium channel blockers (verapamil, diltiazem, nifedipine; Seoane et al., 2009).

Interfering with intracellular signalling pathways that play an important role in Prh synaptic plasticity produces recognition memory impairments: recognition memory is impaired at 24 hours by blocking CaMKII (Tinsley et al., 2009) while blocking CaMKK has been shown to impair consolidation (Tinsley et al., 2011). Of interest, blocking BDNF expression via intra-Prh infusion of antisense oligodeoxynucleotides blocks visual recognition memory acquisition tested at 24 h (Seoane et al., 2010).

Viral transduction of Prh neurons prevented the phosphorylated CREB (pCREB)-mediated signalling, which impaired visual recognition at 24 hours but not at 20 min (Warburton et al., 2005). Another recent study showed that viral transduction of Prh neurons with a lentivirus expressing a peptide considered able to block AMPAR internalization, impaired visual recognition at both 24 hours and 5 min (Griffiths et al., 2008). When the Prh is conditioned by direct infusion of a transfecting virus, it is not possible to distinguish if the deficit seen is related to impairments in acquisition, consolidation or retrieval, since the transfection is a stable modification and requires days after the infusion to be expressed. Nonetheless, the advantage of evaluating memory deficits in an animal model locally transfected with a virus remains in the ability to evaluate how the expression of a gene, or the integrity of a pathway in a specific brain region, affects memory in a wild-type animal. The only alternative would consist in a transgenic animal model, which carries a mutation affecting every tissue of the

organism and therefore all brain areas. The non specificity of the transgene expression could possibly affect the experiment in a way not directly related to the functions of the brain area under examination.

1.3.1.2. Perirhinal cortex and visual recognition memory: electrophysiological recordings

As previously described (section 1.2), synaptic plasticity is thought to represent the cellular correlate of memory and learning. In order to better clarify the cellular and molecular mechanisms underlying visual recognition memory, *in vitro* electrophysiological recordings on Prh acute slices were carried out by several research groups, providing a collection of data complementing and enriching behavioural observations already described in section 1.3.1.1.

Both LTD and LTP can be observed in Prh after the application of the appropriate stimulation protocol (Bilkey, 1996; Ziakopoulos et al., 1999; Cho et al., 2000; Massey et al., 2001, 2004; Aicardi et al., 2004). Input specific LTP in Prh is NMDAR dependent (Bilkey, 1996). Also, LTP induction is strictly layer dependent: HFS (100 Hz) of the superficial layer does not cause LTP induction, whereas stimulation of layer II/III induces a robust LTP in the Prh and is strictly NMDAR-dependent (Ziakopoulos et al., 1999). Further studies have shown that the NR2A subunit containing NMDAR is necessary for LTP induction in Prh, whilst NR2B is deemed necessary in LTD induction alone (Massey et al., 2004). In addition, LTP induction in Prh requires BDNF-mediated activation of TrkB and the application of HFS (100 Hz) determines an activity-dependent increase of the basal secretion of BDNF in horizontal Prh slices (Aicardi et al., 2004). Finally, inhibition of pCREB-mediated signalling blocks LTP induction in Prh (Warburton et al., 2005).

LTP may be involved in refining stabilized patterns in Prh network: such synaptic changes may be necessary for long-term maintenance of visual

information, essential for familiarity discrimination processing: note that NMDAR activity (Barker et al., 2006a) and pCREB signalling (Warburton et al., 2005) are necessary for acquisition of visual recognition memory with a delay of 24 hours but not of 20 min, suggesting that LTP may indeed have a role as the cellular correlate of this acquisition process. Assuming that LTP plays a role in visual recognition memory, we have to consider that electrophysiological recordings in the Prh of animals performing visual recognition tasks showed a decrease in the neuronal responsiveness (Brown and Wilson, 1987; Fahi et al., 1993; Li et al., 1993; Miller et al., 1993; Xiang et al., 1998). Thus, LTD may well overrule the importance of LTP as cellular correlate of visual recognition memory. Some elegant computational studies show how LTD could represent, in an *in vitro* model, the decreased long-term neuronal responsiveness necessary to encode the information concerning familiarity (Brown and Bashir, 2002). Supporting this hypothesis, a recent study showed that both LTD and depotentiation are prevented in Prh slices obtained from the 'familiar' brain hemisphere of an animal that underwent the paired-viewing protocol. Interestingly, the possibility to induce both LTD and depotentiation was restored in this hemisphere by bath application of the muscarinic antagonist scopolamine, indicating a pivotal role for cholinergic neurotransmission in both visual recognition memory and synaptic plasticity in Prh (Massey et al., 2008). Several other studies have focused on the cellular mechanisms involved in LTD induction and expression in the Prh, leading to interesting results. LTD in Prh can be induced with both pharmacological (Massey et al., 2001) and electrical stimulation (Cho et al., 2000; Ziakopoulos et al., 2000); its induction relies on the activation of NMDAR, mGluRs, KAR, muscarinic receptor 1 (M1), depending on age, excitation level of the network and stimulation protocol. As observed for LTP, one form of LTD requires glutamate receptors: at

variance with other brain areas, LTD in Prh requires both mGluRs and NMDAR activation (Cho and Bashir, 2002; Cho et al., 2000; McCaffery et al., 1999). An elegant study by Cho et al. (2000) showed that the activation of both group I and II mGluRs in combination with the co-activaction of NMDAR may be necessary for LTD induction in Prh slices. This mechanism is voltage dependent: in resting conditions (-70 mV), the application of LFS (1 Hz) induced LTD relying on the co-activation of group I and II mGluRs and NMDAR, whereas depolarization of the postsynaptic membrane (-40 mV) generated the condition where LTD induction required only group I mGluRs and NMDAR co-activation. From these results, it was suggested that in resting conditions the Ca^{2+} influx via NMDAR activation and intracellular Ca^{2+} mobilization after group I mGluR activation is insufficient for LTD induction, requiring the contemporary activation of group II mGluRs. In depolarized conditions, NMDAR are sufficiently activated in order to allow through enough Ca^{2+} influx to trigger the molecular machinery necessary for LTD induction (Cho et al., 2000). It has also been shown that LTD of KAR-dependent synaptic transmission (that is different from AMPAR-dependent synaptic transmission) requires mGluR5 activation, increased intracellular Ca^{2+} from internal stores, PKC activation, protein interacting with kinase C 1 (PICK1) and PDZ domain interactions (Park et al., 2006). Another study showed that application of LFS consisting of 3000 pulses delivered at 5 Hz induces a robust LTD in Prh relying on the activation of mGluR5 in neonatal rats (p7-12). This induction mechanism undergoes a developmental switch in juvenile rats (p28-35) where the same stimulation protocol induces LTD relying on the activation of M1. This molecular switch is mediated by sensory information resulting from the opening of the eyes and relies on the subsequent increase in M1 expression; in fact, M1 is poorly expressed in neonatal rats Prh. Both M1 expression in Prh and the switch from mGluR5 to M1 dependency of the

LFS 5Hz-induced LTD are blocked if neonatal rats are kept in the dark during development (Jo et al., 2006). This introduces another important factor for the induction of LTD: the cholinergic pathway. As previously discussed, cholinergic neurotransmission appears to play a pivotal role in visual recognition memory (see section 1.3.1.1). It has been shown that bath application of the cholinergic agonist charbachol (Cch) at 50 μM for 10 minutes in Prh slices induces robust LTD relying on M1 activation, Ca^{2+} release from intracellular stores and protein synthesis, and is independent from PKC and protein phosphatase activation (Massey et al., 2001). Cholinergic and glutamatergic dependent synaptic plasticity within Prh are probably both synergistic and independent in influencing different facets of object recognition memory processes (Massey et al., 2008). Furthermore, it has been shown that L-type VGCC are involved in both LTD and depotentiation but not in LTP (Seoane et al., 2009).

Recent studies tried to clarify the cellular processes involved in LTD induction downstream to the activation of different receptors. It was found that LTD in neonatal rats (p7-13) can rely on the activation of NMDAR or mGluR5 depending on the stimulation protocol applied. NMDAR-LTD is induced by application of LFS (1 Hz) in depolarized neurons (-40 mV) in Prh slices, while mGlu-LTD is induced by 5Hz-LFS. In both cases an increase in cytosolic Ca^{2+} concentration is required: Ca^{2+} acts as a second messenger, activating different Ca^{2+} sensors: NMDAR-LTD requires calmodulin, mGluR-LTD requires neuronal cell sensor protein 1 (NCS-1) that binds to PICK-1 through its bar domain. NCS-1/PICK-1 and PKC activation has been shown to be strictly involved in mGluR LTD (Jo et al., 2008). Furthermore, it has been demonstrated that the internalization of AMPARs is necessary for 5Hz LTD induction in Prh of adult animals (7-12 weeks). In this study adult rats were intra-Prh transfected with a recombinant lentivirus expressing

a peptide which blocks the interaction between the AMPAR subunit GluR2 and the clathrin adaptor protein 2 (AP2), necessary for endocytosis. These animals were both impaired in visual recognition memory and in LTD induction, but LTP induction was normal (Griffiths et al., 2008). Notice that in these animals 5Hz LTD was NMDAR dependent and not M1 dependent as observed in juvenile (p28-35) rats by Jo et al. (2006), suggesting a further developmental switch between adolescence and adulthood. Finally, it has been shown that both 1 Hz LFS and 5 Hz LFS determine an activity-dependent decrease in the basal secretion of BDNF in juvenile rat Prh slices (Aicardi et al., 2004).

These evidences strongly confirm the role of LTD as a plausible *in vitro* model for visual recognition memory acquisition, although LTP may play a role as well, possibly in encoding long-term modifications necessary for familiarity discrimination.

1.3.1.3. Perirhinal cortex and visual recognition memory: immunohistochemical studies

The study of visual recognition memory and its proposed cellular correlate, LTD in Prh, has been extended to the investigation of the molecular mechanisms underlying these processes. The basic question was: which changes in gene expression are induced by acquisition and consolidation of a visual recognition memory or by protocols inducing synaptic plasticity? The expression of C-fos, an IEG mainly expressed in activated neurons, was used as an indicator of neuronal activity (Dragunow et al., 1996; Herdegen et al., 1998): interestingly, neurons were found to be activated in the Prh by the novel rather than by the familiar stimulus (reviewed by Brown and Aggleton, 2001), and intra-Prh infusion of scopolamine blocked the decrease in c-fos expression observed in the 'familiar' hemisphere (Warburton et al., 2003); a similar effect was observed with the L-type VGCC

antagonist verapamil (Seoane et al., 2009), or by blocking the pCREB signalling pathway (Warburton et al., 2005).

The results coming from this technique, together with behavioural measures and electrophysiological recordings, have so far confirmed the association between visual recognition memory and neuronal activity in the Prh, expressed as a decrease in neuronal responsiveness in the Prh network. LTD, the best *in vitro* model underlying this cognitive function, is induced by the activation of different neurotransmitter receptors depending on the state of excitation of the network, the developmental stage and the induction protocol. These phenomena also involve changes in IEG expression; in particular, visual familiarity is encoded by the decreased levels of c-fos expression within Prh neurons.

1.4. Nitric oxide (NO)

NO is a ubiquitous amphiphilic highly diffusible molecule, synthesized intracellularly. It is a free radical, and because of its instability and ability to freely diffuse through the plasma membrane it mainly acts as a paracrine modulator. NO is involved in many physiological and pathological processes. It emerged as a neuronal messenger about 20 years ago, when searches focused on endogenous modulators showed that NMDAR activation caused increases in cGMP concentration in surrounding neurons (reviewed by Garthwaite, 2008). NO is involved in the regulation of peripheral organs (digestive, urogenital, respiratory) through nitrergic nerves, and acts as a modulator mediating relaxation of smooth muscle tissue (reviewed by Rand and Li, 1995; Toda and Okamura, 2003; Toda and Herman, 2005). It plays a major role in the regulation of blood flow: it is produced from endocytes and it primarily acts within smooth muscle tissue of blood vessels as a vasodilator. In addition, it is produced by many blood cells involved in immunity such as neutrophiles, macrophages and circulating

monocytes, eosinophiles and even platelets: it is involved in both the physiological regulation of the metabolism of these cells and as an inflammatory mediator. In the vertebrate CNS, NO-dependent transmission is involved in several neuronal functions and complex behaviours such as learning and memory formation, sensory and motor function, sleeping, feeding, and reproductive behaviours. Indeed, NO-dependent transmission is highly conserved in evolution as a modulator of behaviour. For instance, NO synthesis in the jellyfish induces cGMP production via activation of a receptor very similar to the mammalian soluble guanilate cyclase (sGC), and cGMP plays a role in swimming patterns associated with feeding (Moroz et al., 2004). In molluscs and insects, NO mediates olfactive, feeding and learning related behaviours (reviewed by Davies, 2000). In the last few years many studies clarified the role of NO in vertebrate CNS functions like neurogenesis, neural development and differentiation (Mize and Lo, 2000; Contestabile and Ciani, 2004; Estrada and Murillo-Carretero, 2005), memory and learning (Susswein et al., 2004), and neuropathology (Contestabile et al, 2003). In the sections to come, I have reviewed NO-dependent transmission and its importance in memory and learning.

1.4.1. Nitric oxide synthesis, receptors and downstream signalling

NO is intracellularly synthesized by a group of enzymes known as NO-synthases (NOS). NOS are complex enzymes expressed in 3 different isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). The first two isoforms (nNOS and eNOS) are constutively expressed in the CNS and they are mainly involved in physiological processes, whilst the third one (iNOS) is prototypically expressed in macrophages and glia in the CNS as a consequence of immunological activation. All three isoforms synthesise NO and L-citrulline from L-

arginine, but they have different structural and functional features (reviewed by Alderton et al., 2001; Stuehr et al., 2004).

nNOS was the first isoform to be cloned and purified (Bredt and Snyder, 1990; Bredt et al., 1991) and was first identified in the CNS, but it is widely expressed also in the peripheral nervous system and in skeletal muscles. nNOS is expressed in four splicing variants (α, β, γ and μ; see Fig 1.9.). The splice variant nNOSα is the most abundant in CNS, and is activated by Ca^{2+} associated to calmodulin. It is mainly localized at postsynaptic densities, where it binds the postsynaptic density protein 95 (PSD-95) through a PDZ domain located at the amino-terminal such as observed for the NMDAR subunit NR2B (Brenman et al., 1996). The physical association of nNOSα with NMDAR explains the preferential functional association between NMDAR activation and NO production (Garthwaite et al., 1988). nNOSβ does not have a PDZ domain so it shows less functional association with NMDAR activation (Gyurko et al., 2002). nNOSγ shows little or no enzymatic activity, while nNOSμ is similar to nNOSα with an insert around the calmodulin binding motif and is mainly expressed in skeletal muscles but also in the CNS where it represents 10% of total nNOS. nNOS has allosteric modulation sites other than Ca^{2+}/calmodulin binding sites. For instance, nNOS shows different putative phosphorylation sites for PKC, PKA, PKG and CaMKII, but they are characterized by mild effects on functionality (Nakane et al., 1991; Bredt et al., 1992; Dinerman et al., 1994). CaMKII is usually co-resident with NMDAR and nNOS at postsynaptic densities (Kennedy et al., 2000); it inhibits NO production by about 50%, via phosphorylation of nNOS at Ser-847, which most likely affects the binding with Ca^{2+}/calmodulin (Komeima et al., 2000). The phosphorylation process on Ser-847 takes about 15 min in cultured hippocampal neurons, suggesting that this system is a form of long-term control. NO increases the degree of nNOS

phosphorylation at low concentrations (5 μM) and decreases it at higher concentrations (100 μM; Rameau et al., 2004).

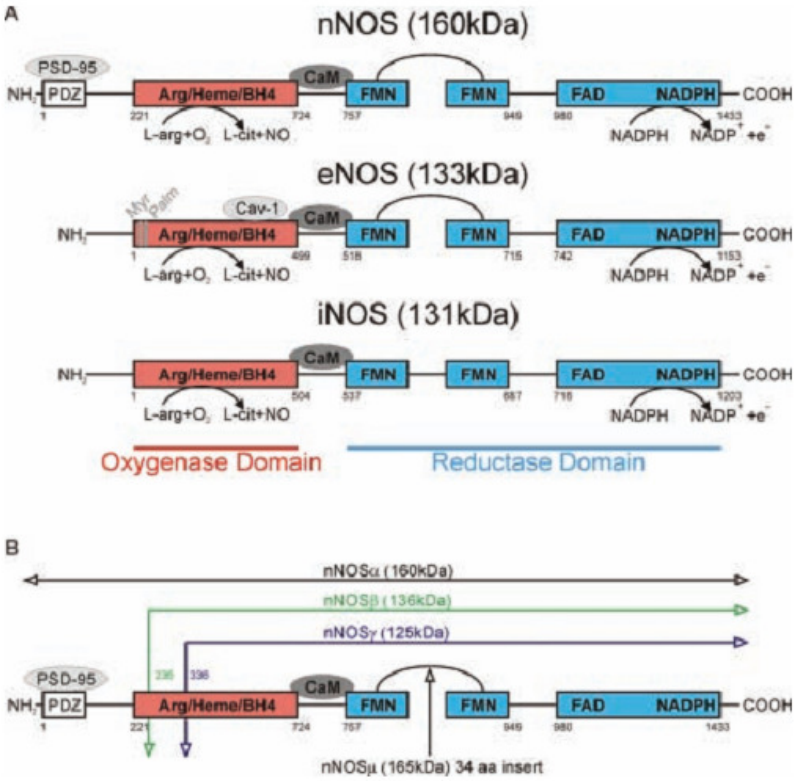


Fig 1.9. A. Schematic representation of the genes encoding for the three isoforms of the NOS. Note that only nNOS has the PDZ domain for interaction with PSD-95, responsible for the co-localization of the enzyme with the NMDAR. B. Schematic representation of the sequence differences between the three isoforms (Modified from Steinert et al., 2010).

Protein kinase Akt was found to phosphorylate nNOS on Ser-1412 in cultured hippocampal neurones (Rameau et al., 2007); this phosphorylation is NMDAR-dependent and it leads to an increase in nNOS activity. The dephosphorylation is subsequent to AMPAR and L-type VGCC activation. Phosphorylation at both Ser-847 and Ser-1412 also occur *in vivo* (Hayashi et al., 1999; Rameau et al., 2007). Other phoshorylation sites are summarised in Table 1. nNOS has also been shown to interact with other proteins that may influence its activity, such as the intra-membrane serotonin transporter, suggesting a possible coupling between serotonin uptake and NO production. Also, the inhibitory protein CAPON competes

with PSD-95 for the binding to the PDZ-domain, without directly affecting nNOS catalytic activity, but interfering with coupling of NMDAR activity with nNOS activation and its intracellular localization (Jaffrey et al., 2002). Some studies highlighted that nNOS activation can also be mediated by G_q-coupled receptors like group I mGluRs in the striatum (Calabresi et al., 1999) or M3/M1 in the rat retina (Borda et al., 2005).

eNOS is expressed in endocytes both peripherally and in the CNS (Seidal et al., 1997; Blackshaw et al., 2003), but many studies suggested that it is also expressed in astrocytes (Reviewed by Lin et al., 2007). As nNOS, eNOS is Ca^{2+}/calmodulin dependent. Notwithstanding the well known role of eNOS in blood flow regulation (reviewed by Toda and Okamura, 2003; Ortiz and Garvin, 2003), emerging evidence suggests that eNOS is also involved in vasculo-neuronal communication, exerting a key role in neuronal excitability as observed in the optic nerve (Garthwaite et al., 2006), neurogenesis (Chen et al., 2005) and synaptic plasticity (Bon and Garthwaite, 2003; Hopper and Gathwaite, 2006; Sergeeva et al., 2007). Control of eNOS is multifactorial (reviewed by Cirino et al., 2003): in endocytes eNOS is bound to plasma membrane through palmitoylation and it is localized in specialized invaginations called caveoli by interacting with caveolin-1 and the heat-shock protein 90 (hsp-90). Ca^{2+}/calmodulin association to eNOS determines dissociation from caveolin-1, which is necessary for eNOS activation. Hence, eNOS is usally tonically active in endocytes for the regulation of the smooth muscle tone of arterioles, both peripherally and in the CNS. The tonic activation of eNOS is due to the phosphorylation of Ser-1179 by the protein kinase Akt (a modification that is structurally and functionally comparable to the phosphorylation of Ser-1412 in nNOS) enabling eNOS activation at resting intracellular Ca^{2+} concentrations (reviewed by Garthwaite, 2008). In addition,

eNOS is regulated by NO itself, which inhibits its activity in resting endothelial cells via S-nitrosylation at Cys-94 and Cys-99 of the zinc tetrathiolate cluster, blocking eNOS dimerization (Erwin et al., 2005).

iNOS expression relies on inflammatory and disease conditions; expression is low in physiological conditions and it has little role in maintaining vascular tone (Ortiz and Garvin, 2003). iNOS activation determines NO production at micromolar levels (whereas eNOS and nNOS produce NO at nanomolar levels); it's a Ca^{2+} independent process involved in many pathological processes, comprising neurodegeneration (reviewed by Garthwaite, 2008).

The activation of the soluble guanylate cyclase (sGC) has been recognized as the main transduction pathway through which NO physiologically exerts its functions, even before the discovery that the so called "endothelial-derived relaxing factor" corresponds to NO (Arnold et al., 1977; Miki et al., 1977). Latter studies on NO showed the preminence of this mechanism in nitrergic transmission (Krumenacker et al., 2004). Even if sGC is the most commonly used name for this receptor, it does not have much meaning in a cellular context (Chrisman et al., 1975). It is more correct to define NO-receptors as enzyme-linked proteins, sometimes indirectly associated with membranes. As other receptors, NO-receptors have a ligand-binding domain and a transduction domain. The ligand-binding domain consists in a heme group similar to the one inserted in haemoglobin for molecular oxygen (O_2) binding; once incorporated in the NO-receptor protein, it shows a stunning preference for NO, notwithstanding the chemical similarity between the two ligands. The heme prosthetic group inserted in the protein component of NO-receptors allows cellular NO-dependent transmission in the presence of 100,000-fold higher O_2 concentration (Martin et al., 2006). The protein component of the NO-receptor is a αβ heterodimer expressed in two

isoforms: α1β1 and α2β1. Both are comprised of a heme-binding domain, a dimerization domain and a catalytic domain where the guanosine triphosphate (GTP) is converted into cyclic guanosine monophosphate (cGMP). In the ligand binding domain, the heme group is coordinated to the protein by a histidine bond (Zhao et al., 1998), whilst in the inactive state the catalytic domain (that is very similar to the catalytic site of adenyl-cyclase) is in an open state (Dessauer et al., 1999).

Phosphorylation Site	Effect on NOS	Kinase	Reported in
nNOS			
Ser-741	Inhibition	CamKI	nNOS transfected HEK cells
Ser-847	Inhibition	CamKII	Cultured cortical neurons, NG108-15
Thr-1296	Inhibition	PP1/PP2A inhibition	Neuroblastoma cells (NG108-15)
Ser-1412	Activation	Akt/PKB	Cultured cortical neurons
	Activation	ERβ-mediated Src kinase activation	Primary hypothalamic neurons
eNOS			
Tyr-81	Activation	Src kinase	BAEC
Ser-114	Inhibition	Enhanced association with Cav-1	BAEC
Thr-495	Inhibition	PKC	HCMVEC
Ser-615	Activation, modulatory action	PKA, Akt/PKB	BAEC
Ser-633	Activation	PKA	BAEC
Ser-1177	Activation	Akt/PKB, PKA, AMPK, PKG, PKC, CamKII	HUVEC, HCMVEC, human platelets

Fig 1.10. Different phosphorylation sites on the three isoforms of NOS, effects of the phosphorylation, kinases involved and cells in which the observations were made (Modified from Steinert et al., 2010).

The binding of NO to the heme group determines a pivot of the haem, ensuing dislocation of the histidine to which it is coordinated, inducing a conformational change that ultimately results in the closing of the catalytic domain and in the conversion of GTP into cGMP (Roy and Garthwaite, 2006). The two isoforms of

NO-receptors are both widely expressed in the CNS within different cell-types (Friebe and Koesling 2009) and subcellular localizations (Budworth et al., 1999; Szabadits et al., 2007). α2β1 is mainly localized at synapses, thanks to its association to PSD-95 through its PDZ domain (Russwurm et al., 2001) whereas α1β1 is mainly cytosolic but it can be transported near to the plasma membrane as a consequence of increased intracellular Ca^{2+}. Immunocytochemistry of NO-receptors (Ding et al., 2004) and of cGMP after neurons exposure to NO (deVente et al., 1998) underlies different levels of expression of this enzyme throughout the different areas of the CNS, that are complementary to nNOS expression. Different sites on NO-receptors have been identified as possible allosteric targets for the modulation of NO-cGMP signalling. In addition, many phosphorylation sites have also been identified on the NO-receptors, but the physiological role for such modifications has still to be clarified (reviewed by Pyriochou and Papapetropoulos, 2005). It is of particular interest a recent study that shows that activation of M2 receptors for acetylcholine on intestinal smooth muscle cells decreases cGMP production through Src kinase-dependent Tyr phosphorylation of the NO-receptor (Murthy, 2008).

cGMP actions are exerted by direct binding on the agonist or regulatory sites of cyclic nucleotide gated ion channels (CNG) (reviewed by Kaupp and Seifert, 2002) or hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channels (reviewed by Craven & Zagotta, 2006). Another action of cGMP is mediated by its binding to phosphodiesterases (PDE), a family of 11 enzymes responsible for the hydrolysis of cyclic nucleotides: most of them can bind and hydrolyse cGMP, in particular PDE 1, 2, 3, 5, 6, 9, 10 and 11 (reviewed by Bender and Beavo, 2006). PDE 2 and 5 once bound by cGMP have higher activity states with increased cGMP breakdown. The binding of cGMP to PDE3 (since this protein does not

hydrolyse it) results in decreased cAMP clearout and, in turn, potential increases in cAMP levels (reviewed by Bender and Beavo, 2006). PDE5 is mainly involved in hydrolysis of cGMP in cerebellum (Shimizu-Albergine et al., 2003) while PDE2 is mostly involved in cGMP hydrolysis in the hippocampus (van Staveren et al., 2001; Wykes et al., 2002; Boess et al., 2004). There is an availability of selective inhibitors for different isoforms of PDEs that are precious tools for investigating the functional meaning of nitrergic transmission in different systems, comprised learning and memory (reviewed by Blokland et al., 2006).

Activation of cGMP-dependent protein kinase (PKG) is the signalling system activated by cGMP mostly responsible for the NO-dependent cellular downstream signalling. There are three isoforms of PKG: PKG1a and PKG1b (splice variants) are derived from the expression of a gene located on human chromosome n° 10 and finally PKGII, whose gene is located on human chromosome n° 4. While PKG1a and PKG1b are principally expressed in the hippocampus, dorsal root ganglia, cerebellum and the olfactory bulb, PKGII (which is anchored to the plasma membrane through myristoilation) is expressed throughout the entire CNS (reviewed by Feil et al., 2005; Vaandrager et al., 2005; Hofmann et al., 2006). PKGI is a homodimer of 76 kDa and PKGII is an homodimer of 86 kDa. All the PKGs contain an amino-terminal domain with 5 regulatory sites: 1) a dimerization site 2) self-inhibition sites, involved in the inhibition of the catalytic domain in absence of cGMP 3) self-phosphorylation sites that in presence of cGMP increase the basal catalytic activity and the affinity of the PKG for cAMP 4) a site that regulates the affinity and cooperativity between the cGMP binding sites 5) an intracellular localization site. The regulatory domain of PKG contains two binding sites for cyclic nucleotides that mediate the full activation of the enzyme just after their full occupation by two cGMP molecules. The catalytic site at the carboxi-

terminal contains binding sites for Mg^{2+}, ATP and the target protein (reviewed by Domek-Lopacinska and Strosznajder, 2005). The main target substrates for PKG are phosphatases, leading to the variation of phosphorylation levels of effector proteins (reviewed by Schlossmann and Hofmann, 2005). cGMP was also found extracellularly in the brain with concentration levels changing according to NO production (reviewed by Vincent et al., 1998; Pepicelli et al., 2004). After intracellular synthesis, cGMP can be transported extracellularly via multidrug resistance proteins (reviewed by Sager, 2004) where it can possibly act as an intercellular messenger (Touyz et al., 1997; Poulopoulou & Nowak, 1998; Montoliu et al., 1999).

There is some evidence of cGMP independent NO-dependent transmission (Jacoby et al., 2001; Lev-Ram et al., 2002) suggesting other downstream signalling pathways triggered by NO. For example, it has been suggested that S-nitrosation (or nitrosylation) of thiol groups on cysteine residues can be an alternative signal transduction system. S-nitrosation is induced by exposing proteins to exogenous concentrations of NO (in the order of magnitude of μM) or nitrosating compounds of oxygen (as N_2O_3) or high concentrations of Ca^{2+} (reviewed by Hogg, 2002) but to date, there is no evidence of a physiological role for S-nitrosation dependent transmission. *In vivo*, S-nitrosation has been shown to be involved in pathological states (Zhang and Hogg, 2005). High concentrations of NO (in the order of magnitude of μM), deriving from iNOS expression and activation, can lead to the binding of NO to the ferrous haem on the cytochrome oxidase in mithocondria, causing the blockade of cellular respiration and exerting cytotoxic effects. This phenomenon is important in chronic neurodegenerative diseases such as Alzheimer's disease (AD) Parkinson's disease (PD), multiple

sclerosis and other amyloid disease (reviewed by Smith and Lassmann, 2002), as described below (section 1.4.5.).

Considering its reactivity, NO is usually regarded as a messenger that does not need a specific scavenging mechanism. At physiological concentrations (in the order of magnitude of nM) NO is poorly reactive (Ford et al., 1993), but it rapidly reacts with lipid peroxyl radicals, underlying its role in protecting the organism from lipid peroxidation subsequent to oxidative stress (Keynes et al., 2005). Remarkably, in the brain there is a mechanism for NO scavenging more efficient than in the rest of the organism, as evidenced by the measure of the rate of consumption of NO in cerebellar slices, that is significantly higher than that observed in dispersed cells (Hall and Garthwaite, 2006).

Because of its amphiphillic nature and dimensions, NO freely diffuses through membranes, accessing any cell compartment and hence acting both as an intra- and inter-cellular messenger. NO acts on target neurons as a result of volume transmission (Steinert et al., 2008), integrating neuronal activity over a volume limited by diffusion, coordinating synaptic transmission and plasticity of the neurons comprised in that volume (Gally and others 1990). A single source that emits NO for 10-20 seconds generates a diameter of influence of about 200 μM that corresponds to ~2 x 10^6 synapses. For multiple sources of NO, its concentration linearly increases with time affecting a population of active and inactive cells (Jacoby et al., 2001; Steinert et al., 2008).

1.4.2. targets of NO signalling

Not much is known about targets mediating NO/cGMP/PKG signalling. In fact, modulation of synaptic transmission, LTP and LTD do not represent 'targets', but rather integrated functions influenced by various alterations of structure/function of intracellular or membrane proteins, such as ion channels at both pre- and post-

synaptic levels (reviewed by Steinert et al., 2010). Increasing evidence points to the fact that synaptic plasticity can also be achieved by changes in the function of voltage-gated ion channels, influencing neuronal excitability and, in turn, the efficacy of information transfer, without directly affecting synaptic strength. Phosphorylation or S-nitrosation of VGNC, VGCC, Ca^{2+} activated and ATP-sensitive potassium (K^+) channels have been proposed as possible targets (reviewed by Biel et al., 1998; Ahern et al., 2002; Sanders and Kho 2006; Kawano et al., 2009). The targeting of K^+ channels to change neuronal excitability has been observed in the auditory brainstem (Steinert et al., 2008) with suppression of Kv3 channels and consequent increases in the neuronal excitability and duration of the action potentials. This action is triggered by NO-evoked increases in cGMP that activate PKG which in turn leads to the downstream dephosphorylation of the Kv3 channel expressed in CHO (Moreno et al., 2001); it has to be established if the same mechanism is also observable in a native preparation. It is worth noting that AMPAR, VGNC and NMDAR are influenced by NO. NO transmission has also been shown to inhibit Kv4 channels in human atrial and mouse ventricular myocites (Gomez et al., 2008). Kv1 activity is also inhibited by NO via cGMP and S-nitrosylation mediated mechanisms (Nunez et al., 2006). Furthermore, NO is known to interfere with AMPAR trafficking, a crucial process in LTP and LTD expression mechanisms, characterized by the insertion or internalization of AMPAR into or from the plasma membrane, respectively (Malinow and Malenka 2002; Song and Huganir 2002). GluR1 subunit phosphorylation on Ser-845 by PKGII leads to increased insertion of AMPARs at the cell surface (Serulle et al., 2008) as does direct nitrosylation of GluR1 and GluR2 (Huang et al., 2005; Selvakumar et al., 2009). On the other hand, cGMP

has been demonstrated to induce phosphorylation-independent inhibition of the AMPAR (Lei and others 2000).

Later findings in the peripheral nervous system and at the junction between nitrergic nerves and the effector organs suggest new possible targets for NO/cGMP signalling (reviewed by Toda and Okamura, 2003; Toda & Herman, 2005). Also, sampling of extracellular fluids suggests interactions with other transmission systems (reviewed by Prast by Philippu, 2001). Serotoninergic transmission is heavily influenced by NO/cGMP/PKG in the pond snail, Lymnaea stagnalis (Straub et al., 2007). It is of remarkable interest the interaction between NO/cGMP/PKG pathway and cholinergic neurotransmission in the brain (reviewed by de Vente, 2004) and in particular the role of NOS/sGC/PKG in the M1-dependent LTD in rat prefrontal cortex slices (Huang et al., 2009). Recent studies highlighted the role of another important component in this machinery, the endocannabinoids (eCBs): in the vertebrate (lizard) neuromuscular junction, activation of muscarinic (M3) and consequent transient decrease in acetylcholine relies on eCB secretion activating the NO/cGMP/PKG pathway (Newman et al., 2007). This mechanism is presumably presynaptic, since CB1 receptors are located presynaptically. In addition, depolarization-induced suppression of inhibition in the hippocampus in the presence of a cholinergic agonist relies on both NO/cGMP and eCBs (Makara et al., 2007). Furthermore, a close interplay between eCBs and NO in long-term plasticity at the corticostriatal excitatory synapses (Sergeeva et al., 2007) and spinal locomotor circuitry (Kyriakatos and El Manira, 2007) has been previously demonstrated. These latter evidences represented a key feature in the development of this project.

The activation of the NOS/sGC/PKG pathway can also result in the modification of gene expression: for instance, pavlovian fear conditioning in rats

relies on the activation of the nNOS/sGC/PKG pathway in the lateral amygdala, where it activates ERK/MAP kinase (Ota et al., 2008). Furthermore, NOS/sGC/PKG activation mediates the late phase of LTP and induces phosphorylation of CREB in the hippocampus (Lu et al., 1999), while YC-1, a potent sGC agonist, induces the enhancement of LTP at SC-CA1 synapses and the phosphorylation of CREB and ERK (Chien et al., 2003).

Even if most of the studies are focused on the action of NO on neurons, glia can also play an important role in NO/cGMP dependent transmission. For example, in the cerebellum astrocytes have the highest concentration of cGMP (de Vente et al., 1990; Southam et al., 1992; Southam & Garthwaite, 1993). The synapses are frequently interconnected with astrocyte processes, so the NO produced at a neuronal level may affect astrocyte metabolism. Forebrain cultures briefly (100 ms) exposed to NO are characterized by increased Ca^{2+} concentrations in glia (Willmott et al., 2000). Furthermore, NO/cGMP in astrocytes has been shown to regulate the expression of glial fibrillary acidic protein (GFAP) (Brahmachari et al., 2006). Fig 1.11. summarizes the main pathways of synthesis and signal transdution of NO.

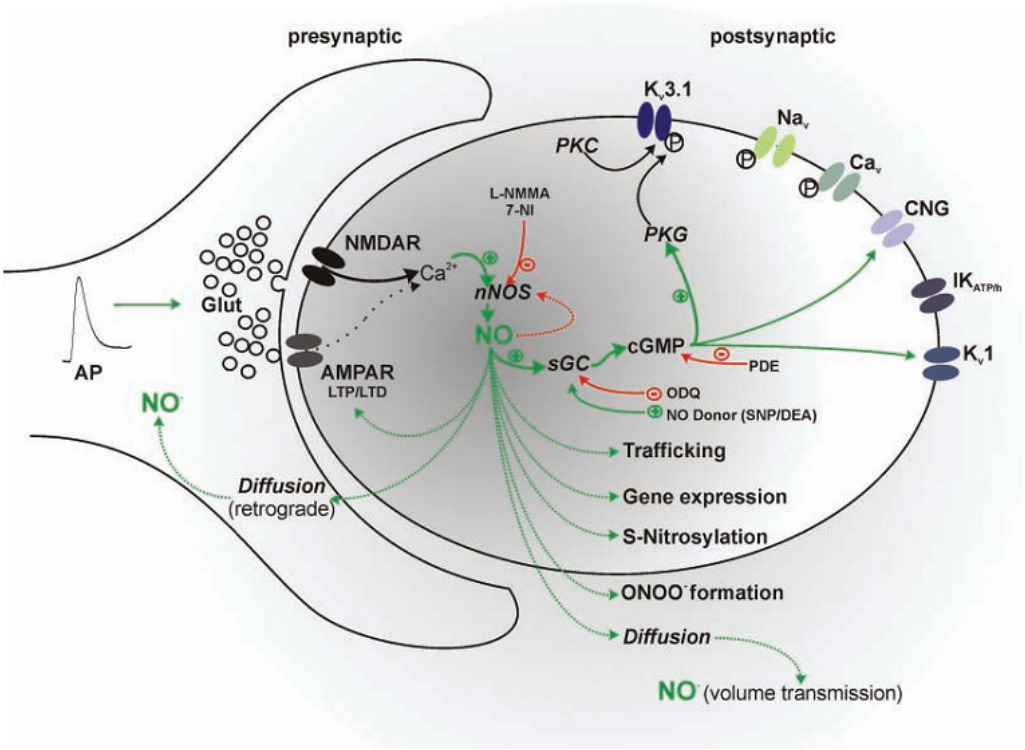


Fig 1.11. Schematic representation of NO production and downstream signalling at a glutamatergic synapse. The main pharmacological tools used to study NO-dependent transmission consist in antagonism of the nNOS such as L-NMMA, 7-NI or L-NAME (not shown). Similarly, sGC represents a main target antagonized by molecules such as ODQ or NS2028 (not shown). sGC can be also be activated by the application of NO donors as DEA/NO or SNP. nNOS is activated by the Ca^{2+}/calmodulin complex as a consequence of the NMDAR or g_q-coupled receptor activation (not represented). NO can act both post- and presynaptically, affecting neuronal function via S-nitrosylation or via the NO-receptor sGC. S-GC synthesizes the second messenger cGMP that can directly act on CNG or Kv1 channels or indirectly by activating PKG. cGMP activity is negatively regulated by PDEs. Abbreviations: AP = action potential; Cav = voltage-gated calcium channel; CNG = cyclic nucleotide-gated ion channels; DEA/NO = diethylamine nonoate; IKATP/h = ATP-sensitive potassium channel/hyperpolarizing potassium channel; Kv3.1 = voltage-gated potassium channel; L-NAME = L-ω-nitro-arginyl-methylesther; L-NMMA = NG-methyl-L-arginine; LTD = long-term depression; LTP = long-term potentiation; Nav = sodium channel; NS2028 = 4H-8-Bromo-1,2,4-oxadiazolo[3,4-d]benz[b][1,4]oxazin-1-one ODQ = 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; ONOO– = peroxynitrite; PDE = phosphodiesterase; SNP = sodium nitroprusside; 7-NI = 7-nitroindazole. (Modified from Steinert et al., 2010).

1.4.3. NO and synaptic plasticity

Since synaptic plasticity is described as a variation in synaptic strength following a specific pattern of activity of the neural network, this concept implies the existence of a messenger coordinating pre- and post-synaptic changes. Because of its diffusibility, NO was considered a good candidate for such a function since the beginning; after several years of studies generating contradictory evidences, it is now well accepted that NO plays a key role as a retrograde messenger mediating both LTP and LTD induction in different brain areas (Garthwaite & Boulton, 1995; Holscher, 1997; Calabresi et al., 1999; Prast & Philippu, 2001; Susswein et al., 2004; Garthwaite et al., 2008; Steinert et al., 2010).

The role of NO in LTD in the cerebellum at excitatory synapses from parallel fibres to Purkinje cells (PC) is well established. In brief, NO is produced in parallel fibres or in interneurons after activation of NMDAR by parallel fibres activation (Shibuki and Kimura, 1997; Shin and Linden, 2005). Purkinje cells have been shown to express high levels of the so called PKG substrate that once phosphorylated works as a phosphatase inhibitor (Endo et al., 2009). This, with PKC activation, leads to persistent AMPAR phosphorylation, resulting in AMPAR internalization (Launey et al., 2004; Steinberg et al., 2006). As mentioned before, cerebellar LTD also relies on eCBs release that, in turn, induce NO production (Safo and Regehr, 2005). Carbachol (Cch)-induced LTD in rat prefrontal cortex relies on the activation of NO/cGMP/PKG causing a presynaptic decrease release of neurotransmitter (Huang et al., 2009). Hippocampal LTD at SC-CA1 synapses requires NO/cGMP/PKG and release of Ca^{2+} from ryanodine sensitive intracellular stores, possibly mediated by cyclic ADP-ribose (Reyes-Harde et al., 1999). In addition, hippocampal NMDAR-dependent, but not mGluR1-dependent LTD, relies

on reduction of glutamate release mediated by NOS activity (Zhang et al., 2006). In the hippocampus, LTP is shown to involve AMPAR insertion in the postsynaptic membrane (reviewed by Collingridge et al., 2004) and the NO/cGMP/PKG pathway is known to play a pivotal role in the insertion of AMPAR on the postsynaptic membrane, via the phosphorylation of Ser-845 on the AMPAR subunit GluR1 (Antonova et al., 2001; Wang et al., 2005; reviewed by Garthwaite et al., 2008). NO acts as a retrograde messenger in synaptic plasticity in hippocampal LTP acting via cGMP/PKG (Arancio et al., 1995; 2001), coordinating the increase in both post-synaptic AMPAR insertion and presynaptic neurotransmitter release (Wang et al., 2005). LTP induction in rat visual cortex requires NOS activation and cGMP production (Haghikia et al., 2007). Bath application of the NO donor DEA/NO (300 μM) on acute hippocampal slices has been demonstrated to mediate a transient depression (presumably due to the cytotoxic effects of the exogenous NO on the mythocondrial oxidative phosphorylation) followed by a stable potentiation dependent on cGMP production (Bon and Garthwaite, 2001). In addition, it has been previously shown that a weak tetanic stimulation consisting of 5 pulses delivered at 50 Hz does not induce plasticity, although, if coupled to the bath application of DEA/NO (3 μM; at this concentration it does not affect basal synaptic transmission) it induced a robust LTP in hippocampal SC/CA1 synapses (Bon and Garthwaite, 2003). NO/cGMP/PKG activation was found to potentiate glutamate release in rostral ventral medulla neurons acting on N-type VGCC (Huang et al., 2003). In the cerebellum, parallel fibres-PC synapses undergo a form of presynaptic LTP requiring NO and cAMP but not cGMP production (Jacoby et al., 2001). Also, in the cerebellum, mossy fibre–granule cell synapse LTP, relies on NO/cGMP production for increased presynaptic excitability (Maffei et al., 2003). In the

hypothalamus, NO-cGMP pathway activation leads to the reduction of extracellular serotonin (Kaehler et al., 1999) presumably due to the phosphorylation of a threonine residue located on the serotonin transporter, followed by the subsequent increase in serotonin uptake (Ramamoorthy et al., 2007). Furthermore, alterations of intrinsic neuronal excitability mediated by NO/cGMP can be responsible for synaptic plasticity (Smith & Otis, 2003).

1.4.4. NO signalling in memory and learning

Blocking the NO production impairs the habituation of the proboscis extension reflex in the honeybee (Müller and Hildebrandt 2002). In mammals, it affects spatial learning which is under the control of the hippocampus (Böhme et al., 1993; Mogensen et al., 1995), and motor learning which is processed in the cerebellum (Allen and Steinmetz 1996; Nagao et al., 1997). The effects of NO blockade on hippocampal- and cerebellar-dependent learning behaviour underlie the role of NO in LTP and LTD in these brain areas. In other forms of learning, even if subsequent memory formation can be affected, blocking NO production does not affect the associated behaviour. For instance, NO is not involved in classical conditioning in the proboscis extension reflex of the honeybee (Müller et al., 1996) and in various learning feeding paradigms in Aplysia (Katzoff et al., 2002) and Helyx (Teyke; 1996). NO is also involved in many forms of short-term memory. Short-term memory lasts not more than ten minutes following the learning phase, and relies on structural and functional changes of already synthesized proteins (phosphorylation, S-nitrosylation etc.; Kandell, 2001). These modifications are triggered by the production of second messenger systems, and when associative learning paradigms are applied, two or more second messenger systems are activated influencing the target neurons (Byrne 1987). The possible role of NO/cGMP in short-term memory was first explored and confirmed in the

Aplysia feeding behaviour and in the passive avoidance task in the chick (Rickard et al., 1998; Katzoff et al., 2002).

Long-term memory lasts from hours to the entire lifespan of the organism and it is derived from the consolidation of short-term memory. It relies on the production of newly synthesized proteins induced by the activation of transcription factors, stably changing the metabolism and the structure of neurons. NO has been shown to affect long-term memory. In mice, blockade of NO production after training inhibits the consolidation of associative spatial memory tested with an inhibitory avoidance task (Baratti and Kopf, 1996). In Aplysia and Helyx, learning paradigms affecting feeding behaviour show that blockade of NO during training, but not after it, inhibits long-term memory formation (Katzoff et al., 2002; Teyke 1996). NO is also associated with the consolidation of olfactive memories: in fact, the olfactory bulb is particularly rich in NOS. In rat pups, the association of an odour to a tactile stimulus results in the situation by which their mother's handling generates a preference for the associated odour versus other odours. This kind of learning is blocked by blocking the NOS before, but not after the application of the paired stimulus (Samama and Boehm 1999). NO has also been shown to be involved in the Bruce effect in rat, whereby the pregnancy of the female rat caused by the mating with a novel male mate is blocked and the subsequent allowance of the pregnancy with a familiar male that impregnated the female before is known as the Bruce effect. This mechanism relies on an odour recognition memory: the female rat remembers the odour associated to the pheromones of a male that impregnated it beforehand. Injection of an NO donor instead of mating and the simultaneous exposure to the associated pheromones results in the unblockage of the pregnancy meaning that NO acts as a reinforcer of the original pheromone (Okere et al., 1996). After olfactory learning, it is possible to observe an increase

in the nNOS mRNA (Okere and Kaba 2000). It has been shown that intracerebroventricular administration of the sGC agonist YC1 into rats performing in the Morris water maze, a well known test of spatial learning, resulted in decreased escape latencies, whereas in avoidance tasks it resulted in increased latencies in the passive variant and decreased latencies in the active variant; furthermore, all these effects were reversed by the NOS antagonist L-NAME and the PKG antagonist KT5823, exemplifying the role of the whole NO/cGMP/PKG pathway in spatial memory and learning (Chien et al., 2005). The NOS inhibitor NO-Arg was shown to impair the retention for a single-trial step-down inhibitory avoidance task in rats (Bernabeu et al., 1995, 1996, 1997). A limitation to the interpretation of these studies is that all these effects may be non-specific to the learning, i.e. they are mediated by the effect of NO on cerebral blood flow.

NOS inhibition blocks long-term memory just within a very narrow time window around the initial training (Müller 1996, 2000). The late phase of LTP in hippocampal SC-CA1 is blocked by inhibitors of gene transcription or protein translation (Frey et al., 1988). The late phase of LTP relies on the activation of inducible transcription factors via phosphorylation by kinases such as PKA (Abel et al., 1997) or PKG. In fact these kinases can phosphorylate CREB to pCREB directly (Lu et al., 1999) or via ERK (Chien et al., 2003). In particular, blocking NO blocks the late phase of hippocampal LTP only if the inducing protocol consists in three spaced pulses but it is minimally affected if there are four pulses (Lu et al., 1999). Knock-out mice for an isoform of PKG display a reduced late phase of LTP, but hippocampal dependent memory and learning functions are intact (Kleppisch et al., 2003). Thus, it is possible that the late phase of LTP relying on the NO/cGMP/PKG pathway underlies only a subset of learning and memory tasks depending on hippocampal activity. Furthermore, studies on day-old chicks, using

a weaker variant of the passive inhibitory avoidance task that does not allow memory consolidation, showed that the co-application of the PDE5 inhibitor Zaprinast allowed memory consolidation (Campbell and Edwards, 2006). This result is consistent with the evidence, on the same model, that administration of L-NAME at 40 minutes post training in a single trial passive inhibitory avoidance task blocked memory retention (Rickard et al., 1998). In addition, also cerebellar LTD is characterized by a protein synthesis–dependent late phase, thus also NO/cGMP/PKG might be involved in motor memory consolidation (Linden 1996). Furthermore, many lines of evidence highlighted a possible role for NO/cGMP in object recognition memory, which is shown to rely on activity of the Prh rather than the hippocampus (reviewed by Brown and Aggleton, 2001). Systemic administration of the PDE5 inhibitor sildenafil prior or during the training phase increased the retention of recognition memory in rats (Prickaerts et al., 2002, 2005) and in mice (Rutten et al., 2005). Rats which were intraperitoneally injected with the unspecific NOS inhibitor L-NAME before or after the training phase demonstrated impaired recognition memory when tested at 24 h but not at 1 h in a spontaneous novel object exploration task. L-NAME administered just before the test phase in rats tested at 24 h rescued the delay-dependent impairment in object recognition memory (Boultadakis et al., 2010a). Another study showed that the systemic administration of a NO donor (NCX2057) antagonized the deficit in visual recognition memory induced by previous systemic administration of the muscarinic antagonist scopolamine (Boultadakis et al., 2010b). Finally, L-NAME was shown to antagonize the object recognition deficit induced by the NMDAR antagonist MK-801 and ketamine (Boultadakis and Pitsikas, 2010). Intra-hippocampal blockade of the NO/cGMP/PKG pathway induced deficits in visual recognition memory suggesting a key role for this pathway in the consolidation of this memory, and

also showing that the NO/cGMP/PKG downstream signalling system caused the activation of β–adrenergic receptors that in turn induced an increase in BDNF expression in the hippocampus (Furini et al., 2009).

1.4.5. Role of NO in neuropathology

NO is a free radical and it can react with many molecules; therefore it is involved in many pathological processes. In particular, the term nitrosative stress is related to the ability of NO and reactive nitrogen species (RNS) to damage proteins, nucleic acids and lipids. NO can react with the superoxide anion O_2^- to form peroxynitrite $ONOO^-$ that also decreases NO bioavailability (Beckman and Koppenol 1996). NOS directly contributes to O_2^- production in cells deficient in the NOS co-factor tetrahydrobiopterin (BH4) or the substrate L-arginine because they inefficiently catalyse the five electron reduction of L-Arg to citrulline with the conversion of O_2 to O_2^- (Xia et al., 1996). BH4 deficiency is associated to both Alzheimer's (AD) and Parkinson's disease (PD) (Kuiper et al., 1994; Foxton et al., 2007).

Eukaryotic cells undergo the production of reactive oxygen species (ROS) because of the electron leakage from the mitochondrial electron transfer chain that reacts with oxygen to produce O_2^-. This is normally metabolised by superoxide dismutase (SOD) to H_2O_2 that is further transformed by antioxidant enzymes. Both NO and $ONOO^-$ inhibit respiratory chain metabolism, resulting in a decreased production of ATP (Heales et al., 1999; Erusalimsky and Moncada 2007). Thus, neurodegeneration shows a complex dependency by oxidative and nitroxidative stress factors such as oxygen availability, antioxidant enzyme production and local metabolic rates (Sims et al., 2004). NO/$ONOO^-$ production determines increased Zn^{2+} from internal stores with the formation of S-nitrosothiols and neurotoxicity (Kroncke et al., 1994; Knoch et al., 2008). A rise in free Zn^{2+} leads to apoptotic

death via p38 MAP kinase activation and caspase-independent K^+ efflux (Bossy-Wetzel et al., 2004). NMDAR over-activation and NO exposure can determine mitochondrial fragmentation and cell death (Yuan and others 2007). This phenomenon in response to NO was reported in Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD; Bossy-Wetzel et al.,2008; Knott and Bossy-Wetzel 2008, 2009; Knott et al., 2008). Also, fragmentation of Golgi after NMDAR activation precedes neuronal death (Nakagomi et al., 2008). nNOS activation is highly coupled to NMDAR activation, so excitotoxicity can be related to nitrergic signalling.

iNOS expression in glia is triggered by the exposure to insult-related stimuli such as pathogens, ischemia, hypoxia, neurotoxic substances (comprised extracellular β-amyloid deposits). Oxidative and nitrosative stress are key factors in neurodegenerative diseases such as AD, PD, ALS, multiple sclerosis (MS), HD, and in brain damage coming from ischemic-reperfusion events (Bennett et al., 2009). Enhanced nitrotyrosine immuno-reactivity and oxidative protein damage are evident in brains from AD patients (Sultana et al., 2006; Danielson and Andersen 2008; Sultana et al., 2009); on the other hand, in β-amyloid (Aβ) mutant mice observations of increased H_2O_2 production and decreased cytocrome c oxidase expression were demonstrated (Manczak et al., 2006). AD patients show high levels of S-nitrosylated proteins in the cerebral cortex (Hensley et al., 1998) associated with Aβ deposition (Sultana et al., 2006) along with nitrotyrosilation of Tau protein (Reynolds et al., 2005) and synaptophysin in AD. Dysfunctions in the cholinergic transmission are also observed in AD (Tran et al., 2003). In PD patients and in experimental models of PD, increased expression of iNOS was reported (Gatto et al., 2000; Barthwal et al., 2001; Singh et al., 2005). In the human CNS, iNOS is mainly expressed in astrocytes rather than microglia.

Reports of increased iNOS astroglia immunoreactivity in post-mortem brain samples from patients affected by MS (Bo et al., 1994), AD (Wallace et al., 1997), and PD were demonstrated (Hunot et al., 1996).

Since NO is also involved in many physiological functions, it has to be clarified what the determinant factor is that decides its respective role in either pathological or physiological functions. Other than factors such as malfunctioning in NO generation or compensatory cellular responses, vascular dysfunction is an important condition that importantly contributes to the pathogenesis of many diseases. The involvement of nitrergic signalling in neurodegenerative diseases relies then on the generation of RNS, immune signalling, spill-over from endothelium and its involvement in oxidative stress. Chronic cerebral hypoperfusion and microvascular abnormalities can contribute to AD (Marlatt et al., 2008; Aliev et al., 2009; de la Torre 2009). Hypoperfusion and enhanced ROS generation in AD (Sultana et al., 2009; 2010) can be both responsible for increased iNOS expression as a consequence of a subclinical increase in inflammatory tone. In fact Aβ causes vasoconstriction of human cerebral arteries, with inflammation as a direct consequence, including the synthesis of other vasoconstrictors such as PGE2 and PGF2a (Townsend et al., 2002). In late-onset AD it is possible to observe increased plasma concentrations of the vascular cell adhesion molecule–1 (VCAM-1), related to endothelial activation (Zuliani et al., 2008). Furthermore, in the cerebrospinal fluid of AD patients it is possible to observe increased concentration of vascular-endothelial growth factor (VEGF; Tarkowski et al., 2002; Yang et al., 2004) that is pro-inflammatory (Ryu et al., 2009) and is involved in β-amyloidogenesis (Burger et al., 2009). VEGF activates microglial cells increasing NO production to micromolar levels by increasing iNOS expression (Moncada and Bolanos 2006; Kurauchi et al., 2009). Analogue

inflammatory pathways contribute to the neurodegeneration of nigro-striatal dopaminergic fibres in PD (Miller et al., 2009; Nanhoe-Mahabier et al., 2009).

1.5. Endocannabinoids (eCBs)

eCBs or endogenous cannabinoids (eCBs) take their name from the major psychoactive compound present in marijuana, Δ^9- tetrahydrocannabinol (THC; Gaoni et al., 1964). The effect of THC is due to its action on the CB1 and CB2 receptors: while CB2 are mostly expressed in the peripheral nervous system, CB1 is the most expressed G-coupled receptor (GPCR) in the CNS, in structures such as the hippocampus, Prh, postrhinal and temporal cortices (Liu et al., 2003c). CB1 was first isolated, cloned and characterized as a GPCR associated to $\alpha_{i/o}$ (Devane et al., 1988; Matsuda et al., 1990). A few years later, the eCBs anandamide (AEA) and 2-arachydonyl glycerol (2-AG) were isolated and characterized (Devane et al., 1992; Stella et al., 1997). The role of eCBs in brain function soon emerged, with central roles in the modulation of synaptic transmission and plasticity, mainly acting as retrograde messengers at CB1 (reviewed by Freund et al., 2003). In the last 10 years the role of eCBs in short- and long-term depression has been well characterized, consistent with their involvement in the modulation of many cognitive functions and behaviours, comprised memory and learning (Reviewed by Heyfetts and Castillo, 2009). The actions of eCBs are usually associated to LTD mechanisms, but recent evidence has demonstrated a possible role of eCBs also in LTP (see below section 1.5.1.). Furthermore, certain studies have shown that many neuropathologies such as PD and AD are correlated to alterations in eCB-dependent neurotransmission: therefore eCB receptors now represent a new pharmacological target for the treatment of these diseases (reviewed by Micale et al., 2007).

1.5.1. Endocannabinoid-mediated long term depression (eCB-LTD)

The first evidence of eCB-mediated LTD (eCB-LTD) emerged in excitatory synapses in the dorsal striatum (Gerdeman et al., 2002). eCB-LTD is also present in the amygdala (Marsicano et al., 2002), nucleus accumbens (Robbe et al., 2002), hippocampal CA1 at both inhibitory (Chevaleyre and Castillo, 2003) and excitatory inputs (Yasuda et al., 2008) and in many other areas as summarized in Fig 1.12.

1.5.2. Induction of eCB-LTD

eCB-LTD is usually triggered by the increase in glutamate release from afferent fibres and the consequent release of eCBs from the target neuron (postsynaptic). The released eCBs work in a retrograde manner, acting at presynaptic CB1 (homosynaptic eCB-LTD) and on CB1 located on nearby afferents (heterosynaptic eCB-LTD). The induction protocols of eCB-LTD vary between different brain areas, with frequencies of stimulation comprised between 1 and 100 Hz, including theta burst stimulation (TBS) as shown in Fig 1.12. Moreover, alternating induction of action potentials at both post- and presynaptic neurons with variable time intervals in between can induce the so called spike timing-dependent plasticity (STDP). STDP can consist of both potentiation and depression paradigms (t-LTP and t-LTD), depending on the time intervals and brain areas under consideration (reviewed by Caporale and Dan, 2008). In the case of CB1 dependent STDP, only t-LTD is observed (eCB-t-LTD). This form of eCB-LTD is not distinguishable by the one purely induced by afferent stimulation (Chevaleyre et al., 2007).

Brain structure	Synapse	Induction protocol
Neocortex		
Visual	Excitatory inputs, L5 pyramidal cell pairs	STDP (postsynaptic bursts)
		STDP and LFS
	Excitatory inputs, L4 → L2/3 pyramidal neurons (immature visual cortex)	TBS
Somatosensory (barrel cortex)	Excitatory inputs to L2/3 pyramidal neurons	STDP (postsynaptic bursts)
Prefrontal	L2/3 → L5/6	Moderate 10 Hz stimulation for 10 min
Hippocampus	Inhibitory inputs to CA1 pyramidal cells	HFS, TBS
	Excitatory inputs to CA1 pyramidal cells (immature hippocampus)	HFS
Amygdala	Inhibitory inputs to basolateral amygdala	LFS
Dorsal striatum	Excitatory inputs to medium spiny neurons	LFS, STDP
Nucleus accumbens	Excitatory inputs to medium spiny neurons	Moderate 13 Hz stimulation for 10 min
Cerebellum	Excitatory inputs to stellate interneurons	Four bouts of 25 stimuli at 30 Hz, delivered at 0.33 Hz
Ventral tegmental area (VTA)	Inhibitory inputs to dopamine neurons	Moderate 10 Hz stimulation for 5 min
Dorsal cochlear nucleus	Excitatory inputs to cartwheel cells	STDP
Superior colliculus	Inhibitory inputs to tectal neurons in vitro	HFS

Fig 1.12. eCB-LTD: brain structures, synapses and stimulation protocols (modified from Heifetts and Castillo, 2009).

eCBs release is mediated by two distinct processes: postsynaptic neuron depolarization and neurotransmitter release. For example, glutamate release from the presynaptic site can trigger eCB release from the postsynaptic neuron by stimulating group I mGluRs, that are $G\alpha_{q/11}$ coupled receptors (Varma et al., 2001; Jung et al., 2005). On the other hand, postsynaptic neuron depolarization has shown to generate increases in intracellular Ca^{2+} concentration via VGCC and NMDAR activation that, in turn, stimulates eCBs synthesis and release in the intersynaptic cleft through a mechanism that is not yet well understood (reviewed by Piomelli, 2003). Many evidences underlie that eCB release does not strictly depend on the activation of a particular metabotropic receptor or on a specific source of Ca^{2+}; other than group I mGluRs, metabotropic dopamine 2 (D_2), muscarinic acetylcholine 1/3 (M1/3), metabotropic serotonin 2 ($5HT_2$), orexin and cholecistochinin receptor activation are all effective stimuli for eCBs production.

Most of these receptors are coupled to $\alpha_{q/11}$, engaging phospholipase C (PLC) that generates the substrate for diacylglycerol lipase (DGL) which results in the production of 2-AG. Moreover, other mechanisms have been proposed; in the amygdala, AEA is synthesized after mGluR1-dependent adenylate cyclase (AC) activation which generates increases in cAMP production and PKA activation (Azad et al., 2004). In cortico-striatal synapses, induction of eCB-LTD relies on D_2 activation, that is associated to $\alpha_{i/o}$ and selectively induces AEA synthesis through a mechanism not yet understood (Giuffrida et al., 1999; reviewed by Piomelli, 2003). Ca^{2+}-dependent eCB release is not always required, but it still covers a pivotal role in eCB-LTD (Gerdeman et al., 2002). Ca^{2+} influx from NMDAR, L-type and T-type VGCC and from internal stores was reported to drive to eCB release (Nevian and Sackman, 2006; Bender et al., 2006; Isokawa and Alger 2006; Beierlein and Regehr, 2006; Adermark and Lovinger, 2007; Ohno-Shosaku et al., 2007). The two mechanisms can both operate independently and synergistically (Ohno-Shosaku et al., 2002; Kreitzer and Malenka, 2005; Brenowitz and Regehr, 2005; Hashimotodani et al., 2005).

The degradation of eCBs is mediated by enzymes that may represent a good target for the modulation of eCB-LTD; these include monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH) (reviewed by Piomelli, 2003). MGL is highly expressed in the presynaptic neuron near the CB1, supporting the view that this enzyme plays an important role in eCB-LTD. In fact, the inhibition of MGL leads to the induction of eCB-LTD after sub-threshold stimulation in the prefrontal cortex (Lafourcade et al., 2007). Recently, the topic of a putative postsynaptic membrane eCB transporter (EMT) responsible of eCBs efflux (an important step in eCBs mediated plasticity) has been raised. In the dorsal striatum, after the loading of the postsynaptic medium spiny neurons (MSN) with eCBs, the application of

paired stimuli (0.1 Hz) was shown to induce eCB-LTD not dependent on group I mGluR mediated postsynaptic Ca^{2+} increases or postsynaptic membrane depolarization (Ronesi et al., 2004; Ademark and Lovinger, 2007). This phenomenon was also observed in the somatosensory cortex (Bender et al., 2006).

Presynaptic activity is an important regulating factor for eCB activity. For example, eCB-t-LTD induced in L5 pyramidal neurons in prefrontal cortex requires presynaptic NMDAR activation: application of exogenous eCBs induces LTD only if the presynaptic neuron is activated at a relatively high frequency; this form of depression is blocked by the NMDAR antagonist D-APV (Sjöstrom et al., 2003). It is still to be clarified how presynaptic NMDAR and CB1 co-operate in the induction of eCB-LTD. Furthermore, many lines of evidence underlie that direct stimulation of CB1 alone is not enough to induce eCB-LTD; it requires simultaneous presynaptic activity (Sjöstrom et al., 2003; Bender et al., 2006). This data suggests the need of some sort of signal integration on the presynaptic terminal for eCB-LTD induction, probably in order to provide a control system under eCB spill-over conditions (Singla et al., 2007). The depolarization-driven stimulation of the afferent fibres could be necessary for eCB-LTD induction at the test synapse as supplementary activity or to release a co-factor. In the hippocampus, maximal eCB release is not enough to allow eCB-LTD induction, unless CB1 stimulation is paired to the activation of interneurons connected to the homologous synapse (Heyfets et al., 2008). The presynaptic activation, coming from both afferent firing and interneuron activation, is then necessary for eCB-LTD induction presumably because it mediates increases in presynaptic Ca^{2+} concentration, via presynaptic NMDAR and VGCC. It is still to be clarified how this Ca^{2+} increase mediates decreases in neurotransmitter release. It was hypothesised that presynaptic Ca^{2+}

increases integrates the CB1 signalling by activating the phosphatase calcineurin (reviewed by Heyfets and Castillo, 2009).

To sum up: 1) eCB induction relies on the synthesis and secretion of eCBs from the postsynaptic neuron as a consequence of a postsynaptic metabotropic receptor activation (usually a $G\alpha_{q/11}$ coupled receptor) and after activity-dependent Ca^{2+} increases at the same site 2) the eCBs spreading within the synaptic cleft bind CB1, causing decreased cAMP levels at the presynaptic terminal 3) in order to be effective, CB1 activation must be coupled to presynaptic activation, meaning presynaptic NMDAR or VGCC activation with consequent presynaptic increases in Ca^{2+} concentration 4) the combined activation of CB1 and a target of Ca^{2+} in the presynaptic site (presumably calcineurin) leads to long-term suppression of neurotransmitter release (summarised in Fig 13.).

1.5.3. Expression of eCB-LTD

Expression mechanisms of eCB-LTD have just begun to be explored. It has been shown that CB1 activation is not necessary for eCB-LTD long-term consolidation (Chevaleyre and Castillo, 2003; Sjöstrom et al., 2003; Ronesi et al., 2004). Long-term modifications determining decreased neurotransmitter release probably consist in the modified excitability of the afferent fibre, resulting in decreased Ca^{2+} inlet during action potential firing or changes in the downstream release machinery. In eCB-LTD, CB1 activity lasts several minutes (Chevaleyre et al., 2006) unlike what happens in short-term eCB mediated plasticity, such as depolarization dependent suppression of inhibition/excitation (DSI/DSE). In fact, CB1 dependent DSI in the hippocampus relies on decreased activity of VGCC, likely mediated by $G_{\beta\gamma}$ subunits (Wilson et al., 2001; Varma et al., 2002). For eCB-LTD of inhibitory synapses (I-LTD), the activation of $G\alpha_{i/o}$ is necessary and may reflect the need of prolonged activation of the receptor and subsequent inhibition

of the cAMP/PKA pathway (Howlett et al., 1986; Childers and Deadwyler, 1996). Continuous activation of AC has been shown to inhibit I-LTD but not eCB-dependent DSI (Chevaleyre et al., 2007). In addition, the postsynaptic inhibition of PKA and calcineurin do not affect both I-LTD and DSI, thus these modifications on the balance between phosphatases/kinases occur only presynaptically in eCB-dependent plasticity. The dependency of eCB-LTD expression on the cAMP/PKA pathway involvement was observed in many brain areas like the striatum (Calabresi et al., 1994), hippocampus, amygdala (Azad et al., 2004) and the nucleus accumbens (Mato et al., 2008).

As in many other forms of synaptic plasticity, the modulation of the cAMP/PKA pathway influences the downstream release machinery. For example, the active zone protein RIM1α is a target for PKA. Its phosphorylation is involved in many forms of synaptic plasticity such as LTP at mossy fibres/CA3 (Castillo et al., 2002) and at the SC/CA1 (Huang et al., 2005) synapses in hippocampus or parallel fibres/PC synapses in the cerebellum (Castillo et al., 2002). RIM1α is necessary for eCB-LTD in the hippocampus and the amygdala as shown by experiments on RIM1α knock-out mice (Azad et al., 2004). According to the PKA/RIM1α model, prolonged CB1 activation would lead to the dephosphorylation of RIM1α and the consequent decrease in neurotransmitter release. However, a recent study showed that in transgenic mice in which RIM1α was mutated (Ser413Ala) rendering RIM1α insensitive to PKA-dependent phosphorylation, eCB-LTD was still present in the hippocampus. Therefore, PKA regulation of neurotransmitter release in eCB-LTD relies on PKA activity on other proteins besides RIM1α. In the nucleus accumbens, it has been shown that blockade of P/Q-type, but not L- or N-type VGCC occluded eCB-LTD (Mato et al., 2008), whereas in the amygdala the

amphetamine-induced eCB-LTD was coupled to the suppression of P/Q-type VGCC (Huang et al., 2003).

A third mechanism that can underlie the expression of eCB-LTD is the reduction of presynaptic excitability. Heterosynaptic eCB-LTD in CA1 hippocampal synapses of the developing rat is associated with a decreased amplitude of fibre volleys; this phenomenon is blocked by K^+ channels antagonists (Yasuda et al., 2008).

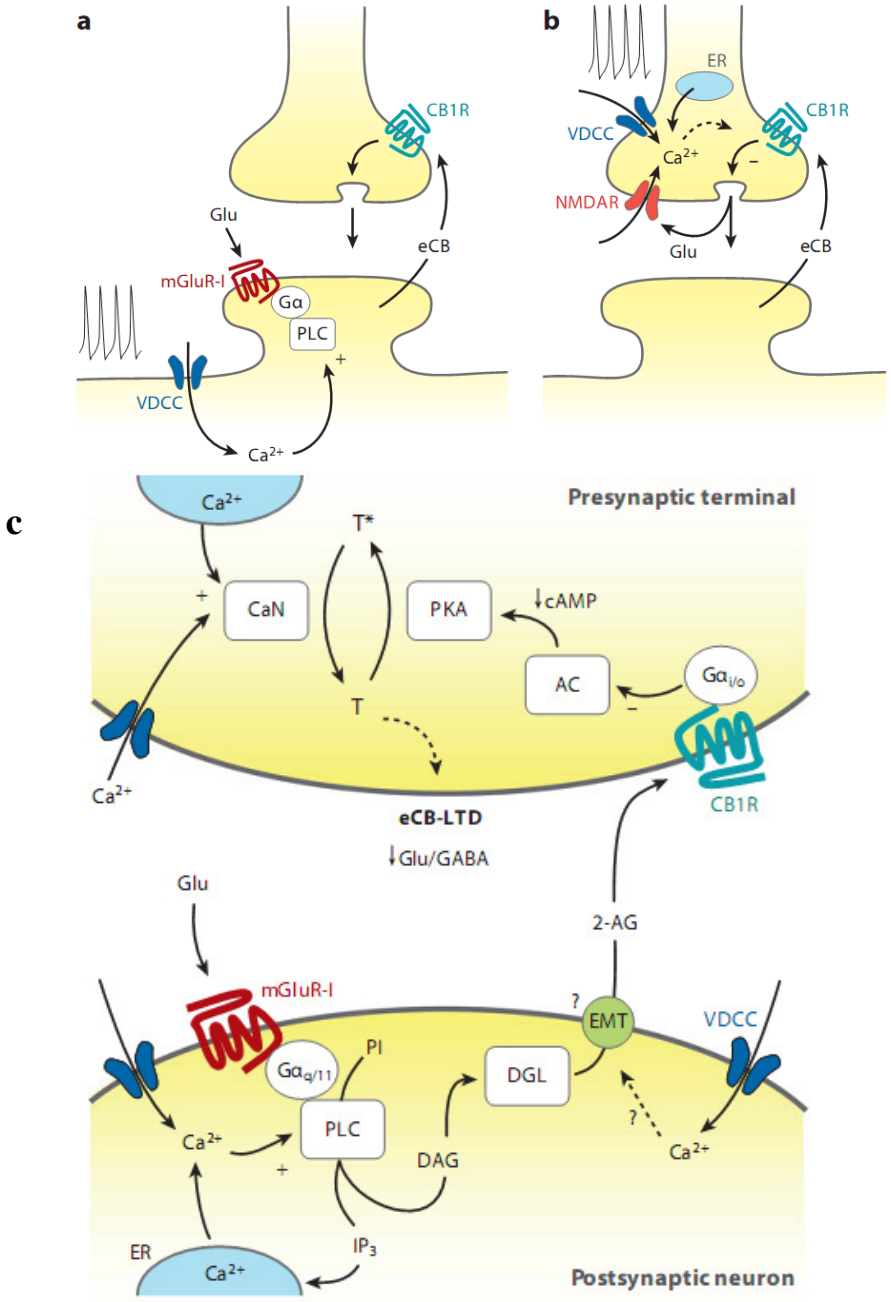


Fig 1.13. a) Calcium influx into the postsynaptic cell through VDCC as a consequence of depolarization triggers ECB release b) presynaptic firing and simultaneous activation of CB1 determines ECB-dependent decrease in neurotransmitter release; c) schematic representation of the cellular mechanism mediating eCB-LTD: the activation of a g_q-coupled receptor determines activation of DGL with production of ECBs and subsequent release in the inter-synaptic cleft through the putative EMT. The activation of presynaptic CB1 determines a decrease in PKA activity resulting in turn to decreased neurotransmitter release (modified from Heyfetts and Castillo, 2009)

1.5.4. eCBs in memory and learning

The ubiquitous expression of CB1 in the CNS indicates that exogenous ligands of this receptor may interact with many brain functions, ranging from homeostatic regulation of feeding to associative memory (Herkenham et al., 1990; Tsou et al., 1998). The role of CB1 modulation in neuropsychiatric disorders and neurodegenerative diseases has also been investigated (see below section 1.5.5.).

LTD is the main model to explain the loss of connectivity after sensorial deprivation in the related brain area (Glazewski and Fox, 1996; Rittenhouse et al., 1999; Allen et al., 2003; reviewed by Chklovski et al., 2004; reviewed by Hensch 2005). The LTD-like phenomena observed in denervated somatosensory and visual cortex resembles the induction and expression mechanisms observed in eCB-LTD. Whisker removal in the rat determines weakening of L4 and L2/3 synapses in barrel cortex by the decrease in presynaptic activity as a result of sensory information deprivation (Bender et al., 2006). Furthermore, monocular deprivation (MD) is known to depress visually evoked responses in the visual cortex (Trachtenberg et al., 2000); this phenomenon is probably related to the LTD induced at L4/L2-3 synapses in visual cortex that relies on CB1 activation (Crozier et al., 2007). In addition, short-term CB1 driven depression (DSI/DSE) could underlie these forms of cortical plasticity (Fortin et al., 2004; Bodor et al., 2005).

Many lines of evidence underlie the role of eCBs and eCB-LTD in hippocampal and amygdale-dependent associative memory. The first studies employed the Morris water maze and cued fear conditioning on CB1 -/- transgenic animals or wild-type animals treated with CB1 agonists/antagonists (Marsicano et al., 2002; Varvel and Lichtman, 2002). These studies demonstrated that CB1 activation is necessary for the extinction of these memories but not for their acquisition. Also,

FAAH inhibitors were found to improve extinction of a cued-shock association and of the memory of the position of a hidden platform in Morris water maze (Chhatval et al., 2005; Varvel et al., 2007). However, eCBs modulate the excitability and the induction of LTP in the amygdala and the hippocampus, a phenomenon mostly related to memory acquisition rather than extinction (Chevaleyre and Castillo, 2003; Azad et al., 2004; Chevaleyre and Castillo, 2004).

eCBs are usually involved in LTD-like phenomena, but recent studies have shown that this is probably a narrow vision. Intraperitoneal administration of the CB1 antagonist AM251, the CB1/2 unselective agonist WIN55,212-2, or the inhibitor of eCBs reuptake and breakdown AM404, result in impairment of LTP induction at SC/CA1 synapses. Furthermore, AM404 was shown to significantly enhance LTD, while AM251 and WIN did not affect it. Finally, intra-hippocampal infusion of AM251 was demonstrated to block extinction in an inhibitory avoidance conditioning and extinction task, while AM404 and WIN55,212-2 facilitated the extinction. It is worth noting that none of these compounds affect the acquisition of this conditioning. Considering the functional antagonism of these compounds, it appears that eCB modulation of synaptic plasticity and of memory and learning is a complex phenomenon which is not limited to a specific inhibitory activity (Abush and Akirav, 2009). A recent study further underlined the multiple roles of eCBs in modulating synaptic plasticity: eCBs definitely induce eCB-LTD by acting on presynaptic CB1, but they can also be responsible for potentiation if they act on astrocytic CB1, the latter of which determines increases in the intra-astrocytic Ca^{2+} concentration which in turn increases the release of glutamate from the astrocyte itself. Astrocyte released glutamate acts on presynaptic mGluR1s determining an increased release of neurotransmitter and subsequent potentiation of synaptic transmission (Navarrete and Araque, 2010). These two latter studies underlie the

complexity of the eCBs modulation of synaptic activity, revealing new directions in the study of the activity of these endogenous molecules.

1.5.5. eCBs in neuropathology

eCB signalling is involved in many neuropathological states, from mood disorders to neurodegenerative diseases (reviewed by Micale et al., 2007). Recent data show the role of eCB dysfunctions in the pathogenesis of neurodegenerative diseases such as PD, AD and HD.

The pathogenesis of PD arises from the neurodegeneration of nigrostriatal dopaminergic neurons. In the striatum, MSNs express D_2 but not D_1 receptors for dopamine. The dominant therapeutic strategy of PD relates to the compensation of the lack of dopamine (i.e. L-DOPA). Considering that corticostriatal eCB-LTD relies on D_2-dependent decrease of neurotransmitter release, this form of synaptic plasticity could represent a major target in PD therapy. The FAAH inhibitor URB597 administered together with a D_2 agonist reduced the motor deficit observed in PD in an animal model of the disease (Giuffrida et al., 1999; Kreitzer and Malenka, 2007).

The eCB system plays a double role in the pathophysiology of AD: it inhibits Ach release (cholinergic loss is one of the main characteristics of AD) whilst having anti-inflammatory properties. Many studies have discussed the possible role of the eCB system in AD because of their role in the modulation of inflammation. CB1 expression in glia is regulated by the exposure to inflammatory stimuli: in particular eCBs exert neuroprotective effects by limiting microglia activation during inflammation (Iravani et al., 2002; Jantzen et al., 2002; Xie et al., 2002; Benito et al., 2003; Ramirez et al., 2005). CB1 are less expressed in the frontal cortex of AD patients (Ramirez et al., 2005) while in the hippocampus increased DGLα activity was seen, resulting in the compensatory up-regulation of

2-AG synthesis in these patients (Farooqui et al., 1988). Considering that CB1 activation increases Erk activity and BDNF expression (Marsicano et al., 2003), eCBs probably exert neuroprotective activity through CB1 activation. The neuroprotective effect of CB1 activation is supported by AEA-dependent inhibition of Aβ42 mediated toxicity in an AD in vitro model (Milton, 2002). On the other hand, activation of CB1 in the hippocampus decreases Ach release generating cognitive deficits: the CB1 antagonist rimobanant decreased the cognitive deficit induced in rodents treated with soluble β-amyloid peptide oligomers. This animal model is characterized by progressive Aβ-plaques deposition, extensive hippocampal damage and decreased retention of newly acquired memories (Mazzola et al., 2003; van der Stelt et al., 2006). Interestingly, CB2 that in physiological conditions are mostly expressed in the peripheral nervous system, are overexpressed in the CNS of AD patients and Aβ-42 treated rats (Benito et al., 2003; Ramirez et al., 2005; van der Stelt et al., 2006), especially in microglia where they may exert a compensatory anti-inflammatory activity. A recent study further highlighted the neuroprotective role of eCBs: repeated administration (12 days) of the eCB reuptake antagonist VDM-11 reversed hippocampal damage and loss of memory retention tested in passive inhibitory avoidance task in rats previously administered with Aβ42 fragments. By contrast, if the administration of VDM-11 is limited to 5 days, starting 7 days after exposition to Aβ42, no neuroprotective effect is observed, once again highlighting the importance of the intervention in the early stages of AD (van der Stelt et al., 2006).

1.6. Alzheimer's Disease (AD): epidemiology, clinical features, pathogenesis, and molecular profile

Alzheimer's disease (AD) is the most common form of dementia in the elderly, accounting for 50-60% of all cases. AD affects less than 1% of individuals aged between 60-64 years, but cases increase exponentially with age: and at 85 years of age, the prevalence is comprised between 24% and 33% in Western countries (Ferri et al., 2005). Thus, AD is becoming more common, representing a major public health concern and a relevant social cost. In 2001, more than 24 million people were diagnosed with dementia and as a direct consequence of the increased life expectancy, it is expected to double every 20 years (Ferri et al., 2005). Other than ageing, epidemiological studies have suggested other not-genetically related risk factors such as low educational and occupational status, low mental ability in early life, reduced mental and physical activity during later life (Mayeux, 2003; Mortimer et al., 2003). Other risk factors are related to vascular diseases, including hypercholesterolaemia, hypertension, atherosclerosis, smoking, obesity and diabetes (Mayeux, 2003).

AD is a multifactorial disorder and thus environmental factors might increase the risk of its sporadic form; although this disease also has a strong genetic background. A large population-based study showed that the extent of heritability for the sporadic disease is almost 80% (Gatz et al., 2007).

First of all, it has to be stated that even if AD is a multifactorial disease, a familial form with a prevalence of less than 0.1% does exist (Harvey et al., 2003). Familial AD is an autosomal dominant disorder with onset typically before 65 years of age. There are several mutations on the amyloid precursor protein (APP) gene on chromosome 21 that explain only a few cases of familial AD. Although, mutations on the genes for presenilin 1 (PS1) and presenilin 2 (PS2) account for

most cases of the familial disease (Sherrington et al., 1995; Levy-Lahad et al., 1995). The sporadic form of AD is the most diffuse one, and it still maintains a strong genetic background. Apolipoprotein E (ApoE) ε4 allele is associated with AD pathogenesis: it increases the risk 3 times in heterozygotes and 15 times in homozygotes (Cordier et al., 1993; Porrier et al., 1993; Farrer et al., 1997). ApoE acts as a cholesterol transporter in the brain and ApoE4 is less efficient in lipid transport and neuronal repair (Poirier, 1994). ApoE is essential for amyloid β (Aβ) deposition, promoting Aβ fibrilisation and plaque formation (Holtzman et al., 2000), possibly acting as a pathological chaperone. The ApoE ε4 allele has been calculated to account for most of the genetic risk in sporadic AD (Raber et al., 2004). Other genetic factors can be mentioned but their involvement is difficult to assess, because this form of the disease is complex and probably several susceptibility genes act in concert, each conferring a minor increase in risk in an integrated interaction with environment factors.

AD is a progressive neurodegenerative disorder, with an ambiguous onset whereby impairments in episodic memory, aphasia, apraxia and agnosia are observed with generalised issues in cognition such as impaired judgement, decision-making and orientation. Based on histopathological observations that plaques and tangles are present in the brains of AD patients and in those with senile dementia, these mutifactorial disorders have been considered one homogeneous disease, in the later phases. The cardinal signs including plaque formation, tangle load and cholinergic deficits in combination with the cognitive impairments described above are more severe in early-onset rather than in late-onset AD (Roth, 1986; Blennow et al., 1991). In younger patients with AD there is a strong correlation between the severity of dementia and plaque and tangle accumulation, which is not found in elderly patients with the disease (Prohovnik et

al., 2006). These early clinical signs highlight the difference between AD and senile dementia. It is still uncertain if these diseases are separate, one, or a continuous intensification of the ageing process (Mann et al., 1984; Brayne et al., 1988).

Neurodegeneration in AD is estimated to start 20-30 years before clinical onset (Davies et al., 1988). During this preclinical phase, the plaque and tangle load increase to a threshold level until the appearance of the first symptoms. The early clinical phase is termed as mild cognitive impairment (MCI; Petersen, 2004), delineated on the basis of subjective reports of memory loss that are verified by close personal informants and by objective measures adjusted for age and education (Petersen, 2004). MCI does not prefigure onset of AD in all patients, but all patients with AD had MCI, highlighting that MCI can also underlie other etiological origins like normal ageing or cerebrovascular disorders (Gauthier et al., 2006). In MCI, the conversion rate to AD is 10-15% per year (Petersen, 2004; Visser et al., 2005).

The pathogenesis of AD is characterized at the microscopic level by hallmark lesions: amyloid plaques and neurofibrillary tangle accumulation (Fig 1.13.) in the medial temporal lobe and prefrontal cortex, with degeneration of neurons and synapses which have particular relevance for cholinergic neurotransmission. These changes are the result of different pathogenetic mechanisms, including Aβ aggregation and deposition with the development of plaques, tau hyperphosphorylation with tangle formation, neurovascular dysfunction and other mechanisms like cell-cycle abnormalities, inflammation, oxidative stress and mitochondrial dysfunction. Plaque deposition is highly related to the severity of the disease (Blessed et al., 1998). β-amyloid is constitutively produced by neurons in physiological conditions (Haass et al., 1992). Aβ is the result of the cleveage of the

amyloid precursor protein (APP) that is processed by two enzymes, β-secretase and γ-secretase (Fig 1.14.).

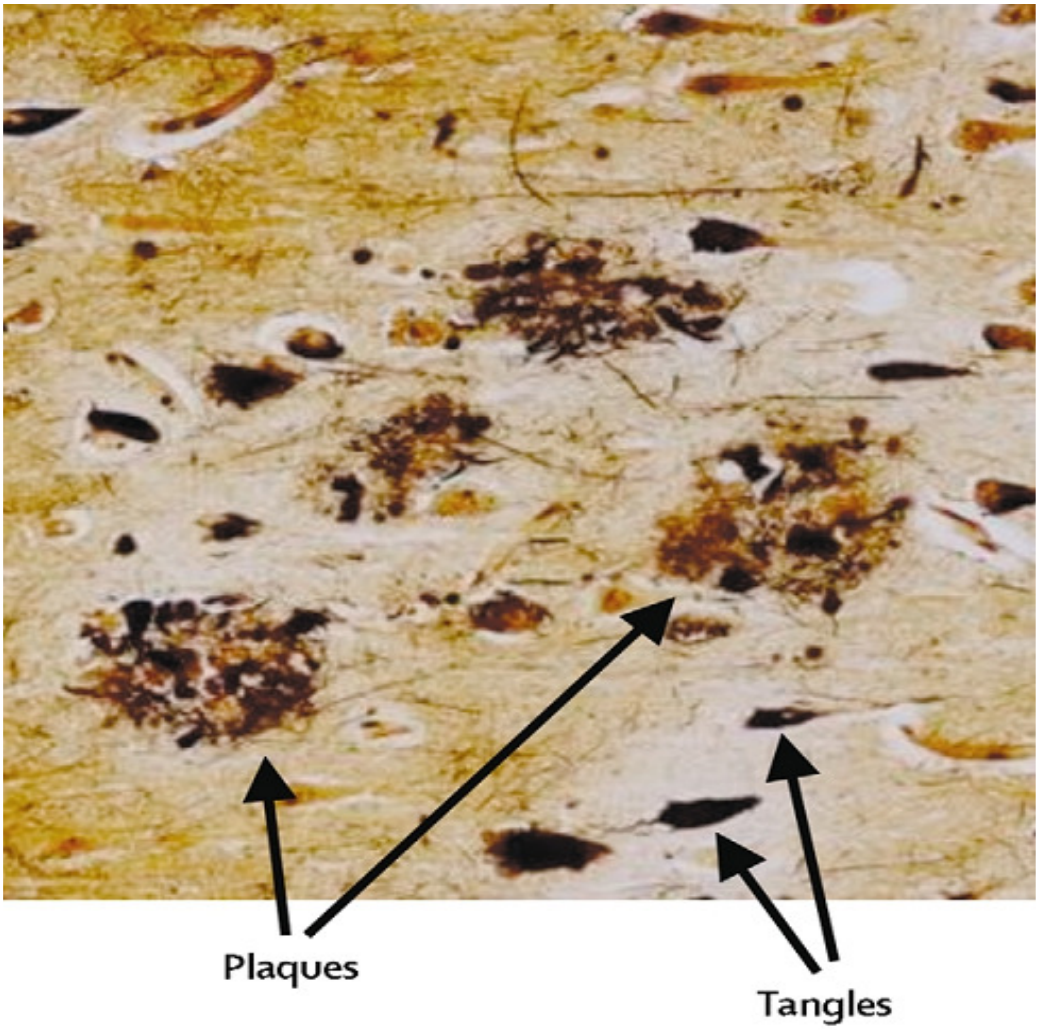


Fig 1.13. Plaques and tangles in the cerebral cortex in AD. Plaques are extracellular deposits of Aβ surrounded by dystrophic neurites, reactive astrocytes, and microglia, whereas tangles are intracellular aggregates composed of a hyperphosphorylated form of the protein tau (modified from Blennow et al., 2006).

γ-secretase is an intramembranous protease complex, composed of four components. β-secretase activity is triggered by an integral membrane aspartyl protease called β-site APP-cleaving enzyme 1 (BACE1) (Vassar et al., 1999). Another APP cleavage pathway is non-amyloidogenic and it's characterized by two proteases with α-secretase activity belonging to the ADAM family of disintegrin and metalloproteinases (Buxbaum et al., 1998; Lammich et al., 1999). In

physiological conditions Aβ is degraded by the following peptidases: insulin-degrading enzyme, neprilysin, and endothelin-converting enzyme (Carson et al., 2002). The clearance of Aβ is mediated by the alteration in the balance between efflux (via low-density lipoprotein receptor-related protein) and the influx (via the receptor for advanced glycation end products) across the blood–brain barrier (Tanzi et al., 2004). The etiopathology of AD is mainly centred on the amyloid cascade hypothesis (Fig 1.14.) stating that an imbalance between Aβ production and clearance exists. This pathogenetic pattern triggers a cascade in which Aβ production is enhanced and keeps accumulating, forming soluble oligomers. Aβ soluble oligomers affect synaptic function (transmission and plasticity) and are therefore probably related to the mild cognitive impairment observed in the early stages of AD (Walsh and Selkoe, 2004; Puzzo et al., 2005; Jacobsen et al., 2006). Aβ oligomers composed of 12 Aβ peptides are related to memory loss in AD transgenic mice models (Lesne et al., 2006). The mutations responsible for the familal form of AD on the APP, PS1 and PS2 genes, have a pivotal role in Aβ generation: this evidence support the amyloidogenic hypothesis of the AD pathogenesis. Mutations on these genes determine increase in Aβ42 (the fibrillogenic isoform of Aβ) production. Duplication of the APP locus in families with familial AD lends support to the notion that life-long APP over-expression results in Aβ deposition. In time, soluble Aβ oligomers keep accumulating, undergoing a conformational change to high β-sheet containing oligomers that are insoluble. Insoluble Aβ oligomers start to aggregate first in fibrils and then in extracellular plaques (Fig 1.15.).

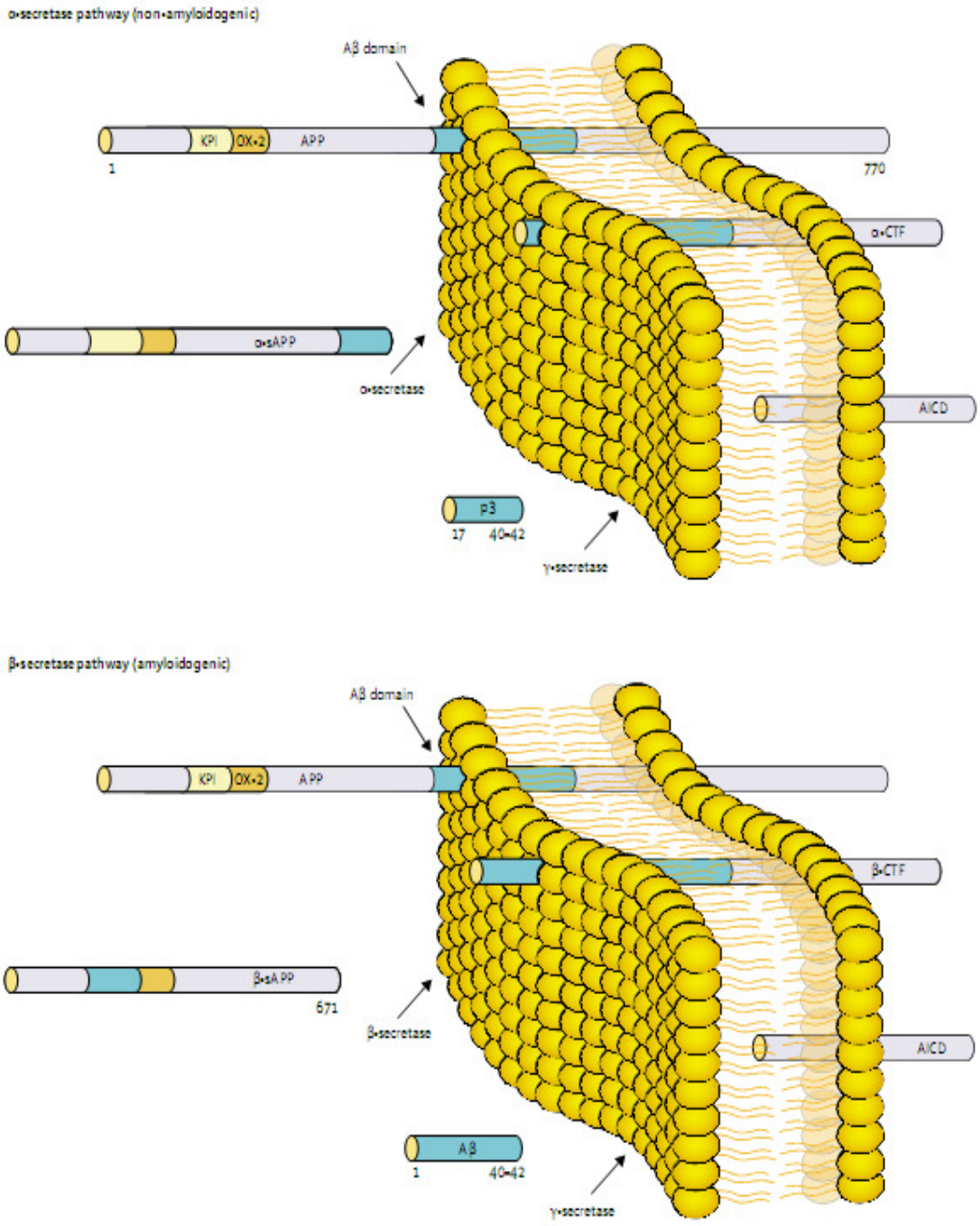


Fig 1.14. APP is a transmembrane protein. The Aβ domain is partly embedded in the plasma membrane and includes the 28 residues just outside the membrane and the first 12–14 residues in the transmembrane domain. APP can be processed along: 1) the α-secretase pathway that precludes Aβ aggregation since the cleveage domain is within the Aβ segment; 2) the β-secretase pathway releasing the β containing APP (βsAPP); γ-secretase cleaves the remaining C-term releasing the free 40 or 42 aminoacid containing Aβ peptide, the amyloidogenic form of amyloid protein (modified from Blennow et al., 2006).

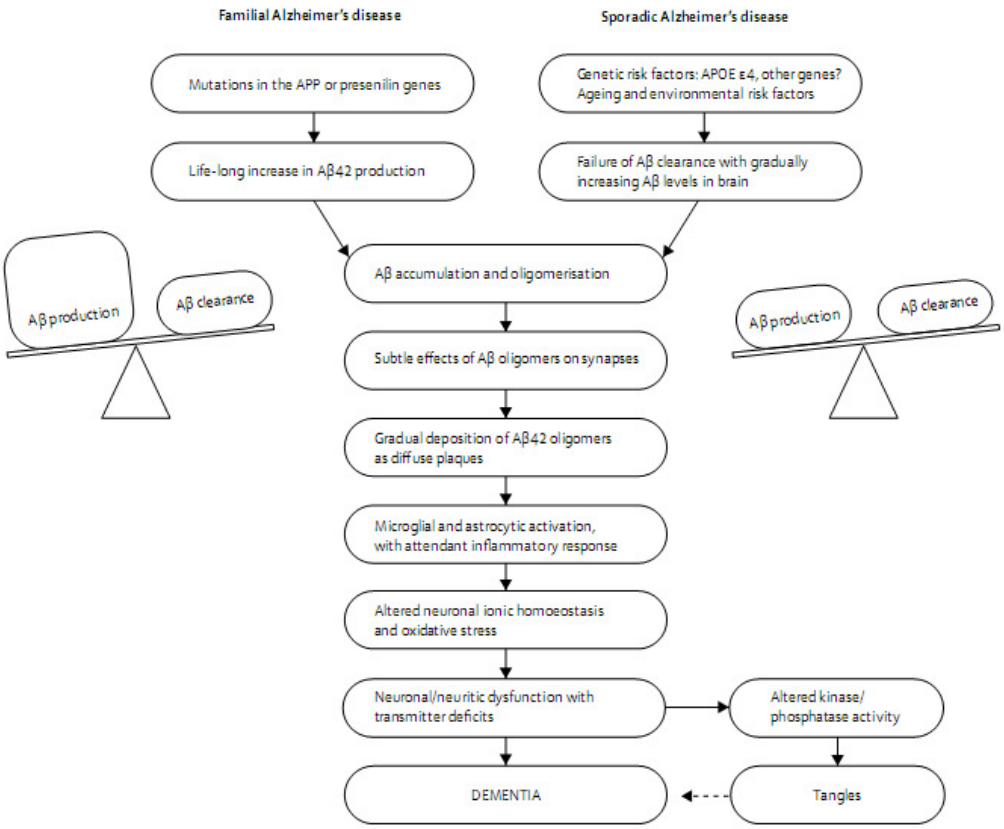


Fig 1.15. Shematic representation of the pathogenetic stages of AD. The central event is the clearance imbalance of Aβ: increased production for the familial and decreased clearance for the sporadic form. Aβ oligomers affect synaptic plasticity: in the later stages, inflammation, oxidative stress and mitochondrial dysfunction participate in the progression of the neuronal damage. In parallel, altered kinase/phosphatase regulation leads to the accumulation of hyperphosphorylated tau tangles.

As observed in other amyloidogenic neuropathologies, fibrillogenic Aβ42 isoform transmits the misfolding to other soluble Aβ peptides, accelerating the plaque formation and deposition (Jarret et al., 1993). In parallel with the accumulation of Aβ plaques, the pathogenesis of AD is characterized by intracellular tangles composed of hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986; Nukina and Ihara, 1986). Physiologically tau is an axonal microtubule binding protein, thus promoting microtubule assembly and stability. Tau phosphorylation is mediated by the balance between multiple kinases (eg, GSK-3β and CDK5) and phosphates (eg, PP-1 and PP-2A; Iqbal et al., 2005). In AD, tau hyperphosphorylation is intracellularly triggered causing microtubule disassembly and in turn compromising

axonal transport, impairing neuronal and synaptic function (Fig 1.16.). The hyperphosphorylation of tau determines its aggregation into insoluble fibrils and then tangles. Tau hyperphosphorylation is an early event in the pathogenesis of AD in the transentorhinal region, spreading to the hippocampus, amygdala and later to neocortical association areas (Braak et al., 1999). It has still to be clarified if tangle formation is a consequence or a cause of AD. Furthermore, many lines of evidence suggest that the Aβ plaque formation cooperates with cerebrovascular alterations in the pathogenesis of AD (Farkas and Luiten, 2001; Mayeux, 2003). The neurovascular hypothesis states that the dysfunction of blood vessels in the CNS can contribute to cognitive dysfunction via impairment of delivery of nutrients to neurons and via the reduction of Aβ clearance (Iadecola, 2004). Cerebrovascular pathology and ischaemia result in the up-regulation of APP expression followed by Aβ deposition (Jendroska et al., 1995; Sadowsky et al., 2004). Other hypotheses on the pathogenesis of AD suggest abnormalities in proteins regulating the cell cycle, inflammatory mechanisms, oxidative stress and mithocodrial dysfunction, especially in the progression of the disease and in the spread of the neuronal degeneration and dysfunction (Aisen, 2001; Webber at al., 2005; Gibson and Huang, 2005; Reddy and Beal, 2005).

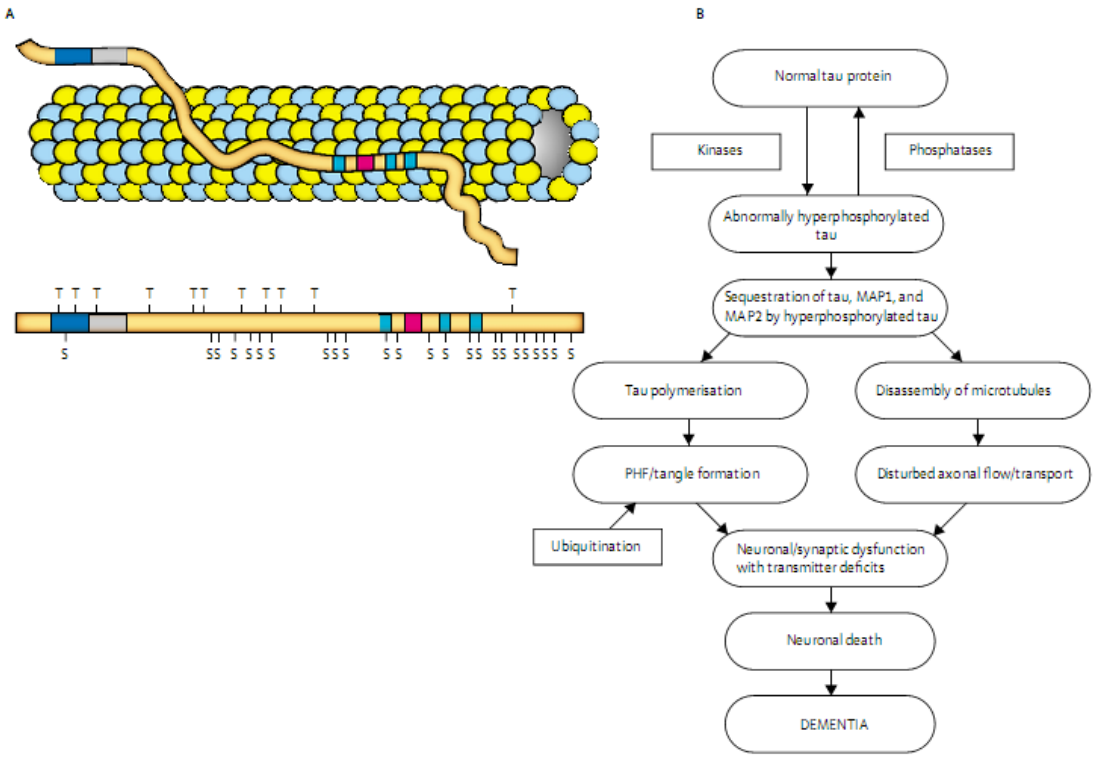


Fig 1.16. A: Schematic representation of tau protein bound to a microtubule through microtubule-binding domains. B: Flow-chart of tau hyperphosphorylation and tangle formation causing disassembly of microtubules and disturbed axonal transport, aggregation into insoluble fibrils (paired helical filaments; PHF) and larger aggregates in tangles (modified from Blennow et al., 2006).

1.6.1. Alzheimer's Disease: animal models

One of the most important experimental approaches for the study of AD is the development of animal models resembling genetic, histological and clinical features of the disease. The multifactoriality of AD represents a major problem for the liability of each model. For this reason, many experimental approaches were used to generate different animals showing different characteristics of the disease in order to get as much information as possible on this pathology. Nowadays there are two main approaches for the development of AD animal models: the generation of transgenic mice expressing human genes correlated to the familial form of AD (La Ferla and Oddo, 2005; Jacobsen et al., 2006; reviewed by Epis et al., 2010) or the induction of neurodegeneration of the cholinergic fibres projecting

from Basal Forebrain Nuclei in wild type rats through intracerebroventricular infusion of the neurotoxin 192 IgG saporin (Aztiria et al., 2007). This latter approach is higly unspecific because the neurodegeneration of cholinergic fibres is a common characteristic of many neurodegenerative disorders, including Parkinson's disease (Liselijn et al., 2008).

The generation of transgenic animal models starts from the evidence of a strong genetic component in AD, both familial and sporadic. The familial form is extremely rare (less than 0.1% prevalence); it is an autosomal dominant disorder, strongly based on mutations of the genes encoding APP, PS1 and PS2. The sporadic form is multifactorial, and its main genetic component is represented by mutations on the APOE protein encoding gene. Given these premises, various trangenic murine models for the study of the disease were developed in order to carry single or multiple human alleles for these genes with single or multiple mutations consistent with the genotype observed in the familial form of the disease. APP, PS1 and PS2 are involved in both the pathological and physiological metabolism of the amyloid protein. Hence, AD transgenic murine models are characterized by the altered proteolysis of the APP. This results in the accumulation of soluble intracellular β-amyloid oligomers (which cause early impairment of synaptic and cognitive functions), leadind to progressive accumulation of extracellular β-amyloid plaques and neurodegeneration. The first transgenic AD mice were developed thanks to the designed constructs of cDNA-based or yeast artificial chromosome, in order to induce expression of the entire human APP in the mouse brain (Buxbaum et al., 1993; Lamb et al., 1993). Other mutant alleles are: human APP751 (Moran et al., 1995; Quon et al., 1991), human APP695 (Yamaguchi et al., 1991), Aβ (Wirak et al., 1991) and/or the C-terminal fragments of APP (Sandhu et al., 1991). The APP transgene was successfully

expressed in the brain, but these mice did not show important neurological changes, with only few β-amyloid deposits. The lack of plaques observed in these models was generally attributed to the low expression level of the transgene. The following experimental approach was a mouse overexpressing mutant alleles of the human APP transgene: it showed an age-dependent AD-like pathology, with amyloid deposits in the brain parenchyma (Hsiao et al., 1996; Moechars et al., 1999). The most known mutations include the V717I “London” mutation (Goate et al., 1991), V717F “Indiana” mutation (Murrell et al., 1991), K670D/M671L “Swedish” mutation (Mullan et al., 1992) andE693G “Arctic” mutation (Nilsberth et al., 2001).

Presenilin knockout mice develop a fast and evident neurodegeneration in the cerebral cortex, memory impairment, and decreased synaptic function (Saura et al., 2004). In order to determine the role of presenilin-1 on APP cleavage, mice models carrying both the wild-type and mutant PS-1 encoding genes were developed (Duff et al., 1996). Both wild-type and mutant alleles carrying mice do not show significant signs of the disease, even if they show high levels of β-amyloid (Borchelt et al., 1996; Duff et al., 1996; Citron et al., 1997; Chui et al., 1999). The double PS1/2 mutant shows both cognitive loss and neurodegeneration (Saura et al., 2004). Crossing PS-1 mutant mice with APP mutant mice generates greatly accelerated β-amyloid aggregation into plaques (Holcomb et al., 1998), indicating that these genes interact synergistically. Because of the high production of β-amyloid plaques and the genetic similarity with the familial form of the disease in humans, the double PS-1/APP transgenic mice are the most used ones for the study of the pathogenesis of the disease (reviewed by Epis et al., 2010). However, these double mutant models do not show neurofibrillary tangles. Another generation of transgenic murine models for

the study of AD is represented by transgenic mice expressing the mutated form of the human gene encoding for tau (MAPT). These mice develop neurophibrillay tangles and show cognitive deficits and neurodeneration (Ramsden et al., 2005), but do not show amyloid plaques. It is important to underlie that so far no mutations have been identified on MAPT in the familial form of the disease: the mutated MAPT allele used in these mice was found in frontotemporal dementia (Ballatore et al., 2007). These studies were then extended by generating a triple transgenic murine model (3xTg-AD), bearing the following mutations: presenilin-1M146V, APPSwe and MAPT (P301L) (Oddo et al., 2003b). This most recent and integrated model presents itself with both extracellular β-amyloid plaque accumulation and intracellular hyperphosphorylated tau tangles which are the main histopathological features of the disease. These lesions are correlated to age-dependent synaptic dysfunctions, including LTP and memory deficits (Oddo et al., 2003a; Billings et al., 2005).

One of the most studied models is the Tg2576 mouse, characterized by the human allele of APP bearing the Swedish mutation (Lys -670-Asn; Met-671-Leu). Six month old Tg2576 mice are characterized by a deficit in spatial memory, a form of declarative memory known to be hippocampal dependent. The cognitive deficit is associated with impairment in synaptic transmission and LTP induction at Schaffer collaterals-CA1 synapses; at this age, this model also shows immunopositivity to intracellular soluble β-amyloid oligomers (Jacobsen et al., 2006). These features correspond to the pathological conditions of early-onset AD patients affected by MCI. Later in life, these animals present increased cytosolic concentration of soluble β-amyloid oligomers; extracellular β-amyloid plaques and neurodegeneration can be observed at around 18 months of age. This model has been widely used to clarify the pathogenetic mechanims of the disease (Dong et

al., 2005; Dong et al., 2008; Sanchez-Ramos et al., 2009; Dong et al., 2009; Tampellini et al., 2010).

1.7. Aims of the research

The main aim of the present study was to identify a potential retrograde messenger involved in synaptic plasticity in the Prh of the rat. NO and eCBs have been extensively studied as modulators involved in synaptic plasticity, memory and learning and their role as retrograde messengers have been demonstrated in various brain structures. Therefore, in the present study I evaluated their role in both LTP and LTD induced in acute perirhinal slices using field potential recordings.

Since Prh plays a major role in visual recognition memory, in the second part of the research I also evaluated the role of NO and eCBs in the acquisition of this form of declarative memory *in vivo*. The performance of adult rats was examined using the spontaneous novel object exploration task after intra-Prh infusions of vehicle or antagonists of nNOS and CB1 (NPA and AM251, respectively).

Finally, since one of the major goals in AD research is to characterize the early stages of the disease in order to improve diagnostic and therapeutic tools, I performed field potential recordings in Prh slices from Tg2576 mice (a model of AD) and from littermate controls at 3 month of age, in order to investigate possible very early in changes Prh synaptic transmission and plasticity.

2. MATERIALS AND METHODS

2.1. Laboratories

This project has been carried out in two different laboratories. It started at University of Bologna, department of Human and General Physiology, with the supervision of Professor Giorgio Aicardi, and it continued at University of Bristol, MRC centre for synaptic plasticity, Department of Physiology and Pharmacology, with the supervision of Professor Zafar Bashir. In both laboratories, field potential recordings were performed, while behavioural experiments were exclusively performed in Bristol. The following section will describe any experimental difference between the two laboratories by indicating in brackets the location (Bologna; Bristol).

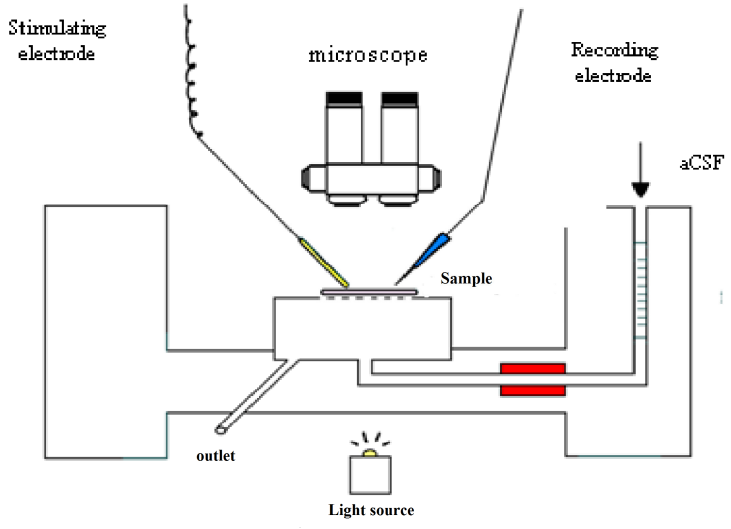
2.2. Electrophysiology

Tissue preparation. Experiments were performed on horizontal brain slices including Prh, lateral entorhinal cortex (LEnt) and hippocampus. Slices were obtained from p21-35 male albino rats (Sprague Dawley strain; Charles River Laboratories, Milano, Italy) (Bologna) and from p28-35 male pigmented rats (Dark Agouti strain; Bantin and Kingman, Hull, UK) (Bristol). All efforts were made to reduce the number of animals used. Animals were treated according to approved European Union guidelines. Animals were anaesthetized with halothane (Bologna) or isoflurane (Bristol) and killed for decapitation. The head was placed in ice cold (2-4°C) cryoprotective artificial cerebrospinal fluid (aCSF) pH=7,4 containing (in mM): 250 glycerol, 2.5 KCl, 1.2 NaH2PO4, 1.2MgCl2, 2.4 CaCl2, 26 NaHCO3 and 11 glucose (Bologna) or aCSF (same composition but with NaCl 125 mM instead of glycerol 250 mM; Bristol) and continuously bubbled with biological gas mixture (95% O_2-5% CO_2). The brain was rapidly removed and the frontal lobes and dorsal

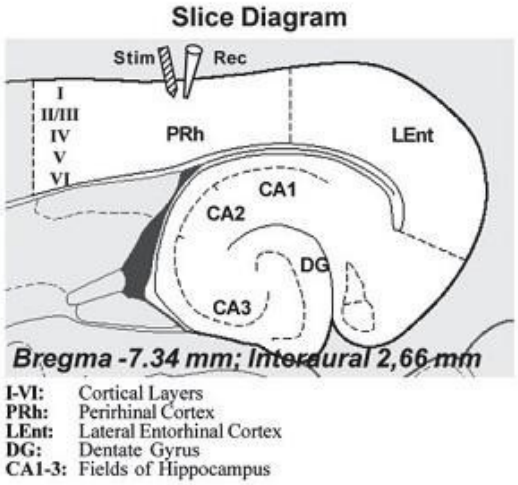
part were resected with two single scalpel cuts. The sample was then glued on the dorsal side (ventral side up) with superglue Loctite on a stainless steel cutting stage. The stage was transferred into the cutting chamber of a motorized vibratome (WPI Europe, Berlin, DE) filled with ice cold, oxygenated, cryoprotective aCSF (Bologna) or aCSF (Bristol). The tissue was then sliced (400μM) at the maximum blade vibration speed and the minimal advance speed. Each slice was immediately transferred for 60-90 min into a recovery chamber filled with aCSF at room temperature (25°C) and gassed with biological mixture.

Field potential recordings. After recovery, a single slice was placed into a recording chamber, continuously perfused with oxygenated aCSF (flow rate of 2/3 ml/min) and maintained at T = 32-34°C. After an acclimatizing period of at least 20 min, extracellular field excitatory post-synaptic potentials (fEPSP) were evoked by application of constant square current pulses (0,2 ms, 20-300 μA, 0,033Hz) on afferent fibers in layer II/III of Prh with a concentric bipolar stainless steel stimulation electrode (40-80 KΩ) connected to a stimulus generator (Master8; AMPI, Israel) through a stimulus isolation unit. Evoked extracellular fEPSP were recorded with a glass micropipette pulled at a horizontal micropipette puller (Sutter Instruments; Novato, USA) with a resistance of 3-5MΩ, filled with 2 M NaCl solution, inserted into the slice in layer II/III, ≈ 500μm distant from the stimulation electrode in caudal direction and connected to a DC amplifier by an Ag/AgCl electrode (Bologna: EPC-7; HEKA, Germany; Bristol: Axopatch 200, Axon Instruments, Foster City, CA). Stimulus intensity was adjusted to induce ≈60-70% of the maximal synaptic response. After at least 30 min of stable baseline recording, long-term depression (LTD) was induced by low frequency stimulation consisting in a single train of 3000 pulses delivered at 5Hz (5Hz-LFS) (Aicardi et

al., 2004). A weak LFS (5Hz-LFS 1350p) was also used, consisting in a single train of 1350 pulses delivered at 5Hz. Chemical LTD was induced via bath application of carbachol (Cch) (50 μM) for 10 min. LTP was induced by theta burst stimulation consisting of four trains delivered every 15 seconds, every train composed by 10 bursts delivered every 150 ms, every burst composed by 5 pulses delivered every 10 ms (100 Hz) (Aicardi et al., 2004).



a)



b)

Fig 2.1. a) Schematic representation of the experimental setup. b) Schematic representarion of a rat brain horizontal slice containing Prh, lateral entorhinal cortex (LEnt), dentate gyrus (DG) and area CA1-3 of the hippocampus. As indicated, the stimulation (Stim) and the recording electrodes (Rec) were placed in layer II/III of Prh.

Data acquisition and analysis. The analogical signal was digitised with an analogical/digital board and transferred to a PC. During experiments, sweeps of 1000 ms comprising artefact, presynaptic afferent volleys and fEPSP were digitally

acquired with the software Axoscope 9.0 (Axon instruments; PA, USA) (Bologna) or WinLTP (University of Bristol, Bristol, UK) each time the stimulus generator delivered a current pulse.

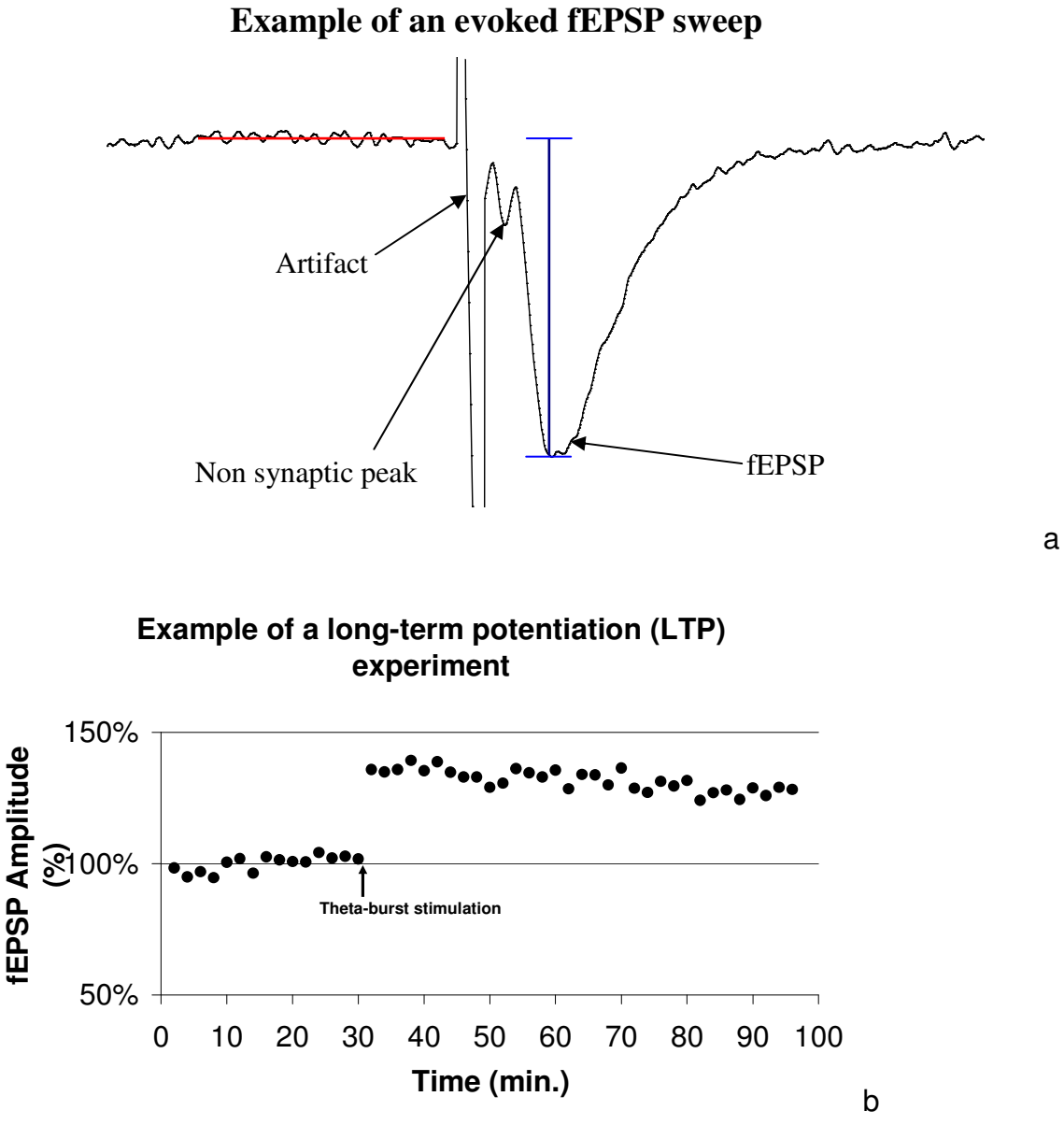


Fig 2.2. a) Example of an extracellular fEPSP recorded sweep. The artifact reflects the square current pulse applied to evoke the fEPSP. The "non synaptic peak" is due to the action potentials directly evoked by the current pulse in presynaptic fibres and/or in postsynaptic neurites. The fEPSP amplitude (blue line) is measured between the baseline (red line) and the minimum value. b) Example of a typical LTP recording plot. After 30 min of a stable baseline, the TBS stimulation protocol was applied and the fEPSP were recorded for more than 60 min.

The fEPSP amplitude is considered the value, in mV, comprised between the average value of the signal recorded 10 ms before the artefact and the minimal value of the fEPSP peak (see Fig 2.2.A). Each sweep was offline analysed with Clampfit (Bologna) or directly on-line analyzed with WinLTP (Bristol); the fEPSP peak amplitude values (mV) were exported in an Excel file. Each amplitude value (mV) was normalized for the baseline value, calculated as the mean of the fEPSP amplitudes recorded in the last 10-30 min of baseline. All the experimental groups were plotted as mean values ± S.E.M. The effects of conditioning stimulation were measured 50-60 min after LTP or LTD induction: any variation of the normalised fEPSP bigger than 10% and significantly different from the baseline was regarded as synaptic plasticity. Significance from baseline was evaluated using paired or unpaired t-test, as appropriate. Comparisons between groups used one way ANOVA where necessary, to examine the effect of treatment with LTD- or LTP-inducing stimuli upon responses.

2.3. Electrophysiological recordings on transgenic Tg2576 mice

Subjects. Heterozygous male Tg2576 mice under the control of the prion promoter and WT littermates were used (Hsiao et al., 1996). Tg2576 mice express a mutated form of the human gene for amyloid precursor protein (APP) bearing the Swedish mutation (Lys-670-Asn; Met-671-Leu). The gene encodes a double amino acid substitution and is associated with a heritable susceptibility to Alzheimer's disease (AD). Resulting phenotypic manifestations in Tg2576 include progressive accumulation of beta amyloid (Aβ) in the brain, analogous to classic "senile plaques" of human AD, and correlated synaptic and cognitive deficits. Tg2576 mice were kindly supplied by Italian National Centre on Aging (I.N.R.C.A.; Ancona, Italy). The original strain of Tg2576, model 001349, obtained on a mixed B6/SJL genetic background, was purchased from Taconic (Hudson, NY).

Subsequently a colony was raised by mating a transgenic male with a non transgenic female with a C57BL/6 genetic background. Three month old heterozigous males and littermate controls were used for the experiments. The transgenic offspring was selected with a genotyping procedure.

Electrophysiological recordings. Field potential recordings were performed in Bologna as described above (section 2.2.) with the following particularities:
1) semi-coronal slices were cut with a 45° angle to the dorsal axis as described elsewhere (Griffiths et al., 2008); 2) the composition of the crioprotective aCSF used during dissection was (in mM): NaCl 124, 3 KCl, 1.25 NaH2PO4, 10 MgSO4 7H2O, 0 CaCl2, 26 NaHCO3 and 10 glucose; the aCSF used for recovery and recording had the same composition except: 1 MgSO4, 2 CaCl2; 3) input/output curves (I/O) were run recording 2 sweeps at each current intensity within 0 and 200 µA with the following steps: 0, 20, 30, 40, 50, 100, 150, 200 µA; 4) LTD was induced after a 20 min stable baseline by application of 5 Hz-LFS; 5) the experimenter was blind as to the genetic condition of the animal.

2.4. Behavioural experiments

All the behavioural experiments were carried out by Dr. Gareth Barker under the supervision of Dr. Clea Warburton; I would like to thank both of them.

Subjects. Male pigmented rats were used (Dark Agouti strain; Bantin and Kingman, Hull, UK), weighing 150–200 g at the beginning of the experiment. The animals were housed under a 12 h light/dark cycle. Behavioural training and testing were performed during the dark phase of the cycle (6:00 A.M. to 6:00 P.M.). All efforts were made to reduce the number of animal used. Animals were treated according to approved guidelines.

Cannulation surgery. After being anhesthetized with isoflurane, the rats were secured in a stereotaxic frame with the incisor bar set at 3.3 mm below the

interaural line. Two stainless steel guide cannulas (26 gauge; Plastics One, Semat, UK) were implanted through burr holes in the skull at an angle of 20° to the vertical, using the following coordinates: antero- posterior, -5.6mm from bregma; lateral, ± 4.47mm; vertical, - 6.7mm from skull surface (Fig 2.3.). Two stainless steel screws and dental cement kept the cannulas anchored to the skull. Between infusions, the cannulas were closed by dummy inserts. After surgery, the rats were allowed for rcovery > 14 days.

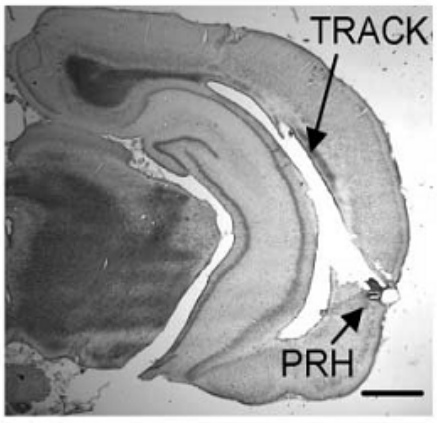
Apparatus. Exploration occurred in an open-topped arena (90 X 100 cm) with 50 cm wood walls and a scaffold covered with black cloth to a height of 150 cm: in this way the animals were isolated from exteranl visual stimuli during the experiment. Sawdust covered the floor of the arena. The animal's behaviour was monitored and recorded via an overhead camera and video recorder, for subsequent analysis. The stimuli were triplicate copies of objects made of plastic that varied in shape, color, and size (10 X 10 X 5cm to 25 X 10 X 5 cm) and were too heavy for the animal to displace.

Training. After handling (1 week), each rat was habituated to the arena without stimuli for 10 min daily for 4 days before the beginning of the spontaneous novel object exploration protocol. This protocol comprised an acquisition phase (training) separated by a delay from a recognition test. In the training phase, duplicate copies of an object (e.g., A1 and A2) were placed near the two corners at either end of one side of the arena (10 cm from each adjacent wall). The animal was placed into the arena facing the centre of the opposite wall and allowed a total of either 40 s of exploration of A1 and A2 or 4 min in the arena. Exploratory behaviour was defined as the animal was directing its nose toward the object at a distance of < 2 cm. Other behaviours (i.e. looking around while sitting on or resting against the object) were not considered as exploration. The delay between the

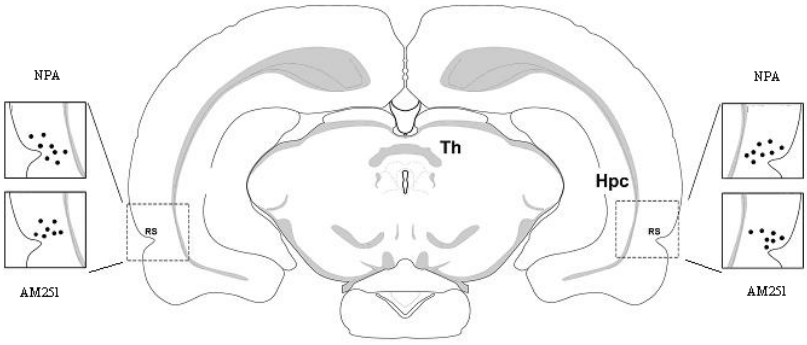
phases was 20 min or 24 h. At test phase (3 min duration), the animal was placed again in the arena with two objects positioned at the same positions as at training: one object (A3) was the third copy of the object used in the sample phase, and the other was a novel object (B3). The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals in a group and between the control and drug-treated groups (Fig 2.4.).

Drug delivery. General procedures have been already described elsewhere (Warburton et al., 2003). Infusions were made into the Prh through a 33 gauge cannula (Plastics One) inserted into the implanted cannula and attached to a 5 µl Hamilton syringe via polyethylene tubing. A volume of 1.0 µl was injected into each hemisphere over a 2 min period by infusion pump (Harvard Bioscience, Holliston, MA). The infusion cannula remained in place for an additional 5 min (Fig 2.3.).

Design. The animals were tested after a 20 min or 24 h delay. Three different groups of rats was used to test the effects of drugs: one for NPA at 24 hours and another one for NPA and AM251 at 20 min and a third one to test AM251 at 24 hours. To examine whether CB1 or nNOS were involved in the acquisition stage of memory, the appropriate antagonist (AM251 and NPA, respectively) or vehicle was infused starting 15 min before the commencement of the acquisition phase. After at least 7 d, the other substance (vehicle or antagonist in a crossover design) was infused, and the animal was tested again. The group sizes (n) were as follows: NPA experiments (24 h delay), n=12; NPA experiments (20 min delay), n=10; AM251 experiments (24 hours and 20 min delay), n=10. Data were analyzed only from animals that completed all phases of an experiment with patent, correctly placed cannulas.



a



b

Fig 2.3. a) Photomicrograph of a coronal brain section showing the track (indicated by arrow) left by a perirhinal cannula. Scale bar, 1 mm. (Modified from Warburton et al., 2003) b) Infusion sites. The sites within the perirhinal cortex at which drugs were infused are shown in the expanded boxes from a schematic brain section. The dots within the expanded boxes are just exemplificative and not necessarily representative of the real distribution of the drug. Hpc, Hippocampus; RS, rhinal sulcus; Th, thalamus (Modified from Barker et al., 2006).

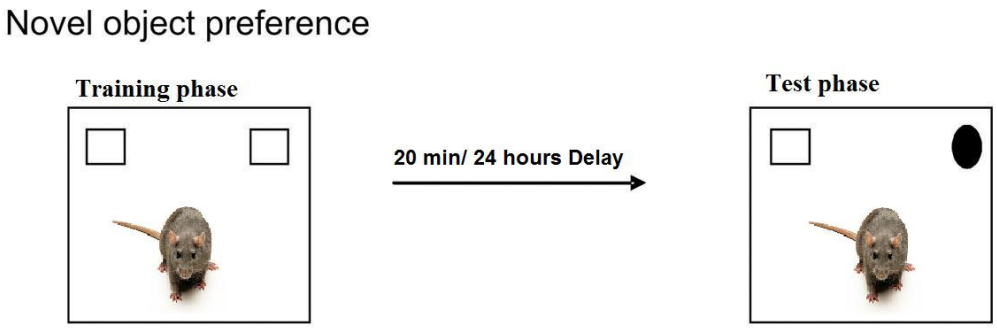


Fig 2.4. Schematic representation of an object recognition memory test. Fifteen min after infusion, the animal was placed in an arena with two identical objects. After a certain delay (20 min or 24h) the animal was placed in the same arena with the familiar and a novel object. If the subject remembers the familiar object, it will preferentially explore the novel one.

Statistical analysis. The experimenter was blind to the treatment during all measures of exploration. The discrimination ratio (DR) was calculated as the difference in time spent by each animal exploring the novel compared with the familiar object, divided by the total time spent exploring both objects. Comparisons

for the vehicle- and drug- treated groups used an ANOVA with repeated measures with treatment (drug or vehicle) as a within- subjects factor and time as a between-subjects factor. One-sample t tests were used to determine the significance of the DR (compared with zero discrimination) for each group. All tests used a significance level of p = 0.05 and were two-tailed.

2.4.Drugs

Electrophysiological recordings. The unselective NO synthetase (NOS) antagonist Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was purchased from Sigma-Aldrich S.r.L., Italy; it was maintained at -20°C. 2 mM, 200 μM and 50 μM L-NAME aCSF solutions were daily prepared and the slice was perfused with it at least 40 min before the application of the induction protocol. The nNOS selective antagonist NPA was purchased from Tocris (Cookson, Ellisville, MO), dissolved in 0.9% saline solution at a stock concentration of 20 mM and kept refrigerated at -20°C. Fresh solutions of NPA 20 μM containing aCSF were daily prepared by 1:1000 dilution of stock solution in aCSF. Slices were perfused with NPA 20 μM 40 min before the application of the induction protocol.

The soluble guanilate cyclase (sGC) antagonist 4H-8-Bromo-1,2,4-oxadiazolo[3,4-d]benz[b][1,4]oxazin-1-one (NS2028) was purchased from Sigma-Aldrich S.r.L., Italy, dissolved in DMSO in a 20 mM stock solution and maintained at –20°C. Fresh solutions of NS2028 0.5 μM containing aCSF were daily prepared by 1:400000 dilution of stock solution in ACSF. Slices were perfused with NS2028 0,5 μM 30 min before the application of the induction protocol as reported (Monfort et al., 2002).

The cGMP-dependent protein kinase (PKG) antagonist (9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl - 1 - oxo - 9, 12 - epoxy - 1 H - diindolo [1, 2, 3- fg: 3', 2', 1' - kl]pyrrolo [3,4i] [1,6] benzodiazocine-10-carboxylic

acid, methyl ester (KT5823) was purchased from Tocris (Cookson, Ellisville, MO) and dissolved in DMSO in a 2 mM stock solution. Fresh solutions of KT5823 2 μM containing aCSF were prepared daily by 1:1000 dilution of stock solution in ACSF. Slices were perfused with KT5823 2 μM 10-30 min before the application of the induction protocol.

The NO donor 2-(N,N-Diethylamino)-diazenolate 2-oxide sodium salt hydrate (DEA/NO) was purchased from Sigma-Aldrich S.r.L., Italy, and it was maintained at –20°C. Because of the pH and temperature dependent stability of the compound, stock solutions 3 mM were daily prepared by dissolving DEA/NO in NaOH 10 mM. DEA/NO 3 μM containing aCSF was prepared immediately before the perfusion by 1:1000 dilution of stock solution in aCSF (t1/2 DEA/NO= 16 min pH=7.4 T=21°C; =2 min pH7.4 T=32°C); the slice was perfused 5 min before until the end of the 5Hz-LFS 1350p.

The acetylcholinesterase (AchE) resistant cholinergic agonist 2-Hydroxyethyl trimethylammonium chloride carbamate (carbachol) was purchased from Sigma-Aldrich S.r.L., Italy, and it was maintained at room temperature. 50 mM stock solutions were prepared by dissolving carbachol in bidistilled H2O and conserved up to 1 week at –20°C. 50 μM Cch containing aCSF was daily prepared by 1:1000 dilution of stock solution in aCSF.

The CB1 receptor selective antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl) - 1 - (2, 4 - dichlorophenyl) - 4 - methyl - 1 H - pyrazole - 3 - carboxamide (AM251) was purchased from Tocris (Cookson, Ellisville, MO), dissolved in pure ethanol in a 2 mM stock solution and maintained at -20°C. Fresh 1 μM AM251 solutions were daily prepared by 1:2000 dilution of stock solution in aCSF. The slice was perfused with AM251 since 20 min before the application of the induction protocol.

The CB1 receptor selective agonist N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) was purchased from Tocris (Cookson, Ellisville, MO), and supplied in a 5 mg/ml stock solution pre-dissolved in pure ethanol. Fresh 1 µM ACEA solutions were daily prepared by dissolving the stock in aCSF. The slice was perfused with ACEA since 20 min before the application of the induction protocol in synaptic plasticity experiments and for 20 and 60 min in basal synaptic transmission experiments.

The CB1/2 non-selective agonist (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de] - 1,4 - benzoxazin -6- yl-1-naphthalenylmethanone mesylate (WIN55,212-2) was purchased from Tocris (Cookson, Ellisville, MO), dissolved in pure ethanol in a 2 mM stock solution and maintained at -20°C. Fresh 2 µM WIN55,212-2 solutions were daily prepared by 1:1000 dilution of stock solution in aCSF. The drug was applied for 20 min and then washed out.

Behavioural experiments. The drugs used were AM251 and NPA (see above for details). Control infusions for NPA experiments consisted of 0,9% saline while for AM251 consisted of 0,1% EtOH containing 0,9% saline. NPA was infused at a concentration of 2 µM/side and AM251 was infused at a concentration of 10 µM/side. For both drugs, saline controls and 0.1% EtOH sham controls the volume infused was 1 µL.

3. RESULTS

3.1. Role of the NO and eCBs in the synaptic plasticity in Prh cortex

In this section are reported the results of electrophysiological recordings performed to clarify the role of NO and eCBs in LTP and muscarinic dependent LTD. Since it was not observed any difference between different kind of controls (external, interleaved, Sham EtOH 0.1%, Sham DMSO 0.1%) in each laboratory, Bologna and Bristol, all the controls are merged together.

3.1.1. Role of the NOS/sGC/PKG pathway in the 5 Hz-induced LTD (Bologna)

The first subset of experiments showed that the application of a 5 Hz LFS induced LTD in the Prh of male juvenile Sprague-Dawley rats in control conditions (Fig 3.1. A, n=11, baseline: 94.5% ± 2.5%; 60 min follow-up: 76.9% ± 6.1%; $p < 0,01$) but LTD was blocked in presence of the NOS antagonist L-NAME (2 mM) (Fig 3.1. B, n=6, baseline: 100.6% ± 1.2%; 60 min follow-up: 94.3% ± 4.6%; $p > 0.05$), of the sGC antagonist NS2028 (0.5 μM) (Fig 3.1. C, n=7, baseline: 97.4% ± 1.7%; 60 min follow-up: 97.9% ± 3.5%; $p > 0.05$), and of the PKG antagonist KT5823 (2 μM) (Fig 3.1. D, n=4, baseline: 99.5% ± 3.7%; 60 min follow-up: 93.6% ± 2.1%; $p > 0.05$). There was a significant difference between the magnitude of the LTD between controls and each of the other group ($p < 0.01$).

In a second subset of experiments it has been shown that the application of a sub-threshold weak 5 Hz LFS, consisting in 1350 instead of 3000 pulses, failed in inducing LTD (Fig 3.2. A, n=9, baseline: 98.0% ± 1.7%; 60 min follow-up: 93.2% ± 4.2%; $p > 0.05$) and the bath application of the NO donor DEA/NONOate (DEA/NO, 3 μM) did not affect basal synaptic transmission (Fig 3.2. B, n=4; $p > 0.05$) but the co-application of the two protocols determined induction of a robust

LTD (Fig 3.2. C, n=9, baseline: 97.5% ± 2.5%; 60 min follow-up: 78.6% ± 5.1%; $p < 0.01$). There was a significant difference between the magnitude of the LTD induced with the weak 5 Hz LFS and with the co-application of that stimulation with DEANONOate 3 μM ($p < 0.01$). These results indicate a pivotal role for the NOS/sGC/PKG pathway in the induction of 5 Hz LTD (see section 4. for discussion).

3.1.2. Role of the NOS/sGC/PKG pathway in the Cch-induced LTD (Bologna)

The bath application of Cch (50 μM) for 10 min (Cch-LTD) in the Prh of male juvenile Sprague-Dawley rats determined the induction of a robust LTD (Fig 3.3. A, n = 21, baseline: 98.8% ± 1.1%; 60 min follow-up: 76.4% ± 4.2%, $p<0.01$). The NOS antagonist L-NAME (2 mM) or the sGC antagonist NS2028 (0.5 μM) co-applied to Cch blocked Cch-LTD induction (Respectively: for L-NAME, Fig 3.3. B, n=8, baseline: 100.4% ± 1.0%; 60 min follow-up: 101.8% ± 4.6%, $p>0.05$; for NS2028, Fig 3.3. C, n=8, baseline: 98.4% ± 1.3%; 60 min follow-up: 94.5% ± 5.1%, $p>0.05$). There was a significant difference in the magnitude of the LTD induced in control conditions and in presence of L-NAME or NS2028 ($p < 0.05$).

These results indicate a role for NOS/sGC pathway in the induction of CCh-LTD (see section 4. for discussion).

The PKG antagonist KT5823 was used in another set of experiments carried out between Bologna and Bristol and that is going to be described in the next section.

3.1.3. Role of the NOS/sGC/PKG pathway in the Cch-induced LTD (Bristol)

In order to confirm the data collected in Bologna, in Bristol I performed field potential electrophysiological recordings to verify the role of NOS and PKG in the Cch-LTD in the Prh of male juvenile Dark Agouti rats (see section 2.2. for methodological details).

The first subset of experiments showed that 10 min Cch (50 μM) bath application determined LTD induction (Fig 3.4. A, n = 21, baseline: 98.8% ± 1.1%; 60 min follow-up: 76.4% ± 4.2%, $p<0.01$) and that the NOS antagonist L-NAME successfully blocked Cch-LTD if applied at 200 μM (Fig 3.4. C, n = 9, baseline: 104.0% ± 2.5%; 60 min follow-up: 101.7% ± 3.3%, $p>0.05$) and 2 mM (Fig 3.4. D, n = 5, baseline: 101.3% ± 2.3%; 60 min follow-up: 101.9% ± 3.8%, $p>0.05$) but not at 50 μM (Fig 3.4. B, n = 7, baseline: 98.6% ± 2.6%; 60 min follow-up: 84.0% ± 5.3%, $p<0.01$). There was a significant difference in the magnitude of LTD induced in control conditions and in presence of L-NAME 200 μM and 2 mM ($p < 0.01$) but not 50 μM ($p > 0.05$). Also, I showed that the selective antagonist of the neuronal isoform of the NOS (nNOS) NPA (20 μM) blocked the induction of Cch-LTD (Fig 3.4. D, n = 5, baseline: 102.8% ± 1.4%; 60 min follow-up: 94.5% ± 5.4%, $p<0.01$). There was a significant difference in the LTD induced in control conditions and in presence of NPA 20 μM ($p < 0.05$).

The second subset of experiments showed that the PKG antagonist KT5823 (2 μM) did not affect Cch-LTD induction (Fig 3.4. E, n = 7, baseline: 101.8% ± 1.2%; 60 min follow-up: 84.6% ± 2.4%, $p<0.01$). There was not any significant difference in the magnitude of LTD induced in control conditions and in presence of KT5823 (2 μM) ($p > 0.05$).

These results confirm the role of NO in the induction of muscarinic dependent LTD, as previously verified in the first part of this study carried out in Bologna.

Also, Cch-LTD does not rely on the PKG activation, unlikely to what observed for 5 Hz LTD, that's still M1 dependent but induced with electrical instead of chemical stimulation (Jo et al., 2006). The possible reasons for this discrepancy are discussed in section 4.

3.1.4. Role of the NOS in the TBS-induced LTP (Bristol)

The bath application of L-NAME at both 200 µM (Fig 3.6. B, n = 3, baseline: 102.1% ± 1.9%; 60 min follow-up: 131.6% ± 5.6%, p<0.01) and 2 mM (Fig 3.6. C, n = 6, baseline: 101.4% ± 1.7%; 60 min follow-up: 120.0% ± 5.3%, p<0.01) did not affect LTP induction compared to controls (Fig 3.6. A, n = 26, baseline: 101.4% ± 1.7%; 60 min follow-up: 114.8% ± 2.8%, p<0,01). There was not any significant difference in the magnitude of the LTP induced in the three different conditions. (p > 0.05). These data show that NOS is not involved in the induction of LTP. For this reason I did not further verify the role of sGC and PKG in TBS-induced LTP since these two enzymes are sequentially activated after NO production.

3.1.5. Effect of the cannabinoid receptors CB1 and CB2 activation on the basal synaptic transmission (Bristol)

The selective CB1 agonist ACEA (1 µM) did not affect basal synaptic transmission in the Prh of male juvenile Dark Agouti rats when bath applied for either 20 min (Fig 3.7. A, n = 6, p > 0.05) or 60 min (Fig 3.7. B, n = 6, p>0.05). The same lack of effect has been observed after the bath application of the CB1/CB2 not selective agonist WIN55,212-2 (2 µM) for 20 min (Fig 3.7. C, n = 4, p > 0.05).

3.1.6. Role of the CB1 activation in 5 Hz-induced LTD (Bristol)

Both ACEA (1 µM) (Fig 3.8. B, n = 7, baseline: 98,5% ± 3.1%; 60 min follow-up: 79.7% ± 2.5%, p<0.01) and AM251 (1 µM) (Fig 3.8. C, n = 5, baseline: 93.3% ± 1.3%; 60 min follow-up: 76.6% ± 7.2%, p<0.01) did not affect the induction of 5 Hz LTD compared to controls run in presence of EtOH 0,1% (Fig 3.8. A, n = 5,

baseline: 92.6% ± 4.9%; 60 min follow-up: 83.9% ± 1.2%, p<0.01). There was no significant difference in the magnitude of LTD induced in presence of EtOH 0.1%, ACEA (1 μM) and AM251 (1 μM) (p > 0.05).

3.1.7. Role of the CB1 activation in Cch-induced LTD (Bristol)

Both ACEA (1 μM) (Fig 3.9. B, n = 6, baseline: 96.5% ± 1.4%; 60 min follow-up: 87.6% ± 3.4%, p < 0.01) and AM251 (1 μM) (Fig 3.9. C, n = 6, baseline: 95.3% ± 4.9%; 60 min follow-up: 81.8% ± 5.5%, p < 0.01) did not affect the induction of Cch-LTD compared to controls (Fig 3.9. A, n = 21, baseline: 98.8% ± 1.1%; 60 min follow-up: 76.4% ± 4.2%, p < 0.01). There was no significant difference in the magnitude of LTD induced in control conditions and in presence of ACEA (1 μM) and AM251 (1 μM) (p > 0.05). The results illustrated in this and the previous section, indicate that EC-dependent transmission is not involved in the induction of muscarinic dependent LTD in the Prh cortex of the rat, induced with both electrical and chemical stimulation (see section 4. for discussion).

3.1.8. Role of the CB1 activation in TBS-induced LTP (Bristol)

Application of TBS determined the induction of a robust LTP (Fig 3.10. A, n = 26, baseline: 99.3% ± 0.7%; 60 min follow-up: 114.8% ± 2.8%, p < 0.01) in the Prh of juvenile male Dark Agouti rats. In order to assess the role of the EC transmission in the induction of TBS-LTP, the CB1 agonist ACEA (1 μM) and antagonist AM251 (1 μM) were bath applied. As shown in Fig 3.10., both ACEA (1 μM) (Fig 3.10. B, n = 12, baseline: 98.9% ± 1.3%; 60 min follow-up: 103.2% ± 4.0%, p > 0.05) and AM251 (1 μM) (Fig 3.10. C, n = 8, baseline: 96.5% ± 0.6%; 60 min follow-up: 97.1% ± 2.8%, p > 0.05) both blocked the induction of TBS-LTP. There was no significative difference in the LTP magnitude between controls and in presence of ACEA (1 μM) (p > 0.05) and between controls and AM251 (1 μM) (p > 0.05) but there was a significant difference between controls and AM251 (1 μM)

. These results indicate a role for EC-dependent transmission in the LTP induction in the Prh cortex of the rat (see section 4. for discussion).

3.1.9. Role of the astrocytes in the TBS-induced LTP (Bristol)

Even if eCBs are usually associated to the depression of synaptic transmission (see section 1.4. for details), recent studies showed that probably this is a narrow vision of the phenomenon, suggesting a double functional role for eCBs in both potentiation and depression of synaptic transmission (Abush and Akirav, 2009; Navarrete and Araque, 2010). Expecially, Navarrete and Araque showed in the CA1 of hippocampal slices that eCBs, that are activity dependently released by neurons, induce depression when they bind the CB1 on the presynaptic terminal, and potentiation when they bind the CB1 on the proximal astrocytes via increase in the astrocyte intracellular Ca^{2+} and, in turn, glutamate release and binding to the presynaptic mGluR1. Since in this study I observed a role for eCBs in the LTP induction in the Prh of juvenile male Dark Agouti rats, in this section I evaluated the role of astrocytes i LTP induction by applying TBS on slices pre-incubated for at least 50 min in aCSF added with the glial toxin fluoracetate 5 mM (FAC) (Henneberger et al., 2010).

Application of TBS determined the induction of a robust LTP (A, n = 26, baseline: 99.3% ± 0.7%; 60 min follow-up: 114.8% ± 2.8%, $p<0.01$) in the Prh of juvenile male Dark Agouti rats. The pre-incubation of the slice in aCSF containing FAC (5 mM; at least 50 min) does not affect the magnitude of LTP (B, n = 6, baseline: 100.1% ± 1.8%; 60 min follow-up: 127.2% ± 10.9%, $p<0.01$). These results indicate that astrocytes are not involved in the induction of LTP (see section 4. for discussion).

3.2. Role of the NO and eCBs in visual recognition memory acquisition

The behavioural experiments were kindly performed by dr Gareth Barker under the supervision of dr Clea Warburton. The drugs were locally infused into the Prh of a rat via bilaterally implanted cannulas (see section 2.4. for methodological details). The recognition memory was quantitatively evaluated via the spontaneous preferential exploration of a novel versus a familiar object (Ennaceur and Delacour, 1988) using delays of 20 min and 24 hours.

3.2.1. Effect of the neuronal NOS (nNOS) in the acquisition of visual recognition memory (Bristol)

The selective antagonist for the neuronal isoform of NOS (nNOS) N-ω- propyl L arginine (NPA, 2 μM) was locally infused into a rat's Prh via bilaterally implanted cannulas (Warburton et al., 2003) (Fig. 2.3.). Recognition memory was assessed with spontaneous novel object exploration task (Ennaceur and Delacour, 1988), using delays of 20 min and 24 h. For each delay, NPA or vehicle was infused 15min before the initial familiarization phase in order to test the effect of blocking nNOS during acquisition. Analysis of the discrimination at test revealed a significant drug by delay interaction [$F(1,20)=12.99$, $p<0.01$] and a significant effect of drug $F[1,20)= 18.18$, $p<0.001$] but no significant effect of delay [$F1,20)= 4.09$, $p>0.05$]. Analysis of the significant main effects revealed that the NPA infused animals were significantly impaired compared to the vehicle infused animals at the 24h ($p<0.001$) but not the 20 min delay ($p>0.1$). Additional analysis confirmed that the vehicle infused animals discriminated between the novel and familiar objects at both delays tested [20min $t(9)= 4.50$, $p<0.001$; 24h $t(11)= 7.07$, $p<0.001$]; in contrast the NPA infused animals only showed discrimination between the novel and familiar object at the 20min delay [$t(9)= 2.76$, $p<0.05$] but not at the

24h delay [t(11)= -1.13, p>0.1]. Analysis of the time taken to complete the sample phase and the amount of exploration completed in the sample and test phases revealed no significant interaction between treatment and delay [for all F<1.0, p>0.1] and no significant effect of drug [time to complete sample phase F(1,20)= 2.78, p>0.1; exploration in sample phase F<1.0, p>0.1; exploration in test phase F<1.0, p>0.1]. However there was a significant effect of delay on the amount of exploration completed in the test phase [F(1,20)= 4.88, p<0.05] which reflected the fact that both vehicle and NPA infused animals spent significantly more time exploring the objects at the 20min delay than the 24h delay; there was no significant effect of delay on the amount of time taken to complete the sample phase [F<1.0, p>0.1] and the amount of exploration completed in the sample phase [F(1,20)=2.36, p>0.1] (See table 3.1. for means).

3.2.2. Effect of the cannabinoid receptor 1 (CB1) in the acquisition of visual recognition memory (Bristol)

AM251 (2 µM) or vehicle was infused 15 min before the training phase, in order to investigate the role of CB1 in the acquisition of new visual recognition memory. Analysis of the discrimination at test revealed a non significant drug by delay interaction [F(1,18)<1.0, p>0.1] a non significant effect of drug [F(1,18)<1.0, p>0.1] and no significant effect of delay [F(1,18)<1.0, p>0.1]. Analysis of the significant main effects revealed that the AM251 infused animals were not significantly impaired compared to the vehicle infused animals at the 24h (p>0.01) and the 20 min (p>0.01). Additional analysis confirmed that the vehicle infused animals discriminated between the novel and familiar objects at both delays tested [20min t(9)= 5.19, p<0.001; 24h t(9)= 8.28, p<0.001], just as the AM251 infused animals [20min t(9)= 2.93, p<0.05; 24h t(9)= 7.66, p<0.001]. Analysis of the time taken to complete the sample phase and the amount of exploration completed in the

sample and test phases revealed no significant interaction between treatment and delay [time to complete sample phase $F(1,18)<1.0$, $p>0.1$; exploration in sample phase $F(1,18)=4.36$, $p>0.05$; exploration in test phase $F(1,18)<1.0$, $p>0.1$] and no significant effect of drug [for all $F(1,18)<1.0$, $p>0.1$]. Also there was not a significant effect of delay on the time taken to complete the sample phase and the amount of exploration completed in the sample [time to complete sample phase $F(1,18)= 2.16$, $p>0.1$; exploration in sample phase $F(1,18)<1.0$, $p>0.1$]; however there was a significant effect of delay on the amount of exploration completed in the test phase [$F(1,18)= 7.42$, $p<0.05$] which reflected the fact that both vehicle and AM251 infused animals spent significantly more time exploring the objects at the 20min delay than the 24h (See table 3.1. for means).

3.3. Early impairment in basal synaptic transmission and LTD in the perirhinal cortex of Tg2576 mice

These results show an early impairment in the basal synaptic transmission and LTD induction in Prh of 3 month old transgenic mice carrying the Swedish mutation on the gene for the APP (Tg2576), an animal model widely used for the systematic investigation of AD (see section 2.3. for methodological details). Field potential recordings were performed on Prh containing brain slices (see section 2.3. for methodological details). I observed a significant impairment in the input/output (I/O) curve at 200 mV of stimulation but not at lower voltages (Fig 3.12. A, at 200 mV, littermate controls, n=7: 1.53 mV ± 0.09 mV; Tg2576, n=8: 1.09 mV ± 0.10 mV, $p < 0.01$). The application of 5 Hz LFS determines the induction of a robust LTD in the Prh from littermate controls (Fig 3.12. B, n = 5, baseline: 96.2% ± 1.2%; 60 min follow-up: 81.9% ± 4.0%, $p < 0.01$); the same stimulation protocol does not induce LTD in the Prh from Tg2576 (Fig 3.12. C, n = 8, baseline: 104.6% ± 2.6%; 60 min follow-up: 97.9% ± 3.4%, $p > 0.05$). The

magnitude of LTD between littermate controls and Tg2576 is significantly different ($p < 0.01$).

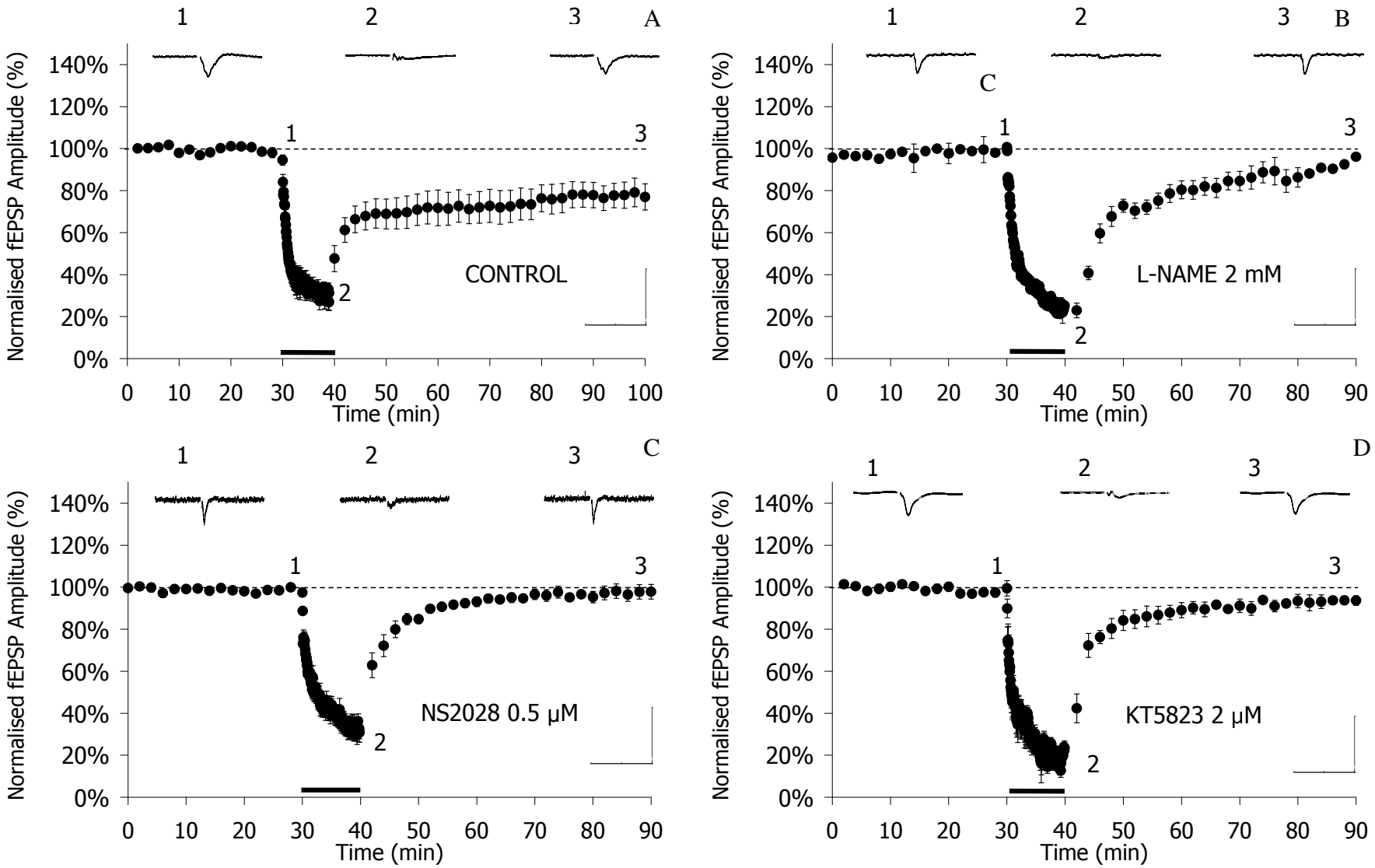


Fig 3.1. Role of NO in 5 Hz-induced LTD in Prh in brain slices from juvenile Sprague-Dawley rats. 5 Hz-induced LTD (A, n=11, p<0.01) is blocked by antagonists of the NOS (L-NAME; 2 mM) (B, n=6, p>0.05), sGC (NS2028; 0.5 µM) (C, n=7, p>0.05) and PKG (KT5823; 2 µM) (D, n=4, p>0.05). Black bar: 5Hz-LFS. In this and in the following figures data are shown as mean values ± S.E.M. Scale bar: 20 ms, 1 mV (in this and following figures)

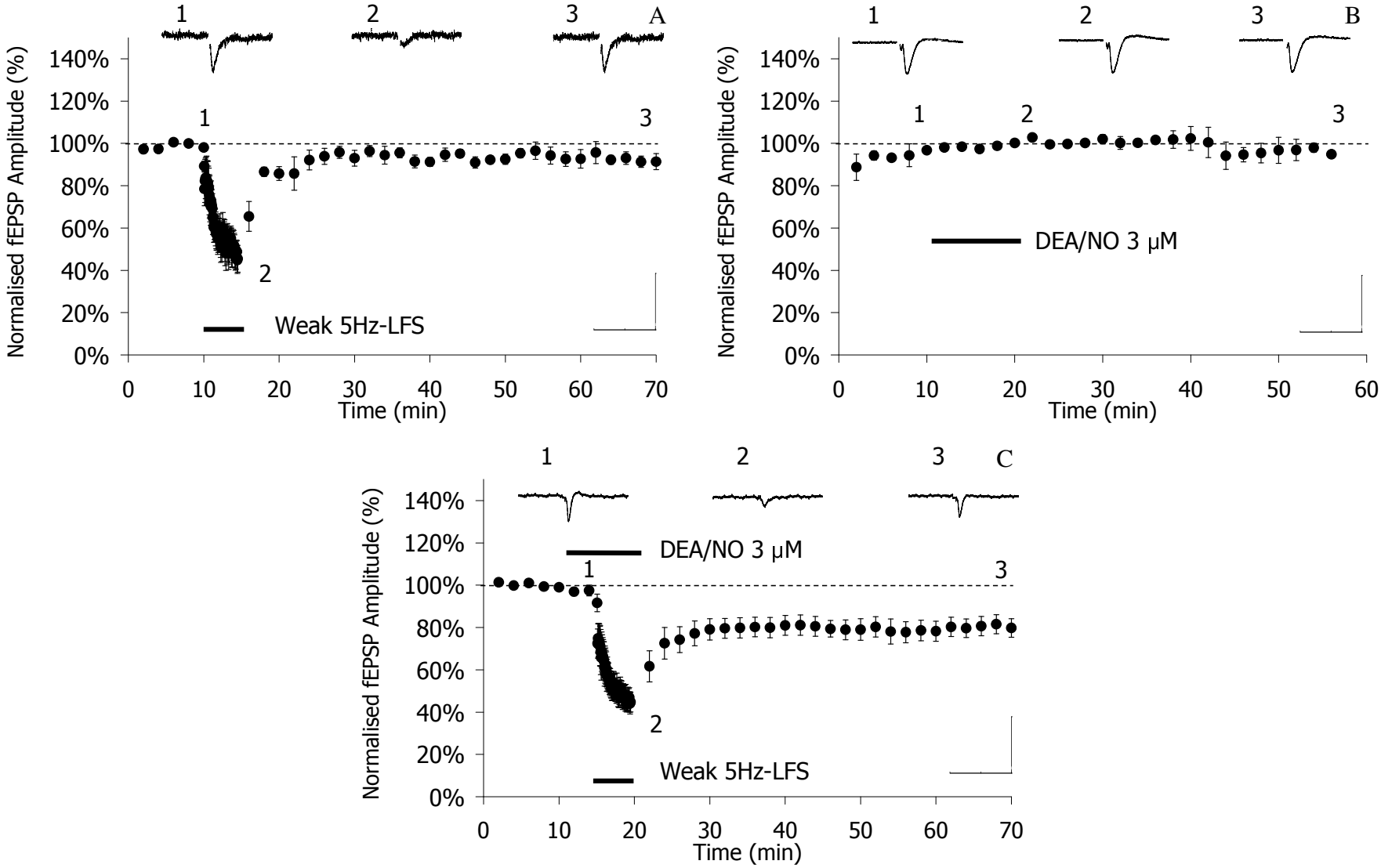


Fig 3.2. Role of NO in 5 Hz-induced LTD in Prh in brain slices from juvenile Sprague-Dawley rats. Neither a weak 5 Hz LFS consisting in 1350 pulses (A, n=9, p>0.05) nor bath application of the NO donor DEA/NONOate (3 µM) (B, n=4, p>0,05) induce LTD, but the co-application of the two protocols induce a robust and persistent LTD (C, n=9, p<0,01).

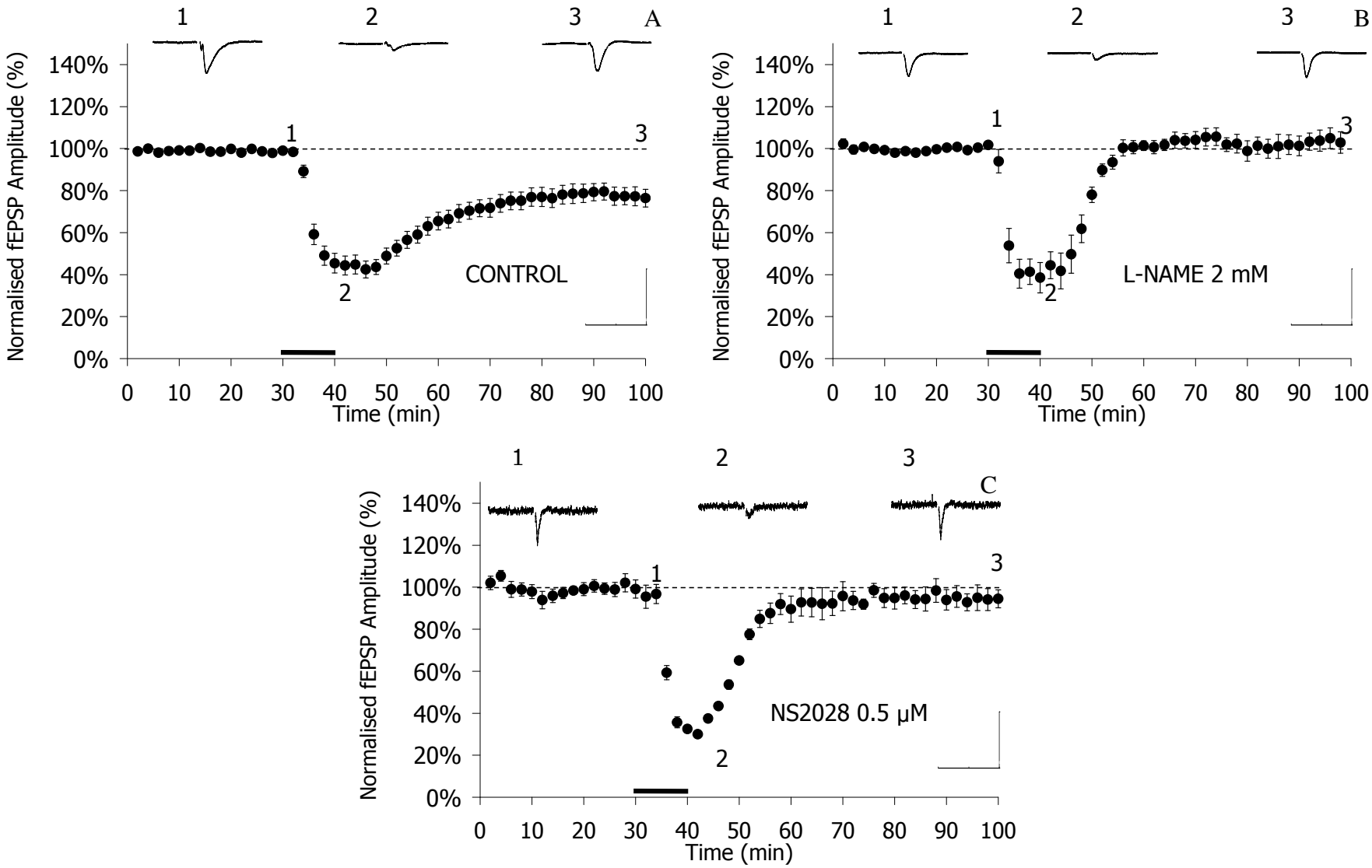


Fig 3.3. Role of the NO/sGC pathway in CCh-induced LTD in Prh in brain slices from juvenile Sprague-Dawley rats. Bath application of Cch 50 µM for 10 min induces LTD (A, n=21, p<0.01) in control slices, but not in slices pre-treated with the NOS antagonist L-NAME (2 mM) (B, n=8, p>0.05) or the sGC antagonist NS2028 (0.5 µM) (C, n=9, p>0,05). Black bar: Cch 50 µM

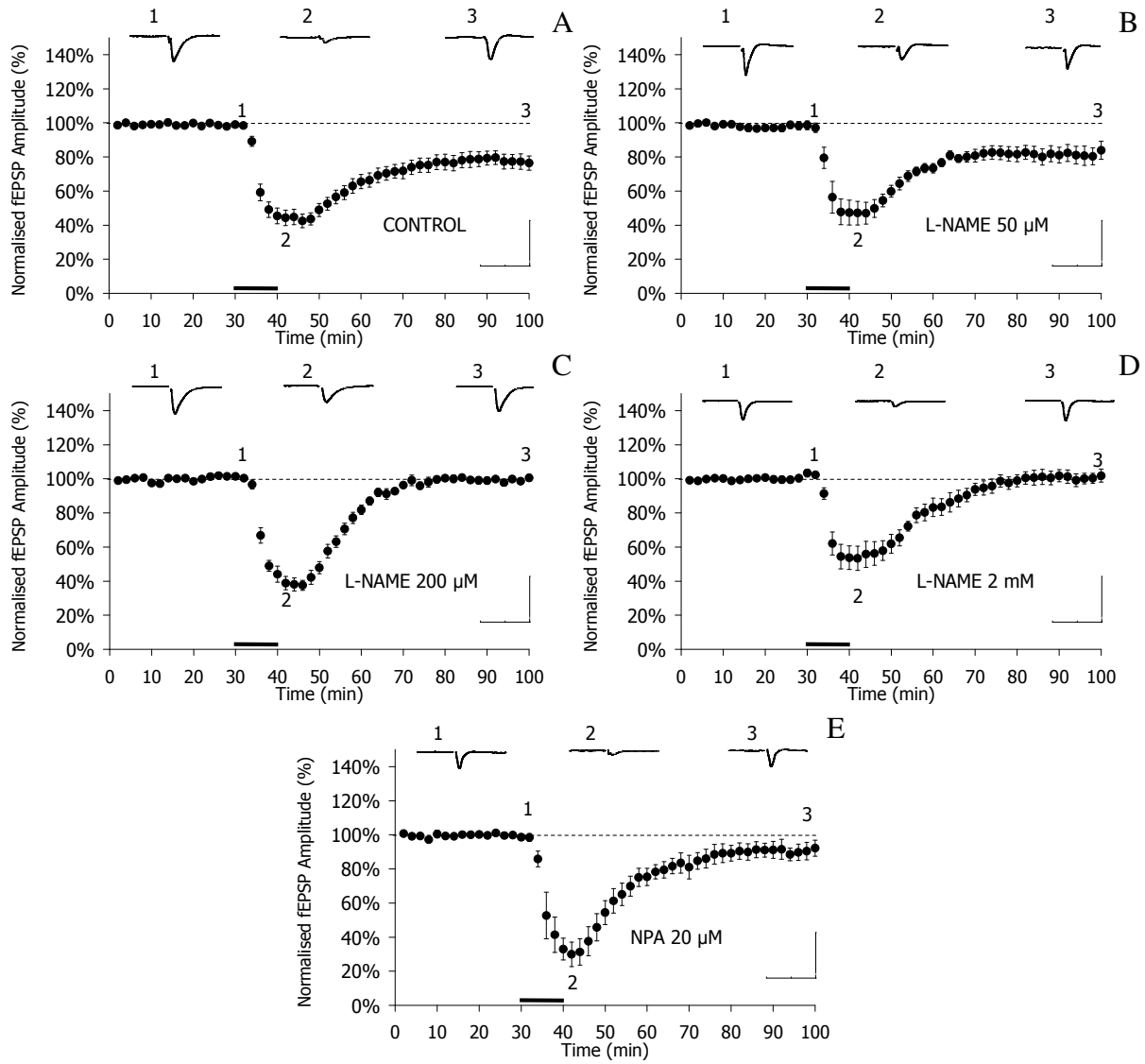


Fig 3.4. Role of NO in CCh-induced LTD in Prh in brain slices from juvenile Dark Agouti rats. Bath application of Cch 50 µM for 10 min induces LTD in control slices (A, n=21, p<0.01) and in slices pre-treated with the NOS antagonist L-NAME 50 µM (B, n=7, p<0.01) but not in those pre-treated with L-NAME 200 µM (C, n=9, p>0.05) or 2 mM (D, n=5, p>0.05) or with the nNOS selective antagonist NPA (E, n=5, p<0.01). Black bar: Cch 50 µM

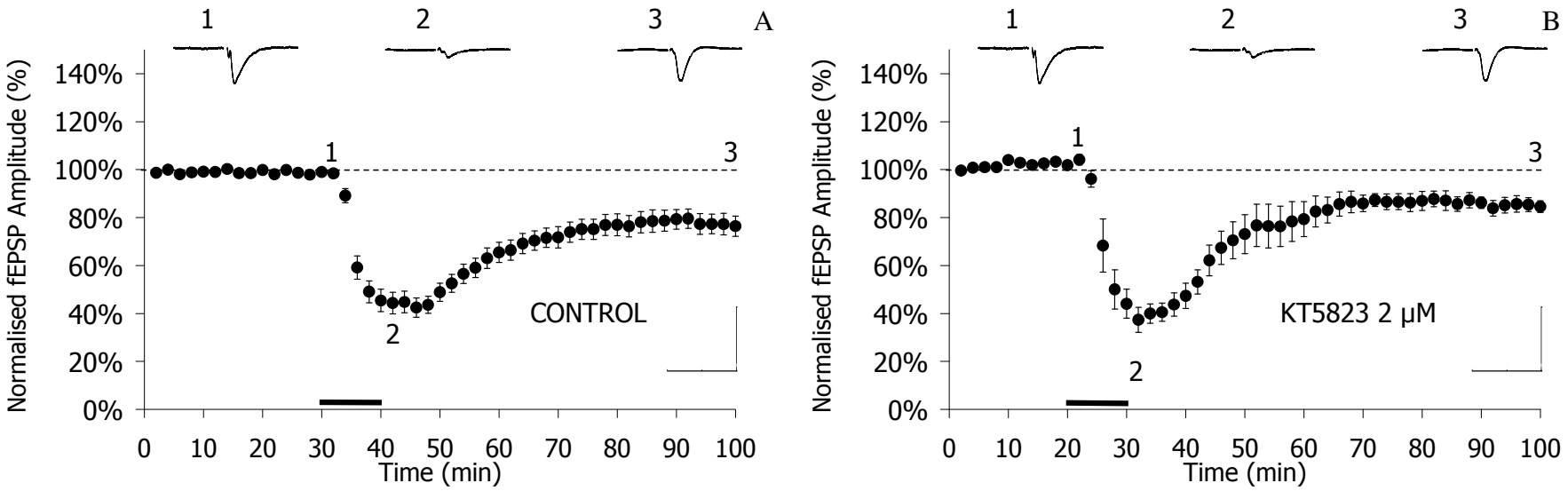


Fig 3.5. Role of PKG in CCh-induced LTD in Prh in brain slices from juvenile Dark Agouti rats. Bath application of Cch 50 µM for 10 min induces LTD in control slices (A, n=21, p<0.01). The PKG antagonist KT5823 (2 µM) does not affect Cch-LTD induction (B, n=7, p<0,01). Black bar: Cch 50 µM

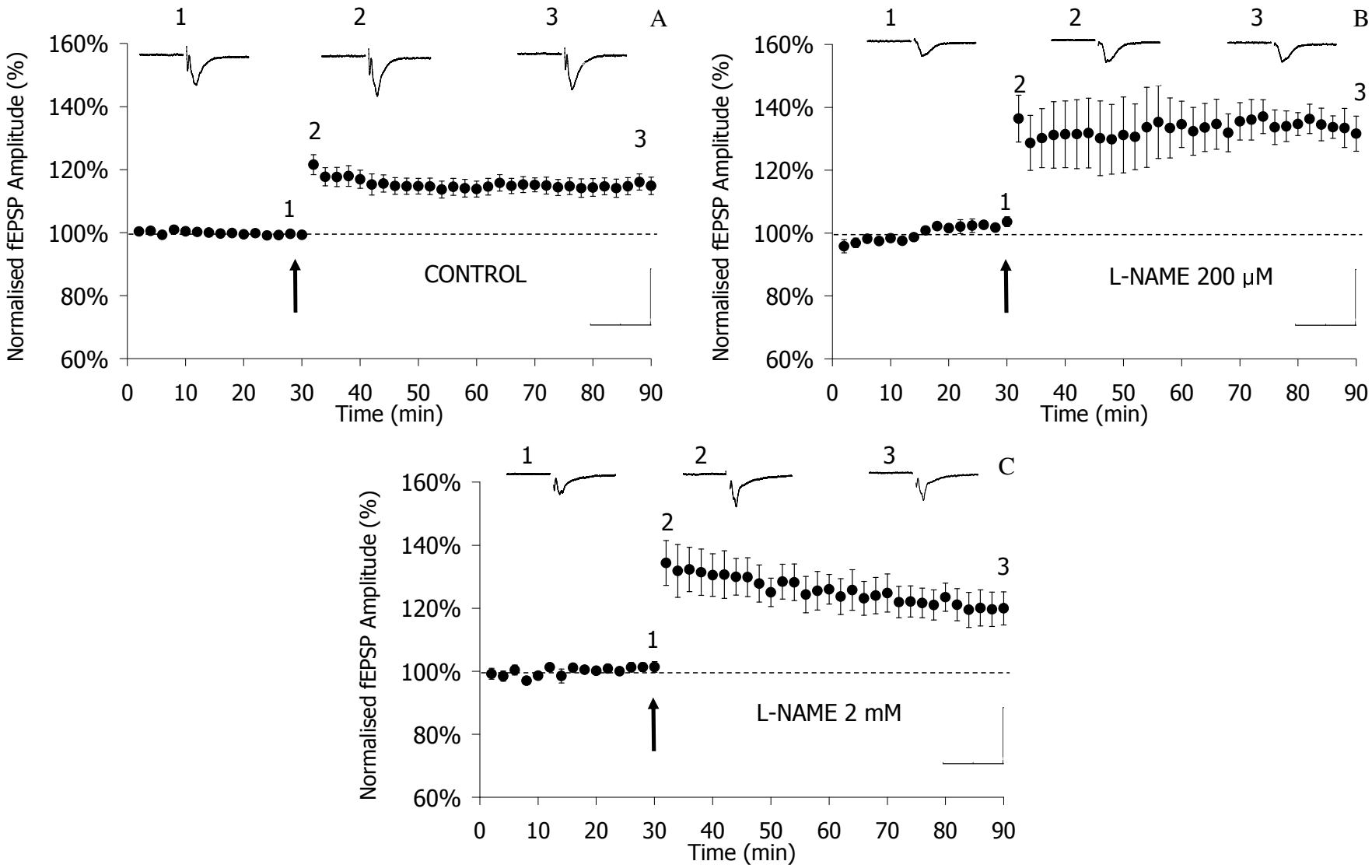


Fig 3.6. Role of NO in TBS-induced LTP in Prh in brain slices from juvenile Dark Agouti rats. TBS induces a robust LTP (A, n=26, p<0,01) in control slices. LTP induction is not affected by the NOS antagonist L-NAME 200 µM (B, n=4, p<0,01) or 2 mM (C, n=6, p<0,01). Black arrow: TBS

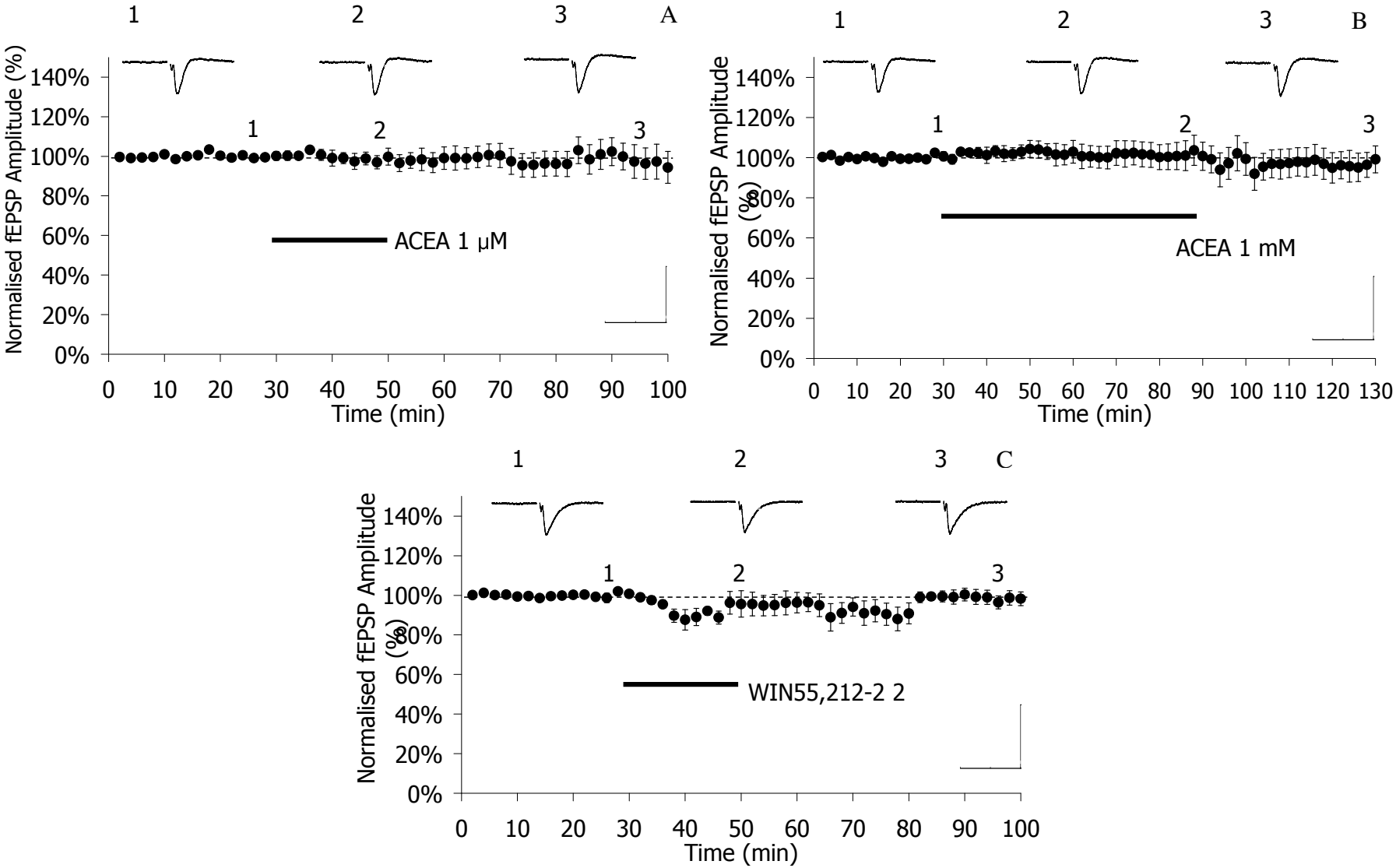


Fig 3.7. Role of CB1 in basal synaptic transmission in Prh in brain slices from juvenile Dark Agouti rats. Basal synaptic transmission is not affected by bath application of the CB1 selective agonist ACEA (1 µM) for 20 min (A, n=6, p>0.05) or 60 min (B, n=6, p>0.05), or the CB1/CB2 agonist WIN55,212-2 (2 µM) for 20 min (C, n=4, p>0.05).

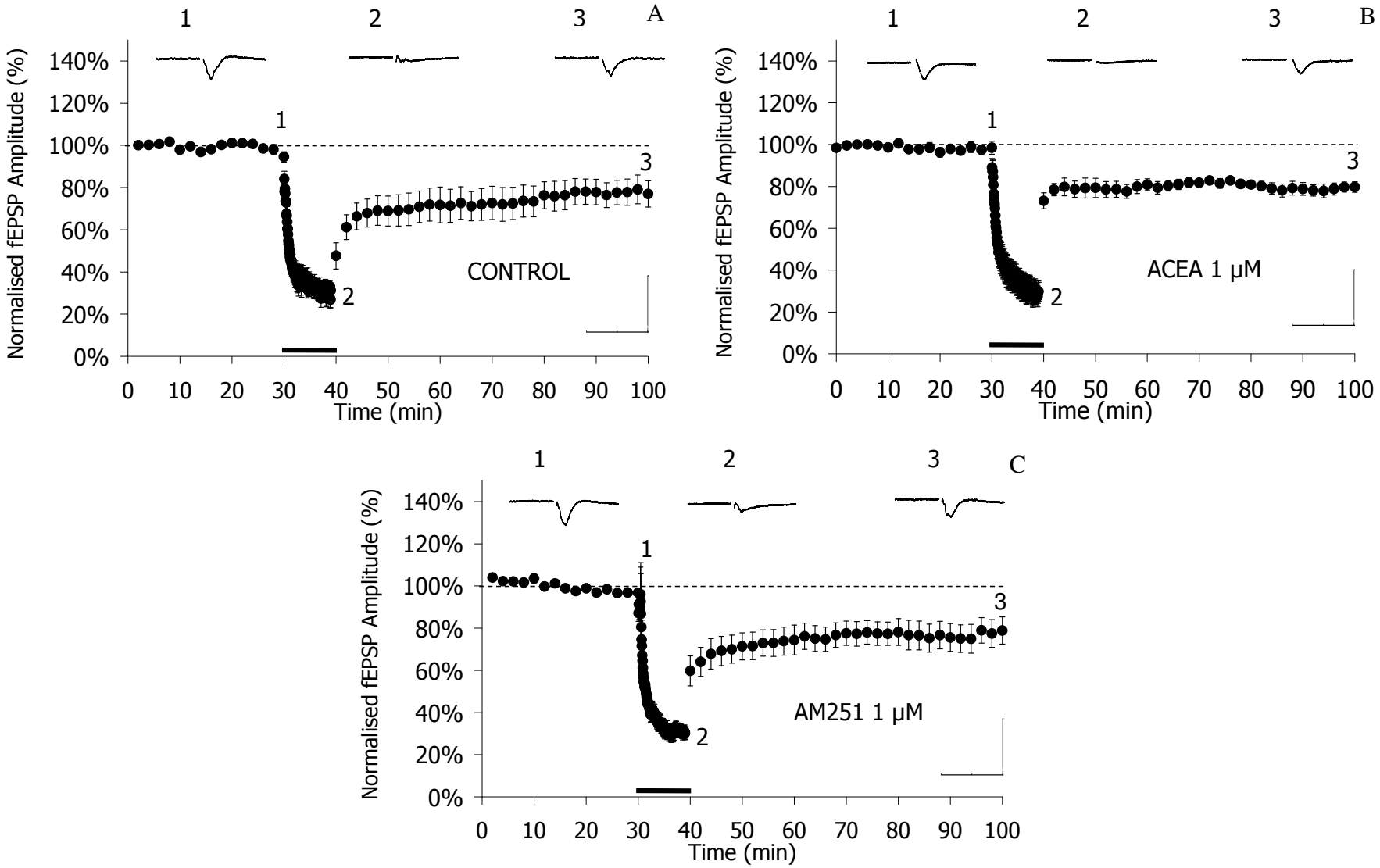


Fig 3.8. Role of CB1 in 5Hz-induced LTD in Prh in brain slices from juvenile Dark Agouti rats. 5Hz-induced LTD (A, n=11, p<0.01) is not affected by bath application of the CB1 selective agonist ACEA (1 µM) (B, n=7, p<0.01) or the CB1 selective antagonist AM251 (1 µM) (C, n=5, p<0.01). Black bar: 5Hz-LFS

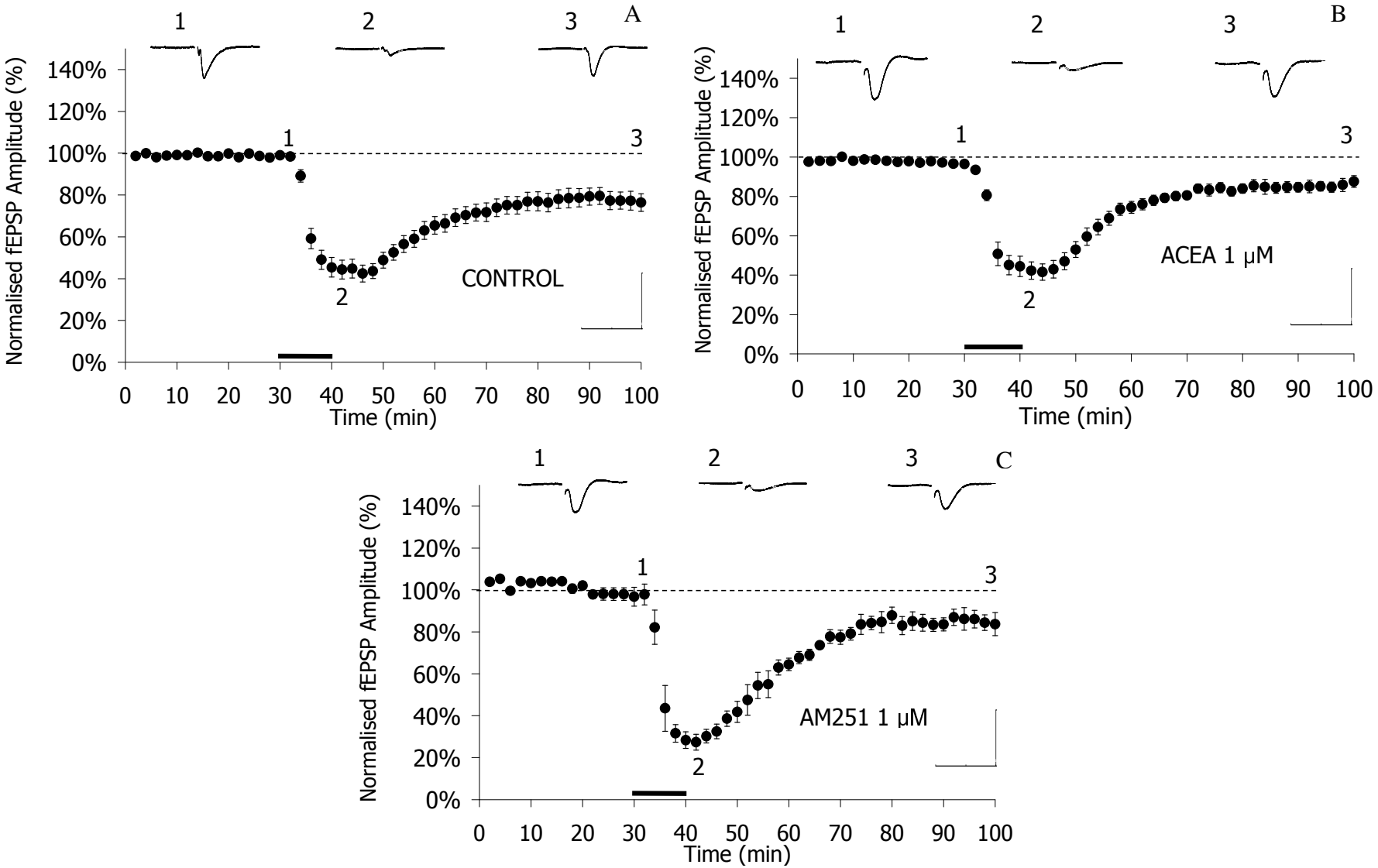


Fig 3.9. Role of CB1 in Cch-induced LTD in Prh in brain slices from juvenile Dark Agouti rats. Cch- LTD (A, n=21, p<0.01) is not affected by bath application of the CB1 selective agonist ACEA (1 µM) (B, n=7, p<0.01) or the CB1 selective antagonist AM251 (1 µM) . (C, n=5, p<0.01). Black bar: Cch 50 µM

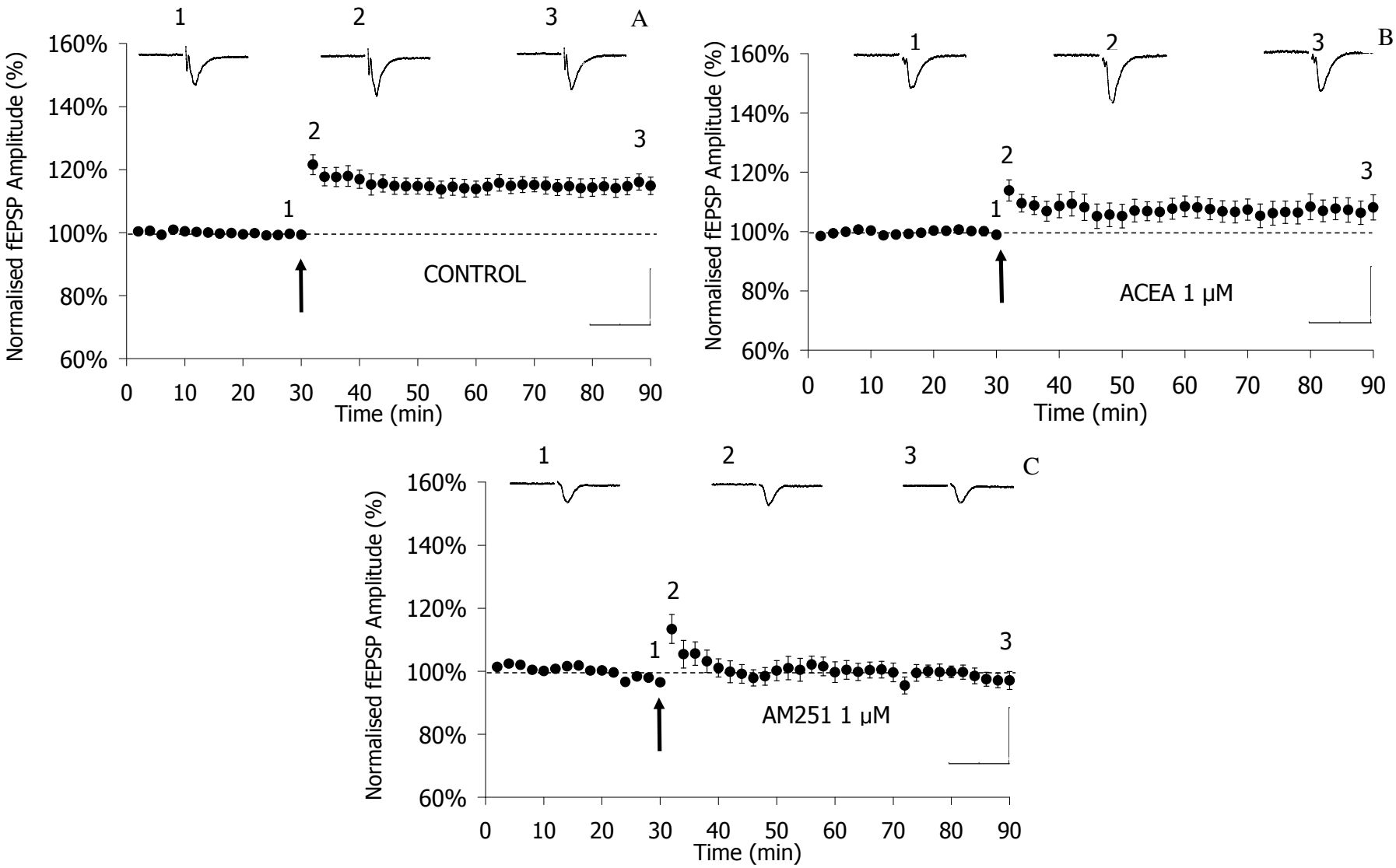


Fig 3.10. Role of CB1 in TBS-induced LTP in Prh in brain slices from juvenile Dark Agouti rats. TBS-induced LTP (A, n=26, p<0.01) is blocked by bath application of the CB1 selective agonist ACEA (1 µM) (B, n=13, p>0.05) or the CB1 selective antagonist AM251 (1 µM) (C, n=8, p>0.05). Black arrow: TBS

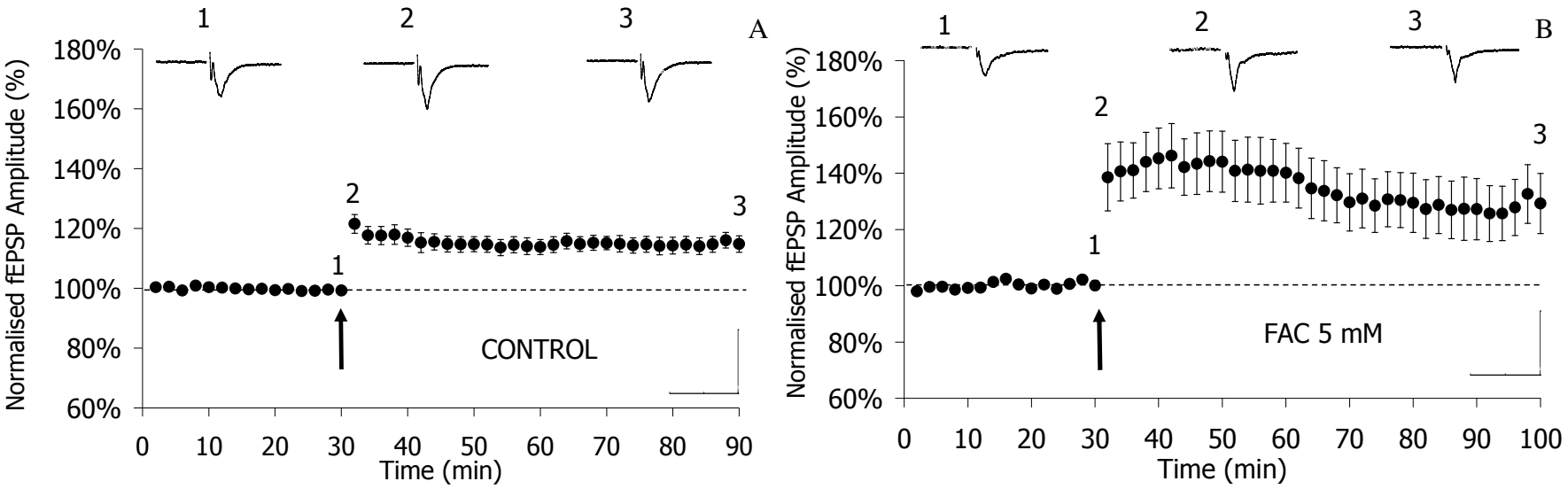


Fig 3.11. Role of astrocytes in TBS-induced LTP in Prh in brain slices from juvenile Dark Agouti rats. TBS application induces a robust LTP (A, n=26, p<0.01); 50 min pre-incubation of the slices in aCSF containing the glial toxin FAC (5 mM) does not affect LTP induction (B, n=6, p<0.01). Black arrow: TBS

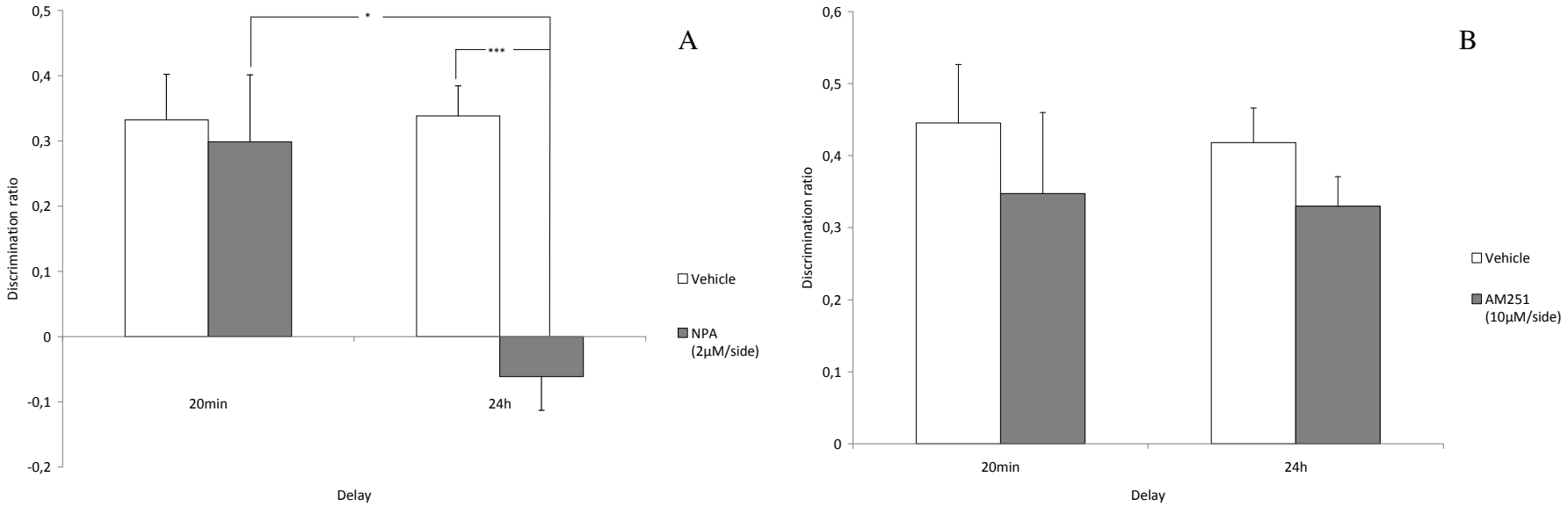


Fig 3.12. Role of NO and endocannabinoids in visual recognition memory acquisition in juvenile Dark Agouti rats. A. Infusion of the nNOS selective antagonist NPA (2 µM) in the Prh impairs longer-term (24 h) but not shorter-term (20 min) recognition memory. Data are presented, for each group, as mean (±SEM) DR (proportion of additional time spent exploring a novel rather than a familiar object). For control animals, DR was significantly different from zero (i.e. they discriminate between novel and familiar) at both delays, whereas for NPA (2 µM) treated animals DR was significantly different from zero at 20 min but not at 24 h. *p < 0.01 difference between the 20 min and 24 h delay within NPA-treated animals; ***p < 0.001, difference between vehicle- and NPA-treated animals at the 24 h delay. B. Infusion of the CB1 selective antagonist AM251 (10 µM) in the Prh does not affect recognition memory at both delays.

Infusion	Delay	Time to complete acquisition phase (s)	Total exploration in acquisition phase (s)	Total exploration in test phase
Vehicle	20 min (n=10/group)	190 ± 14	34 ± 3	33 ± 3
NPA		210 ± 13	34 ± 2	31 ± 2
		$F_{(1,20)} < 1.0$; NS	$F_{(1,20)} < 1.0$; NS	$F_{(1,20)} < 1.0$; NS
Vehicle	24 hours (n=12/group)	214 ± 11	36 ± 1	26 ± 1
NPA		227 ± 6	35 ± 1	27 ± 2
		$F_{(1,20)} < 1.0$; NS	$F_{(1,20)} < 1.0$; NS	$F_{(1,20)} < 1.0$; NS
Vehicle	20 min (n=10/group)	174 ± 15	40 ± 0.1	30 ± 3
AM251		191 ± 17	38 ± 1	34 ± 3
		$F_{(1,18)} < 1.0$; NS	$F_{(1,18)} < 1.0$; NS	$F_{(1,18)} < 1.0$; NS
Vehicle	24 hours (n=10/group)	169 ± 20	36 ± 2	25 ± 3
AM251		154 ± 18	39 ± 0.7	25 ± 2
		$F_{(1,18)} < 1.0$; NS	$F_{(1,20)} < 1.0$; NS	$F_{(1,18)} < 1.0$; NS

Tab 3.1. Role of NO and endocannabinoids in visual recognition memory acquisition in juvenile Dark Agouti rats: effect of the nNOS selective antagonist NPA and CB1 selective antagonist AM251 on general exploration behaviour. No significant (NS) differences in total exploration times were observed between control and treated animals; hence, the drugs had no significant effect on general exploration behaviour.

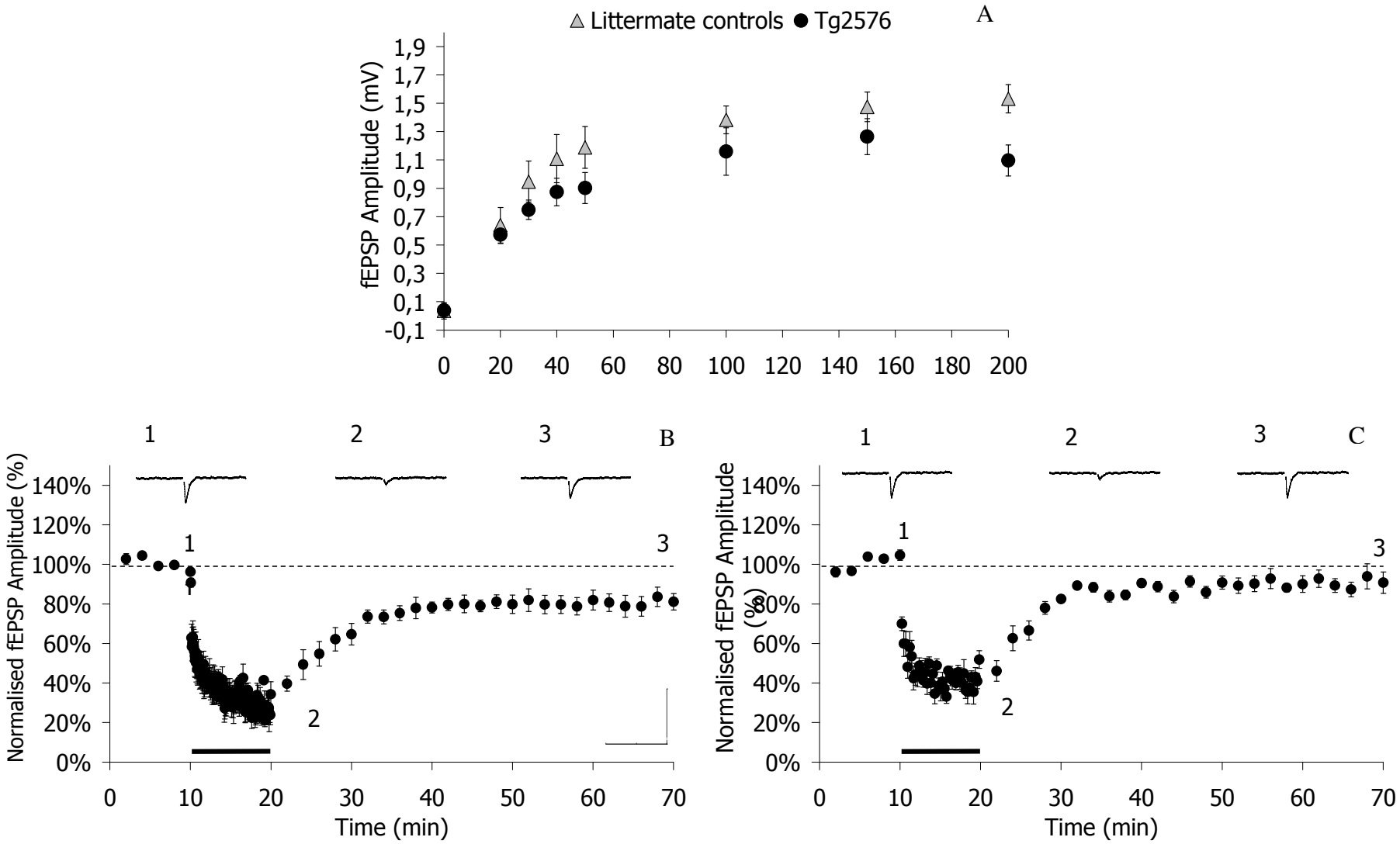


Fig 3.13. Early impairment in basal synaptic transmission and LTD in the perirhinal cortex of Tg2576 mice. Input/output relationship revealing significant differences in basal synaptic transmission at 200 µA but not at lower stimulation intensities (A, △ littermate controls, n=7, ● Tg2576, n=8). 5 Hz LFS induces a robust LTD in Prh in brain slices from littermate controls (B, n=5, $p<0.01$) but not in those from Tg2576 mice (C, n=8, $p>0.05$). Black bar: 5Hz-LFS

4. DISCUSSION

4.1. Role of NOS/sGC/PKG and CB1 in rat perirhinal cortex LTD and LTP induction

In the last decade many studies tried to clarify the cellular correlates underlying synaptic plasticity in Prh (Bilkey, 1996; Ziakopoulos et al., 1999; Cho et al., 2000; Massey et al., 2001, 2004; Aicardi et al., 2004; Jo et al., 2006; Jo et al., 2008; Griffiths et al., 2008; Seoane et al., 2009). The induction of LTD in the rat Prh has been shown to depend on the activation of different receptors, including glutamate ionotropic receptors (NMDAR; KR) (Cho et al., 2000; Park et al., 2006), metabotropic glutamate receptors (mGluRs) (Cho et al., 2000; reviewed by Cho and Bashir, 2002; reviewed by Bashir, 2002; Jo et al., 2006; Jo et al., 2008), and metabotropic acetylcholine (Ach) receptor (muscarinic 1; M1) (Massey et al., 2001; reviewed by Bashir, 2002). Several studies have highlighted that LTD may be the main cellular correlate of visual recognition memory. *In vivo* studies showed that the neuronal activity is reduced in the Prh of an animal performing a visual recognition task (Brown and Wilson, 1987; Fahi et al., 1993; Li et al., 1993; Miller et al., 1993; Xiang et al., 1998). This view has been confirmed by immunohistochemical observations: the expression of the immediate early gene c-fos is reduced in the 'familiar' hemisphere of the Prh of rats that received the paired-viewing protocol (Zhu et al., 1996; Warburton et al., 2003; Massey et al., 2008). Further *in vitro* electrophysiological studies confirmed that LTD in acute Prh slices represents the the neuronal event related to familiarity acquisition (Warburton et al., 2003; Massey et al., 2008, Griffiths et al., 2008).

LTP induction relies on NMDAR activation (Bilkey, 1996) and requires TrkB activation by BDNF (Aicardi et al., 2004), even if the role of this form of synaptic plasticity in visual recognition memory is still not clear.

Cholinergic neurotransmission has been shown to have a pivotal role in visual recognition memory acquisition and LTD induction in the rat Prh (Massey et al., 2001; Warburton et al., 2003; Winters and Prickaerts 2005; Winters et al., 2006a,b; Winters et al., 2007).

There is still lacking evidence of a retrograde messenger able to coordinate pre- and post-synaptic changes occurring in Prh for the induction and consolidation of synaptic plasticity. Here, I investigated the role of NO (and the associated downstream pathway, that is: sGC and PKG) and the cannabinoid receptor 1 (CB1) in both LTD and LTP in the Prh of juvenile rats.

4.1.1. Role of NOS/sGC/PKG in LTD and LTP induction in the rat perirhinal cortex

The first part of this study is focused on the role of NOS/sGC/PKG pathway in LTD induction in the rat Prh. NO is a small amphiphillic molecule physiologically produced by the enzyme NO synthase (NOS) in response to increased intracellular Ca^{2+}. NOS is expressed in three different isoforms: neuronal and endothelial (nNOS and eNOS, respectively), that are constitutively expressed and are activated by the Ca^{2+}/calmodulin complex, and the inducible isoform (iNOS), which is Ca^{2+} -independent and it is physiologically expressed at very low levels in macrophages and glia. Its expression is induced by an inflammatory insult. NO is widely produced throughout the body, and it mainly acts as a relaxant of the smooth muscles and therefore represents a major component in the regulation of the blood flow (reviewed by Toda and Okamura, 2003). In the vertebrate CNS, NO is involved in many functions: i) neuronal proliferation and development (Mize and Lo, 2000; Contestabile and Ciani, 2004; Estrada and Murillo-Carretero, 2005), ii) synaptic plasticity, memory and learning (Susswein et al., 2004; Garthwaite et al., 2008; Steinert et al., 2010) iii) neurodegeneration (Contestabile et al, 2003). NO

mainly acts through the activation of the downstream enzyme soluble guanylate cyclase (sGC), that converts GTP in the second messenger cGMP; cGMP in turn activates many targets, including PKG. cGMP is hydrolised to GMP by 11 different isoforms of phosphodiesterases (PDEs), that therefore act as negative control system on the NO-dependent transmission. For a more extensive overview on NO see section 1.3 and subsections.

NO is involved in the induction of both LTP and LTD in many brain areas including hippocampus, cerebellum and prefrontal cortex (Garthwaite & Boulton, 1995; Holscher, 1997; Prast & Philippu, 2001; Susswein et al., 2004; Garthwaite et al., 2008; Steinert et al., 2010). Hippocampal LTP at SC/CA1 excitatory synapses requires NOS/sGC/PKG activation: here NO acts as a retrograde messenger (Arancio et al., 1996, 2001, Bon and Garthwaite, 2003). Similarly, NO mediates hippocampal LTD via Ca^{2+} release from cyclic ADP-ribose sensitive stores (Reyes-Harde et al., 1999). The activation of the NOS/sGC/PKG pathawy has been demonstrated to be involved in cerebellar heterosynaptic LTD (Shibuki and Kimura, 1997; Shin and Linden, 2005) and homosynaptic LTP (Jacoby et al., 2001).

Cholinergic neurotransmission has been shown to play a central role in the acquisition of visual recognition memory (Massey et al., 2001; Warburton et al., 2003; Winters et al., 2005, 2006, 2007). Recent studies showed the association between M1-dependent LTD and the activation of the NOS/sGC/PKG pathway in the prefrontal cortex (Huang et al., 2009), corticostriatal synapses (Centonze et al., 2003) and lizard neuromuscular junction (Graves et al., 2004). A recent study has shown that NO and eCBs signalling cascades interact in the induction of the corticostriatal group I mGluR-dependent DHPG-LTD (Sergeeva et al., 2007). M1 is a g_q-coupled receptor and hence its activation results in PLC activation that

converts the phosphatidil inositol bisphosphate (PIP_2) in diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG is usually associated with protein kinase C (PKC) activation while IP_3 is responsible for Ca^{2+} release from intracellular stores. It has been recently shown that M1/M3 activation brings to NO production in the rat retina (Borda et al., 2005).

NO synthase is highly expressed in the rat Prh (Liu et al., 2003a,b). Furthermore, the induction of 5Hz-LTD in the Prh of juvenile (p28-35) rats is M1-dependent (Jo et al., 2006). In addition, bath application of the cholinergic agonist carbachol (Cch, 50 μM) for 10 min results in M1-dependent LTD in rat Prh: this form of Cch-induced LTD (Cch-LTD) requires intracellular Ca^{2+} release and protein synthesis but does not require PKC and protein phosphatases activation (Massey et al., 2001). These observations suggest that M1-dependent LTD and NMDAR-dependent LTP in Prh may rely on the activation of the NOS/sGC/PKG pathway.

In the present work, evoked extracellular field potentials were recorded on II/III layer of horizontal Prh slices from juvenile (p21-35) rats. Fig 3.1 shows that the LTD induced by the application of a low frequency stimulation consisting in 3000 pulses delivered at 5 Hz (5Hz-LTD) in juvenile (p21-35) Sprague-Dawley rats (A) is blocked by bath application of: the NOS antagonist L-NAME (2 mM) (B), the sGC antagonist NS2028 (0,5 μM) (C) and the PKG antagonist KT5823 (2 μM) (D). These data confirmed that the NOS/sGC/PKG pathway is necessary for the induction of 5Hz-LTD, suggesting that NO can be the retrograde messenger involved in this form of synaptic plasticity in the Prh of juvenile rats.

To confirm this result, a second subset of experiments was carried out with an opposite approach: instead of blocking NO production, a NO donor, diethylamine NONOate (DEA/NO 3 μM), was bath applied. DEA/NO releases NO in a pH- and temperature-dependent fashion: at a pH=7.4 and T=32°C, the half-life of DEA/NO

is ~ 6 min (Bon and Garthwaite, 2001). Previous studies showed that DEA/NO at 3 μM does not affect basal synaptic transmission at hippocampal SC/CA1 synapses (Bon and Garthwaite, 2003), but it induces a transient depression and subsequent potentiation at 300 μM (Bon and Garthwaite, 2001). DEA/NO (300 μM)-dependent transient depression does not rely on the activation of sGC; probably it is a consequence of the neurotoxic effect of the exogenous NO that, at those concentrations, can interfere with the cell oxydative phosphorylation. However, the subsequent potentiation has been demonstrated to require sGC activity (Bon and Garthwaite, 2001). DEA/NO at 3 μM does not affect basal synaptic transmission but causes an increase in intracellular cGMP levels. DEA/NO co-application with a weak tetanus, unable to induce synaptic plasticity, results in the induction of a robust and prolonged LTP (Bon and Garthwaite, 2003). Consistently, DEA/NO (3 μM) does not affect basal synaptic transmission in rat Prh, as shown in Fig 3.2 B. Fig 3.2 A shows that the application of a weak 5Hz-LFS consisting of 1350 instead of 3000 pulses is not strong enough to result in LTD induction. As shown in Fig 3.2. C, the co-application of the two protocols (DEA/NO 3 μM and weak 5Hz-LFS) results in the induction of a robust and prolonged LTD. These results suggest that low concentrations of NO are necessary but not sufficient for LTD induction, raising the possibility that LFS activates other cellular mechanisms involved in LTD, complementing the depressing activity of NO.

Consistently with the work of Massey et al. (2001), I observed that bath application of the cholinergic agonist carbachol (Cch, 50 μM) for 10 min induced a robust LTD in the Prh cortex of juvenile (p21-35) Sprague-Dawley rats (Fig 3.3. A; 3.4. A). As shown in Fig 3.3. B, the blockade of NOS by the non selective NOS antagonist L-NAME (2 mM) resulted in the inhibition of Cch-LTD induction.

Consistently with the trend observed in the 5Hz-LTD experiments, the bath application of the sGC antagonist NS2028 (0.5 μM) resulted in the blockade of Cch-LTD induction.

As explained in section 2.1, this project has been carried out in two different laboratories in slightly different conditions: in Bristol the experiments were performed on p28-35 Dark Agouti (DA) rats instead of the p21-35 Sprague-Dawley rats used in Bologna (for more details on experimental differences between the two laboratories see section 2.1). Therefore, the first series of experiments carried out in Bristol was performed in order to confirm the role of NOS in Cch-LTD. As shown in Fig 3.4, L-NAME blocked Cch-LTD induction at 200 μM (C) and 2 mM (D) but not at 50 μM (B). Finally, since in physiological conditions both eNOS and nNOS are constitutively active, and since L-NAME is a non selective antagonist of both these constitutive isoforms of NOS, I verified the role of the nNOS in Cch-LTD. Bath application of the nNOS selective antagonist NPA (20 μM) effectively blocked Cch-LTD compared to controls (Fig 3.4. E). This result does not exclude a role for eNOS in Cch-LTD, but it confirms the role of the nNOS, providing an interesting cellular correlate of the behavioural data discussed below (see section 3.2 and 4.2). In order to better define the respective potential role for nNOS and eNOS in Cch-LTD, a good experimental strategy could be to perform experiments on single nNOS, eNOS or double eNOS/nNOS KO mice (Son et al., 1996; reviewed by Steinert et al. (2010). Finally, Fig 3.5 shows that PKG is not involved in Cch-LTD induction, since bath application of KT5823 did not block Cch-LTD induction compared to controls. This is at variance with the results obtained with 5Hz-LFS application, that showed the involvement of the entire NOS/sGC/PKG pathway in LTD induction. It is plausible to assume that Cch-LTD relies on the activity of the second messenger cGMP on different effectors from PKG: for

instance, it could directly act on cyclic-nucleotide dependent channels (CNC) or on voltage gated potassium channels 1(Kv1). Another possible explanation is that the chemical protocol is much stronger than the electrical one, i.e. activates multiple intracellular pathways: thus, even if PKG was activated by the application of both induction protocols, KT5823 did not block Cch-LTD because the activation of other downstream Ca^{2+}-dependent mechanisms compensated the PKG blockade. Further investigation is needed to explain this discrepancy.

NO has been very well characterized as a retrograde messenger in hippocampal NMDAR-dependent LTP induction (Arancio et al., 1996; 2001; Puzzo et al., 2005; Wang et al., 2005). Therefore, I also verified the possible role of NO in Prh LTP. LTP in Prh is NMDAR-dependent (Bilkey, 1996). The NMDAR activation is usually strongly associated with NO production. Indeed, nNOS is intracellularly co-localized at the postsynaptic density with NMDAR (see section 1.4.1. for details). Nonetheless, Fig 3.6 shows that NO is not involved in LTP in Prh, since L-NAME (200 μM or 2 mM) does not block its induction.

4.1.2. Role of CB1 LTD and LTP induction in the rat perirhinal cortex

The next step in this study was to evaluate the role of eCBs in synaptic plasticity in the rat Prh. As described in section 1.5, eCBs are lipidic messengers involved in memory, learning and synaptic plasticity, expecially LTD. Their production is triggered by the activation of several G_q-coupled receptors, includiong group I mGluRs and M1. G_q-coupled receptor activation results in PLC activation as a consequence of Ca^{2+} release from the intracellular stores mediated by IP_3. PLC is also activated by other several sources of intracellular Ca^{2+} increase (i.e. NMDAR and/or VGCC activation). PLC activation results in the production of DAG that is the substrate for diacylglicerol lipase (DGL) that converts DAG in 2 arachidonyl glicerol (2-AG). 2-AG and anandamide (whose synthetic pathway is

still to be clarified) represent the two known eCBs. The eCBs membrane transporter (EMT) mediates the eCBs release in the synaptic cleft. Here they act presynaptically by binding the CB1, mainly expressed in the CNS, or the CB2, mainly expressed in the peripheral nervous system. CB1 is a $G_{i/o}$-coupled receptor that mediates the decreased activation of adenylate cyclase (AC) and, in turn, decreased PKA activity. This should result in decreased glutamate secretion from the presynaptic terminals. This phenomenon is regarded as eCB-mediated LTD (eCB-LTD), described in many brain areas such as striatum, prefrontal cortex and hippocampus (see section 1.5.; reviewed by Heyfetts and Castillo, 2009).

Since it has been observed high expression of CB1 in Prh (Tsou et al., 1998; Liu et al., 2003c), and since M1 is a G_q-coupled receptor, I investigated the possible role of eCBs in M1-dependent LTD (both electrically and chemically induced) in Prh. Although NO is critically involved in this form of LTD in Prh (as discussed in section 4.1.1.), a role of eCBs can not be excluded, since group I mGluR-dependent LTD in corticostriatal glutamatergic synapses relies on the sequential activation of CB1 and NOS (Sergeeva et al., 2007). Fig 3.7 shows that the selective agonist ACEA (1 μM) did not affect basal synaptic transmission in rat Prh slices; the same trend was observed with the CB1/2 non selective agonist WIN55,212-2 (2 μM). Furthermore, Fig 3.8 and 3.9 show that ACEA (1 μM) or the CB1 antagonist AM251 (1 μM) did not affect the induction of both 5Hz-LTD and Cch-LTD, compared to control. These result suggests that eCBs are not involved in Prh LTD in our experimental conditions. Recent studies have shown that CB1 activation may play a role in potentiation of synaptic responses in rat hippocampus (Abush and Akirav, 2009; Navarrete and Arraque, 2010). It has been shown that intraperitoneal injection of AM251 impaired LTP induction at the SC/CA1 synapses, while the inhibitor of the reuptake and breakdown of the eCBs AM404

facilitated it (Abush and Akirav, 2009). Furthermore, a recent elegant study showed that eCBs can both induce potentiation and depression in the SC/CA1 synapses, depending on the cell type on which they act. More precisely, a neuronal depolarization results in activity-dependent release of eCBs, which bind the presynaptic CB1 and induce depression. But if the eCBs bind the CB1 expressed on the astrocytes proximal to the site of neuronal depolarization, this results in increased Ca^{2+} concentration in the astrocytic cytosol, causing release of glutamate on the presynaptic neuron, which in turn increases glutamate release via activation of the presynaptic group I mGluRs (Navarrete and Araque, 2010).

In order to investigate if a similar involvement of CB1 activation in potentiation also occurs in Prh, I carried out LTP experiments in presence of ACEA or AM251. As shown in Fig 3.10,C, AM251 (1 μM) blocked LTP, suggesting that CB1 activation is necessary for LTP induction. On the other hand, Fig 3.10B shows that also the agonist ACEA (1 μM) blocks LTP.. Thus, CB1 activation appears necessary for LTP induction, because LTP induction is compromised when it is blocked. But when CB1 is continuously activated by an agonist (ACEA) LTP is inhibited, possibly due to a form of desensitization. This hypothesis needs to be verified by further experiments.

According to the study cited above on the role of astrocytic CB1 in hippocampal LTP (Navarrete and Araque, 2010), in order to investigate the role of astrocytes in this novel form of CB1-dependent LTP in the Prh cortex of juvenile rats, I carried out a series of experiments on Prh slices preincubated with the astrocytic toxin fluoracetate (FAC, 5 mM) for at least 50 min (as described by Henneberger et al., 2010). As shown in Fig 3.11., the selective astrocytic degeneration did not affect LTP induction in the rat Prh. Hence, it remains to be

clarified the cellular machinery responsible for this new atypical form of synaptic plasticity.

4.2. Role of nitric oxide and endocannabinoids in the acquisition of visual recognition memory

As described in section 1.4.4 and 1.5.4, several behavioural studies on animal models confirmed the role of both NO and eCBs (and the relative signalling cascade) in many forms of memory and learning, consistently with the evidence of their involvement in synaptic plasticity.

The cellular correlate of visual recognition memory has been extensively explored in the last years. The main approach consisted in the direct infusion of drugs into the Prh of bilaterally cannulated rats (reviewed by Brown et al., 2010). These drugs mainly consist in agonists or antagonists of membrane receptors. The visual recognition memory abilities of the animals were then assessed with the spontaneous novel object exploration task. In the training phase of this test, the animal familiarizes with two identical objects in an arena in which the animal should acquire visual memory. After a certain delay (20 min or 24 h), the animal is placed back in the arena where one of the familiar objects is replaced with a novel object: if it has memorized the previous objects, it tends to spend more time exploring the novel object rather than the familiar one. Hence, if visual recognition memory is intact, the ratio of the exploration time between the novel and the familial object is high; when visual recognition memory is compromised, the animal equally explore both the novel and the familiar object, and the exploration ratio will result low. The animals infused with a specific drug are compared with control animals receiving saline: the infusion of the drug at different stages of the experiment (before or after the training phase, or before the test phase) gives information about the stage of visual recognition memory (acquisition,

consolidation, retrieval) affected by the drug. So far it has been observed that the the AMPA antagonist CNQX impairs both acquisition and retrieval (Winters and Bussey, 2005). Futhermore, blockade of NMDAR and group I and II mGluRs results in the impairment of visual recognition memory acquisition when tested at 24 h but not at 20 min (Barker et al., 2006a,b). Blockade of the kainate receptor (KR) impairs visual recognition memory acquisition at 20 min but not at 24 h (Barker et al., 2006b), just as recently observed when M1 is blocked by scopolamine (Tinsley et al., in submission). Also, many lines of evidence underlie the pivotal role of cholinergic transmission in visual recognition memory acquisition (Warburton et al., 2003; Winters and Prickaerts, 2005; Winters et al., 2006a,b; Winters et al., 2007; Massey et al., 2008).

Recent studies showed the involvement of NO in visual recognition memory: the intraperitoneal injection of the non-specific NOS antagonist L-NAME, before and immediately after the training phase, successfully blocked visual recognition memory acquisition in a spontaneous novel object exploration task at 24 hours but not at 1 hour. Furthermore, L-NAME administered before the test phase at 24 hours rescued the delay-dependent deficit in visual recognition memory (Boultadakis et al., 2010a). Another study showed that the systemic administration of a NO donor (NCX2057) rescues the visual recognition memory deficit caused by the previous systemic administration of the muscarinic antagonist scopolamine (Boultadakis et al., 2010b). Moreover, L-NAME systemic administration can rescue the visual recognition memory acquisition deficit induced by the NMDAR antagonist MK-801 and ketamine (Boultadakis and Pitsikas, 2010). All these studies have two major limits: first, L-NAME is a non selective antagonist of NOS, therefore it may affect a broad range of physiological functions, including blood flow (see section 1.4.); second, the administration was systemic and therefore the

drug did not selectively act on Prh. Another recent study showed that intrahippocampal infusion of the antagonists of the NOS/sGC/PKG pathway results in the impairment of the acquisition and the consolidation of visual recognition memory, suggesting that this pathway can exert a critical role in this form of memory. Interestingly, it has been shown that NOS/sGC/PKG activation leads to the activation of β-adrenergic receptors and, in turn, increases BDNF synthesis (Furini et al., 2009). This study has two main limits: the first one is that they used Wistar rats, which are characterized by low visual acuity; second, they worked on hippocampus, that is not specifically involved in visual recognition memory (reviewed by Brown et al., 2001; Brown et al., 2010).

In order to overwhelm these limits, in the present study I included some behavioural results obtained in collaboration with Dr. Clea Warburton. The experiments were kindly performed by Dr. Gareth Barker. These experiments evaluated the role of NO in visual recognition memory. A selective antagonist for the nNOS, N-ω-propyl L arginine (NPA, 2 μM), was bilaterally infused into the Prh of Dark-Agouti adult rats. Since nNOS is selectively expressed in neurons and does not have a role in the blood flow regulation, we used of a specific antagonist for nNOS to avoid possible artifacts due to reduced blood flow in the infused area. Furthermore, we used Dark-Agouti rats which are characterized by a relatively high visual acuity, compared to other strains. Finally, the intra-Prh injections allowed us to evaluate the behavioural effects of the drug selectively acting on the brain area of interest. The dose of 2 μM in 1 μL was chosen in order to selectively act on the nNOS, since at higher concentrations NPA can lose specificity (supplier data, Tocris). As shown in Fig 3.12A, bilateral intra-Prh infusion of NPA (2 μM) 15 min before the training phase successfully blocked visual recognition memory when tested at a delay of 24 hours but not at 20 min. This behavioural evidence is

consistent with present electrophysiological data (nNOS is necessary for Cch-LTD, see Fig 3.4 E) and with previous studies showing that cholinergic neurotransmission in Prh is necessary for visual recognition memory acquistion (Warburton et al., 2003; Winters and Bussey, 2005; Winters et al., 2006a,b; Winters et al., 2007; Massey et al., 2008).

A recent study has shown that intra-Prh infusion of scopolamine generates a deficit in visual recognition memory acquisition when tested at 20 min but not at 24 h; the opposite temporal pattern (deficit at 24 h but not at 20 min) was observed for the α7 nicotinc antagonist MLA (Tinsley et al., in preparation). The reciprocal involvement of muscarinic and nicotinic receptors in visual recognition memory acquisition tested at short and long term, is similar to what observed for glutamate metabotropic receptors (Barker et al., 2006a,b). Assuming that nNOS is activated by M1 activation, my observation that NPA blocks acquisition of visual recognition memory when tested at 24 h but not at 20 min appears in temporal contrast with data obtained with scopolamine.

In a second subset of behavioural experiments it has been evaluated the role of the CB1 receptor in acquisition of visual recognition memory tested at 20 min and 24 h. As shown in Fig. 3.12. B, the CB1 antagonist AM251 (10 μM) did not affect visual recognition memory acquisition tested at both time points. This result is consistent with the electrophysiological evidence that CB1 is not involved in LTD induction. On the other hand, the functional relevance of the observed CB1 involvement in LTP induction remains to be elucidated, because the relationship between LTP in Prh and visual recognition memory is still not defined. Interestingly, Abush and Akirav (2009) observed that intra-hippocampal infusion of the CB1 antagonist AM251 and the CB1/2 agonist WIN55,212-2 does not affect the performance in an inhibitory avoidance test, while the endocannabinoid

reuptake and breakdown antagonist AM404 enhances it. Furthermore, in Morris water maze test, animals treated both intra-hippocampally and systemically with WIN55,212-2, AM251 or AM404, are all impaired in spatial memory compared to controls. Also, many studies investigating the role of eCBs in memory and learning suggest that CB1 is mainly involved in memory extinction phenomena (Chhatval et al., 2005; Varvel et al., 2007). Therefore, the finding that CB1s is not involved in visual recognition memory acquisition it's not surprising. Further investigations are required to evaluate the possible role of eCBs in consolidation, retrieval and delay-dependent impairment of visual recognition memory. Finding an active role for CB1 in visual recognition memory might, in turn, help to understand the physiological role of LTP in this brain region.

4.3. Early deficit in the synaptic transmission and plasticity in the perirhinal cortex of an Alzheimer's disease murine model

Most of the studies on AD carried out on transgenic murine models, including the Tg2576 model, have been focused on testing hippocampal functions with behavioural experiments on spatial memory, *in vitro* electrophysiological recordings and molecular biology studies (immunohistochemistry, western blots, RT-PCR). However, hippocampus is not the only area of the temporal lobe affected by the cholinergic loss and by the accumulation of soluble β-amyloid oligomers. Therefore, hippocampus-related memory is not the only impaired cognitive function in AD patients. In particular, visual recognition memory is highly impaired in the very first stages of the disease in humans affected by MCI (Didic et al., 2010; Viggiano et al., 2008). Prh is highly innervated by cholinergic fibres coming from the forebrain basal nuclei, which exert a pivotal role in Prh activity and visual recognition memory (reviewed by Winters et al., 2008; reviewed by Brown et al., 2010).

For these reasons, in the present study I have investigated synaptic functions (transmission and plasticity) in Prh slices from 3 month old Tg2576 mice and from littermate controls. The input/output curve presented in Fig 3.13A shows a significant deficit in basal synaptic transmission at high intensity of stimulation. Furthermore, Fig 3.13B shows that 5Hz-LFS application results in the induction of a robust LTD in littermate control but not in Tg2576 mice Prh slices. Considering the relationship between LTD in Prh and visual recognition memory, one may suppose that the very early deficit in Prh synaptic plasticity might underlie a deficit in visual recognition memory. A recent study demonstrated that 5 month old Tg2576 mice show a deficit in visual recognition memory when tested in a spontaneous novel object exploration task at 4 and 24 hours, but not at 2 min after the training phase, compared to littermate controls. The deficit observed was successfully reversed by the systemic administration of the calmodulin antagonist MK506 (Taglialatela et al., 2008). Interestingly, these results are in contrast with another study that showed that 14 month old Tg2576 mice did not show any visual recognition memory deficit when tested with the same behavioural protocol at 24 hours (Hale and Good, 2005). This discrepancy was interpreted by Taglialatela et al. as a consequence of the different genetic background of the murine strains used to obtain the mutants. It would be worthwhile to perform the same experiment at 3 months of age, and to try to evaluate molecular differences between Tg2576 and the littermate controls. These preliminary results suggest that the study of Prh alterations in AD can provide a novel experimental platform to better understand the etiopathology of the disease, which may lead to new therapeutic strategies.

CONCLUSIONS

The pivotal role of Prh in visual recognition memory is well established, and in the last 15 years many behavioural, electrophysiological and molecular studies have investigated the cellular and molecular mechanisms underlying this cognitive function. In particular, it has been shown that LTD-like rather than LTP-like phenomena underlie visual recognition memory formation, and that cholinergic neurotransmission is a crucial component. Although many facets of the synaptic functions in Prh have been clarified, there is still lacking evidence of a retrograde messenger coordinating pre- and post-synaptic changes in long-term potentiation (LTP) and long-term depression (LTD). NO and eCBs have been extensively investigated as retrograde messengers in synaptic plasticity in other brain areas, and their role in memory and learning is well estabilished.

In the present study, an integrated behavioural-electrophysiological approach was used to evaluate the role of NO and eCBs in synaptic plasticity in the Prh and in visual recognition memory. The results obtained indicate that NO is selectively involved in LTD induction (both chemically and electrically induced), but not in LTP induction. Conversely, the eCB receptor CB1 is selectively involved in LTP, but not in LTD. The latter was an upredicted result, since previous studies in other brain regions have shown a selective involvement of this receptor in LTD induction. The electrophysiological data were confirmed in behavioural experiments, which demonstrate that nNOS plays an important role in the acquisition of visual recognition memory, whereas CB1 appears not involved in this process. The role of this receptor in LTP induction suggests its possible involvement in other aspects of visual recognition memory, which need further investigation.

Finally, preliminary data from a transgenic murine model of AD (Tg2576) showed a very early impairment (3 months of age) in Prh synaptic transmission and plasticity. This is an interesting result, since one of the major goals in AD research is to characterize the early stages of the disease in order to improve diagnostic and therapeutic tools. Prh may provide a good experimental platform for the investigation of the pathogenesis of this debilitating disorder.

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