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Dissertação orientada pela Doutora Diana Cunha Reis e pelo Doutor Rodrigo Almeida

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Esta dissertação é dedicada a minha família, em especial ao meu pai e ao meu avô Vasquito que mesmo não estando fisicamente presentes me estão no coração.

O trabalho experimental descrito nesta tese foi realizado no Instituto de Farmacologia e Neurociências da Faculdade de Medicina de Lisboa e Unidade de Neurociências do Instituto de Medicina Molecular, sob orientação da Doutora Diana Cunha Reis e do Doutor Rodrigo Almeida (Centro de Química e Bioquímica).

Resumo

O VIP (do Inglês 'vasoactive intestinal peptide') é um péptido neuromodulador que se encontra largamente distribuído no sistema nervoso central e periférico e tem um papel determinante em muitas acções biológicas em mamíferos. No hipocampo, é expresso exclusivamente em interneurónios, sugerindo um envolvimento na regulação da transmissão GABAérgica (GABA, do inglês 'γ-Aminobutyric acid') do hipocampo. O hipocampo é uma estrutura cerebral envolvida fundamentalmente na formação e associação de memórias e orientação espacial. Apresenta um circuito neuronal unidireccional constituído por três áreas principais: giros dentado, com células granulares a constituírem a principal camada (stratum granulosum); área cornu Ammonis 3 (CA3), enervada pelos axónios das células granulares do giros dentado e constituída por uma camada principal de células piramidais (stratum piramidales); área cornu Ammonis 1 (CA1), também constituída por uma camada de células piramidais e enervada pelos axónios projectados da região CA3. Associado às células principais excitatórias está um sistema complexo de interneurónios inibitórios GABAérgicos. Os interneurónios estão envolvidos na regulação do ritmo teta no hipocampo, ritmo relacionado com a aprendizagem. Segundo a classificação de Somogyi e Klausberger existem vários tipos de interneurónios, mas apenas três tipos de interneurónios expressam VIP, sendo estas as únicas células que expressam VIP no hipocampo. Duas das populações de interneurónios que expressam VIP têm como alvo outros interneurónios, que são por sua vez responsáveis pela inibição das dendrites das células piramidais. A outra população (composta por interneurónios denominados células 'basket') enerva preferencialmente o corpo celular das células piramidais. Isto sugere que o VIP pode modular a transmissão sináptica para as células piramidais por duas vias alternativas. O VIP actua através da activação de dois receptores selectivos de afinidades semelhantes, o $VPAC_1$ e o $VPAC_2$, acoplados a proteínas G. Estudos prévios utilizando técnicas de imunohistoquímica, autorradiografia e hibridização in situ, revelaram que estes receptores têm uma distribuição não homogénea pelas diferentes camadas no hipocampo. Os receptores $VPAC_1$ situam-se principalmente no stratum oriens e radiatum do CA1, enquanto que os receptores $VPAC_2$ se encontram principalmente no stratum piramidale. Portanto, os receptores $VPAC_1$ encontram-se em pontos onde se situam as sinapses dos interneurónios que expressam VIP que têm como alvo outros interneurónios, sugerindo um papel regulador destes receptores na área CA1. Os receptores $VPAC_2$ localizam-se nas zonas de contacto entre células 'basket' e as células piramidais. No Sistema nervoso central é necessário existir um equilíbrio entre a actividade excitatória e inibitória, a transmissão glutamatérgica e a transmissão GABAérgica, caso contrário gera-se uma alteração na actividade eléctrica do cérebro (convulsões), designada por epilepsia. A epilepsia do lobo temporal (TLE, do inglês 'Temporal Lobe Epilepsy') é a forma mais comum e tem como consequências dificuldade de aprendizagem e perda de interneurónios no hipocampo. Observou-se, quer em pacientes quer em modelos animais da doença, que há um aumento do VIP endógeno e dos seus receptores no hipocampo. Em estudos prévios no nosso laboratório observou-se que o VIP inibe a potenciação de longa duração (LTP, do inglês 'Long-Term Potentiation') através da activação dos receptores $VPAC_1$, sendo que a LTP é o modelo in vitro mais aproximado no estudo da memória.

Sabendo que a cinase A de proteínas (PKA, do Inglês 'Protein kinase A') é importante na formação de memórias de longa duração e que a modulação da memória pelo VIP no hipocampo foi associada à selecção de memórias de longa duração, o objectivo deste trabalho foi investigar: 1) o envolvimento da PKA na LTP de fase tardia, 2) o envolvimento dos receptores $VPAC_1$ na LTP de fase tardia, e ainda 3) a influência das convulsões in vitro de epilepsia na LTP.

Para tal utilizaram-se fatias de hipocampo de ratos jovens (6-7 semanas) e jovens adultos (12-13 semanas) e efectuaram-se registos extracelulares de potenciais excitatórios pós-sinápticos evocados (fEPSPs, do inglês 'evoked field excitatory postsynaptic potentials'). Foi estimulada a área CA1 no stratum radiatum, ou seja, sobre as fibras colaterais de Schaffer e registaram-se os fEPSPs também no stratum radiatum na zona de contacto entre as fibras colaterais de Schaffer e as dendrites das células piramidais. Foi induzida a late-LTP por estimulação θ –burst utilizando dois protocolos de estimulação diferentes:, strong θ-burst (15x4 pulsos de 100Hz separados por 200ms, durante 3 segundo) e late-LTP θ-burst (3 estímulos strong θ-burst com 6 minutos de intervalo). Usou-se a estimulação weak θ-burst (5x4 pulsos a 100Hz separados por 200ms, durante 1 segundo) como controlo. O efeito na potenciação foi avaliado ao fim de uma hora para todos os protocolos anteriores e ao fim de duas horas para o late-LTP θ-burst. A Late-LTP é uma LTP com uma fase mais tardia, caracterizada no seu todo pela sua persistência de várias horas e dependência de PKA sendo um melhor modelo in vitro para estudo da memória, Para avaliar a influência da PKA e dos receptores $VPAC_1$ na Late-LTP induzida por estimulação θ-burst, foram utilizados os inibidores do PKA, H-89 e PKI 14-22 amida, e o antagonista selectivo dos receptores $VPAC_1$ (PG 97-269). A influência do modelo in vitro de convulsões na plasticidade sináptica, foi avaliada em fatias de hipocampo de rato de 6 semanas de idade com uma pré-incubação de bicuculina (50 µM) durante 30 minutos seguido de uma hora de lavagem. Em seguida foi induzida a LTP utilizando o protocolo de estimulação strong θ-burst, sendo avaliado o efeito na potenciação ao fim de uma hora.

A aplicação do H-89 (1 µM) e do PKI 14-22 amida (1 µM), 20 minutos antes da indução da LTP com o protocolo strong θ-burst em fatias de hipocampo em ratos jovens adultos, não alterou a potenciação quando comparando com a condição controlo (Controlo - H-89: 65,2 ± 10%, n = 4; H-89: 64,4 ± 1,5% n = 4; Controlo - PKI: 69,8 ± 15,5%, n = 3; PKI 14-22 amida: 61,8 ± 4,4%, n = 3). A perfusão do H-89 (1 e 3 µM) e do PKI 14-22 amida (1 µM), 20 minutos antes da indução da LTP com late-LTP θ-burst em ratos jovens adultos, não alterou a potenciação quando comparando com a condição controlo (Controlo: 80,5 ± 8,3%, n = 7, H-89 1µM: 74,2 ± 4,5%, n = 2, H-89 3 µM: n 76,2 ± 9,7%, = 5; PKI 14-22 amida: 75,0 ± 1,5%, n = 2). Estes resultados sugerem que a LTP, induzida tanto pelo protocolo strong θ-burst como pelo protocolo late-LTP θ-burst, é independente do PKA.

O papel dos receptores $VPAC_1$ na LTP foi avaliado com o antagonista do receptor $VPAC_1$ (PG 97-269). A aplicação do PG 97-269 (100 nM) no banho, 20 minutos antes da indução da LTP com o protocolo strong θ-burst em fatias de hipocampo de ratos de 12 semanas de idade, diminuiu a potenciação em 20% quando comparando à condição controlo (Controlo: 67,1 ± 5,6%, n = 5; PG 97-269: n 53,4 ± 4,8%, = 5, $p < 0,05$) e com uma maior concentração de PG 97-269 (300 nM), o efeito foi similar (Controlo: n 44,9 ± 8,2% = 3; PG 97-269: 37,1 ± 13,4%,

n = 2). Quando se avaliou a influência da intensidade do protocolo utilizado e da idade dos animais, a aplicação do PG 97-269 (100 nM), 20 minutos antes da indução da LTP com o protocolo weak θ-burst em ratos de 12 semanas, aumentou a potenciação quando comparando com a condição controlo (Controlo: 44,0 ± 0,2%, n = 3; PG 97-269: 59,7 ± 11,5%, n = 3) e usando um protocolo strong θ-burst em ratos de 6 semanas, o efeito foi similar (Controlo: 35,2 ± 6,6 %, n = 6; PG 97-269: 46,4 ± 4,5%, n = 6, p <0,05). A aplicação de PG 97-269 (100 nM), 20 minutos antes da indução da LTP com o protocolo late-LTP θ-burst em ratos de 12 semanas, diminui a potenciação quando comparando com a condição controlo (Controlo: 80,5 ± 8,3%, n = 7, H-89 1μM: 93,9 ± 7,8%, n = 2). Estes resultados sugerem que a activação dos receptores $VPAC_1$ leva a uma diminuição da LTP, excepto quando aplicado um protocolo de estimulação forte (strong θ-burst e late-LTP θ-burst) em ratos de 12 semanas, em que se observa um aumento da LTP pela activação dos receptores $VPAC_1$.

A potenciação obtida no estudo da influência da actividade epileptiforme in vitro na LTP (46,4 ± 8,9%, n = 2) em fatias tratadas foi semelhante quando comparada com a condição controle (sem tratamento). A temperatura utilizada na condição teste foi superior, sendo por isso impossível tirar qualquer conclusão quantitativa da relação mas os resultados sugerem que a LTP será menor, dado que o aumento de temperatura leva a um aumento da transmissão sináptica.

Em suma, estes resultados sugerem que a LTP induzida por estimulação teta é independente da PKA e o papel do receptor $VPAC_1$ na LTP induzida por um protocolo teta não é clara. Sendo por isso necessários mais estudos sobre a modulação do VIP endógeno na LTP para uma melhor compreensão do papel dos receptores $VPAC_1$ na LTP no hipocampo, porque passados 40 anos após a descoberta do VIP por Mutt e Said, o papel do VIP na transmissão sináptica no hipocampo continua incerto. Ainda assim, os dados obtidos nesta tese sugerem que a modulação pelo VIP poderá funcionar como um filtro na expressão da LTP.

Palavras-chave: VIP, LTP, $VPAC_1$, Interneurónios, PKA, Epilepsia

Abstract

Activation of $VPAC_1$ receptors by endogenous VIP, a known modulator of synaptic transmission to hippocampal CA1 pyramidal cells, was recently reported to inhibit long-term potentiation (LTP) induced by θ-burst stimulation in young adult rats. The role of endogenous VIP in the modulation of late-LTP was never evaluated either in healthy or epileptic adult rats.

The influence of $VPAC_1$ receptor activation by endogenous VIP on late-LTP was examined in the CA1 area of young (6-7 weeks) and adult (12-13 weeks) rat hippocampal slices. Late-LTP was induced by strong θ-burst or late-LTP θ-burst (a weak θ-burst was used as control) and potentiation of fEPSP slope evaluated one hour (all protocols) and two hours (late-LTP protocol) later. To select a LTP protocol that would induce a stable late-LTP, lasting for several hours and PKA-dependent, resembling memory formation processes, we studied the involvement of PKA on these late-LTPs. The influence of in vitro epileptiform activity on LTP was studied in 6-week-old rat hippocampal slices pre-treated with bicuculline (50 µM, 30 min). Late-LTP was induced one hour after bicuculline washout using a strong θ-burst protocol.

PKA inhibitors (H-89 and PKI 14-22 amide) did not change the potentiation obtained for any of the θ-burst stimulation protocols in 12 week-old rats. The presence of the selective $VPAC_1$ antagonist PG 97-269 during the strong θ-burst protocol: 1) decreased the potentiation in 12-week-old rats; 2) increased the potentiation in 6 week-old rats. With the weak θ-burst protocol, the presence of PG 97-269 (100nM) increased the potentiation for both age groups.

When studying LTP induced by strong θ-burst protocol one hour after in vitro epileptiform activity the potentiation was not changed.

Together, these results suggest that the late-LTP induced by different θ-burst protocols is PKA independent and that the role of $VPAC_1$ receptors on these late-LTPs varied with the experimental conditions.

Key words: VIP, LTP, $VPAC_1$, Interneurones, PKA, Epilepsy

List of abbreviations

AC – Adenylyl cyclase

aCSF – Artificial Cerebrospinal Fluid

AMPA – α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ATP – Adenosine triphosphate

CA1-CA4 – Cornu Ammonis, areas 1-4

CaM – Calmodulin

cAMP – Cyclic Adenosine Monophosphate

CaMKII – calmodulin kinase II

CREB – cAMP response element-binding

CCK – Cholecystokinin

CNS – Central Nervous System

ERK – extracellular-signal-regulated kinase

fEPSP – field excitatory postsynaptic potential

GABA – γ-Aminobutiric Acid

$GABA_A$ Receptor – GABA-gated receptor channel (type A)

HFS – high-frequency stimulation

LTP – Long-term Potentiation

E-LTP – early-Long-term Potentiation

L-LTP – late-Long-term Potentiation

MAPK – Mitogen-activated protein kinases

NMDA – N-methyl-D-aspartic acid

PAC_1 – PACAP-specific receptor

PACAP – pituitary adenylate cyclase activating polypeptide

PKA – Protein Kinase A

PKC – Protein kinase C

PKMζ – Protein kinase M, zeta isoform

PPF – paired-pulse facilitation

TLE – Temporal lobe epilepsy

VIP – Vasoactive intestinal peptide

$VPAC_1$ – Vasoactive intestinal peptide receptor 1

$VPAC_2$ – Vasoactive intestinal peptide receptor 2

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Background

The Hippocampus

The hippocampus is a component of the mammalian brain that lies in its central zone, at the base of the temporal lobes of the cerebral cortex. It has the shape of a seahorse, that originated its name, derived from the Greek (Hippo – horse, kampos – sea monster) (Pearce 2001) and is a brain region that is critical to spatial learning, awareness, navigation, episodic and semantic memory and associational recollection (Best et al 2001; Burgess et al 2002; LaBar & Disterhoft 1998).

The hippocampus is one of the components of the limbic system that comprises also cortical structures such as the subcallosal, cingulated and parahippocampal gyri and yet sub cortical structures such as the amygdala, the thalamus, the hypothalamus and the septum (See Figure 1) (Lopes da Silva et al 1990). Most of the available information concerning the cellular organization of the hippocampal formation dates from the studies of Ramón y Cajal (Ramón y Cajal (1911) in (Lopes da Silva et al 1990)).

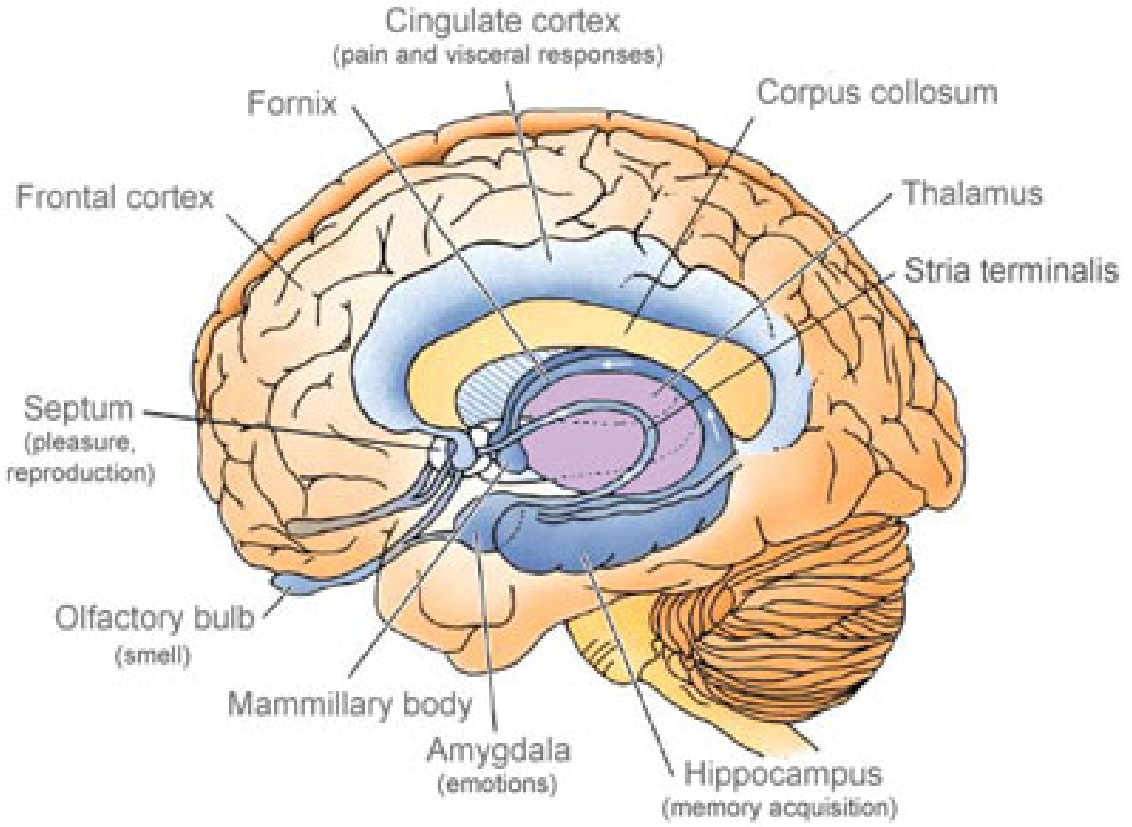

Figure 1. Schematic representation of the limbic system (dark blue) and surrounding structures (Adapted from http://www.daviddarling.info/encyclopedia/L/limbic_system.html).

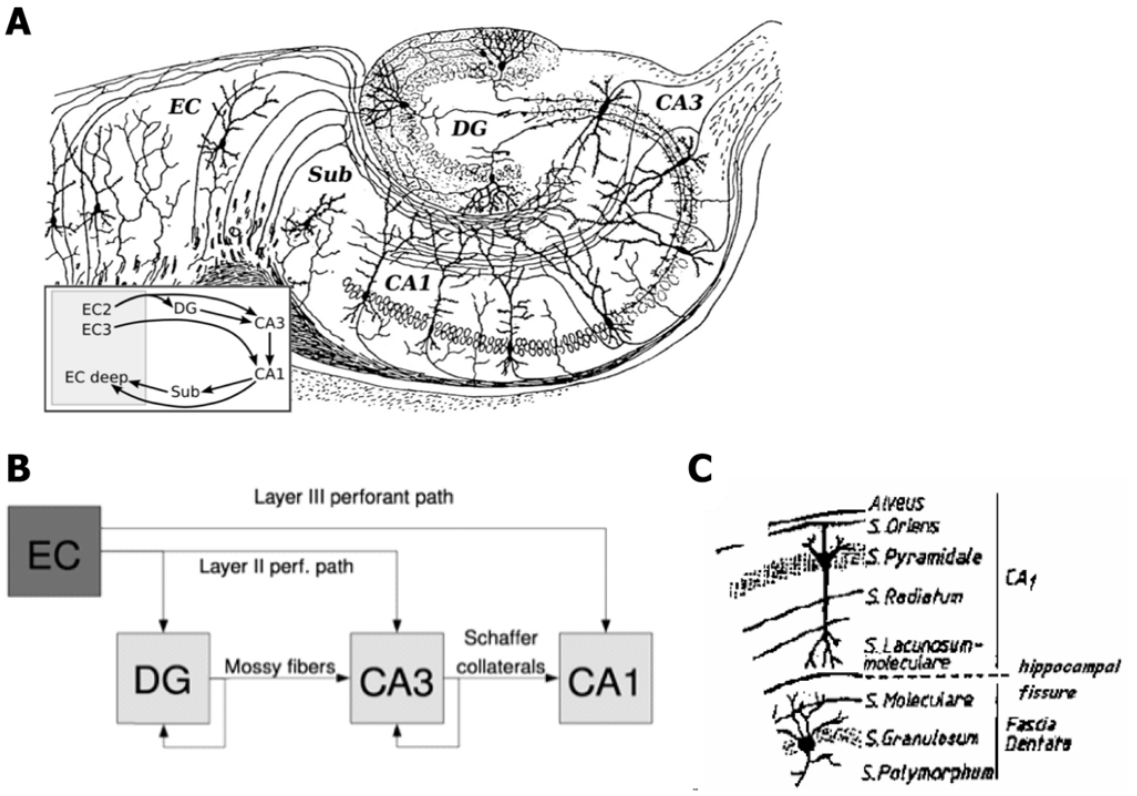

Figure 2. Schematic representation of the hippocampus and trisynaptic circuit. (A) Neuronal organization of the hippocampus. (Adapted from Ramón y Cajal, 1911) (B) Schematic representation of the hippocampal excitatory tri-synaptic circuit DG – Dentate gyrus, EC - Entorhinal cortex, ff - Fimbrial fibers, mf - Mossy fibers, sc - Schaffer collateral fibers (Adapted from www.wikipedia.com) (C) Representation of the different Cornu Ammonis and Dentate gyrus cell layers. (Adapted from Filipe 1991 based on the initial descriptions by Amaral & Witter 1989 and Andersen et al 1969).

The hippocampal formation consists of two C-shaped interlocking cell layers: the granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon´s horn (Cornu Ammonis or hippocampus proper) and the subiculum. The latter is composed by the molecular and pyramidal cell layers. The dentate gyrus has three layers: the molecular or dendritic layer, the granule cell layer and the hilar region characterized by widely scattered, polymorphic neurons (Lopes da Silva et al 1990). The Ammon´s horn is usually further subdivided into CA1 to CA4 areas (from the latin Cornu Ammonis areas 1-4). The first lies the nearest to the subiculum, that confines with the enthorhinal cortex, and the latter is the one directly contacting the dentate gyrus (Lopes da Silva et al 1990). The Ammon's horn consists of seven layers (Figure 2.C): the stratum moleculare that lies directly adjacent to the hippocampal fissure and contains predominantly fibers and dendritic terminals; the stratum lacunosum, which consists mainly of bundles of parallel fibers collaterals of pyramidal cells or extrinsic to the hippocampus. Some authors combine these two layers into the stratum lacunosum-moleculare; the stratum radiatum is characterized by rather sparse cell bodies and several fiber systems, the most important of which is formed by the Schaffer collaterals; the stratum pyramidale is formed by densely packed cell bodies of the pyramidal cells; the stratum oriens is a layer that contains the basal dendrites of pyramidal cells, the cell bodies of GABAergic interneurones and (in CA1) some collaterals of CA3 pyramidal cells; the stratum alveus is formed by axons of pyramidal cells and incoming fibers from pyramidal cells and a few cell

bodies that appear to be displaced from the stratum oriens; the epithelial zone forms the lining of ventricular surface of the hippocampus (Lopes da Silva et al 1990).

The circuits of the hippocampus are organized in a looping trisynaptic excitatory circuit going from the dentate gyrus to the CA1 area that comprises (Figure 2.B): 1) the median perforant path projection, consisting in the axons of layer II cells of the enthorhinal cortex that synapse with the granule cells from dentate gyrus; 2) the mossy fibers, that are the axons of granule cells that synapse with CA3 pyramidal cells; 3) the Schaffer collateral/commissural fibers, that consist in the axons of CA3 neurons that innervate the CA1 pyramidal cells. Besides these main circuits there are two other important inputs from the cortex; the lateral perforant path projection from layer II neurons in the enthorhinal cortex to the CA3 area (now known to be very important for activation of CA3 pyramidal cells dendrites in this area; cf. with mossy fibers) and the projections from the layer III of the enthorhinal cortex to the stratum lacunosum-moleculare of area CA1 of the Ammon's Horn, respectively (Herreras et al 1987).

Hippocampal Interneurones

The hippocampus contains interneurones, that make up 10% of hippocampal neurons (Freund & Buzsáki 1996) and that are cells that use GABA (γ-Aminobutyric acid) as a neurotransmitter. Due to the great diversity of interneurones several attempts have been made to classify them based on anatomical, electrophysiological or neurochemical properties (Per Andersen 2006). Since a detailed description of all interneurone subtypes in the hippocampus would be superfluous in the context of this thesis, we will mention only in more detail those subtypes of hippocampal interneurones that are particularly relevant to the work described in this thesis. Using the classification proposed by Somogyi and Klausberger (2005), interneurones are classified taking into account morphological characteristics - layer location and neuronal projections - and neurochemical properties (Somogyi & Klausberger 2005). For a summary description of the main interneurone subtypes present in the CA1 area of the hippocampus please refer to Figure 3.

Some interneurones have axonal projections to other interneurones (IS, interneurone-specific). IS-interneurones can be divided in three groups according to the layer to which they project and to their neuropeptide content. Two of these (IS-II and IS-III) express vasoactive intestinal peptide (VIP), whose actions on synaptic plasticity are studied in this work. IS-II interneurons cell bodies are at the border of stratum radiatum and stratum lacunosum-moleculare and receive afferents from the temporoammonic pathway, as inferred from the predominant localization of their dendritic tree at the stratum lacunosum-moleculare (Acsády et al 1996). IS-II interneurons innervate mostly stratum radiatum interneurons that control synaptic transmission to proximal dendrites of pyramidal cells. IS-III interneurons are mainly in stratum radiatum - stratum piramidale border and receive multiple afferents because their dendritic tree spans almost all layers (Acsády et al 1996). IS-III VIP-positive interneurones innervate interneurones of stratum oriens projecting to the stratum lacunosum-moleculare (O-LM cells, (Acsády et al 1996; Gulyás et al 1996). O-LM interneurones receive most (≈70%) of the excitatory inputs to come from retrograde projections of pyramidal cells of CA1 and are responsible for the feedback inhibition of pyramidal cells dendrites (See Figure 8).

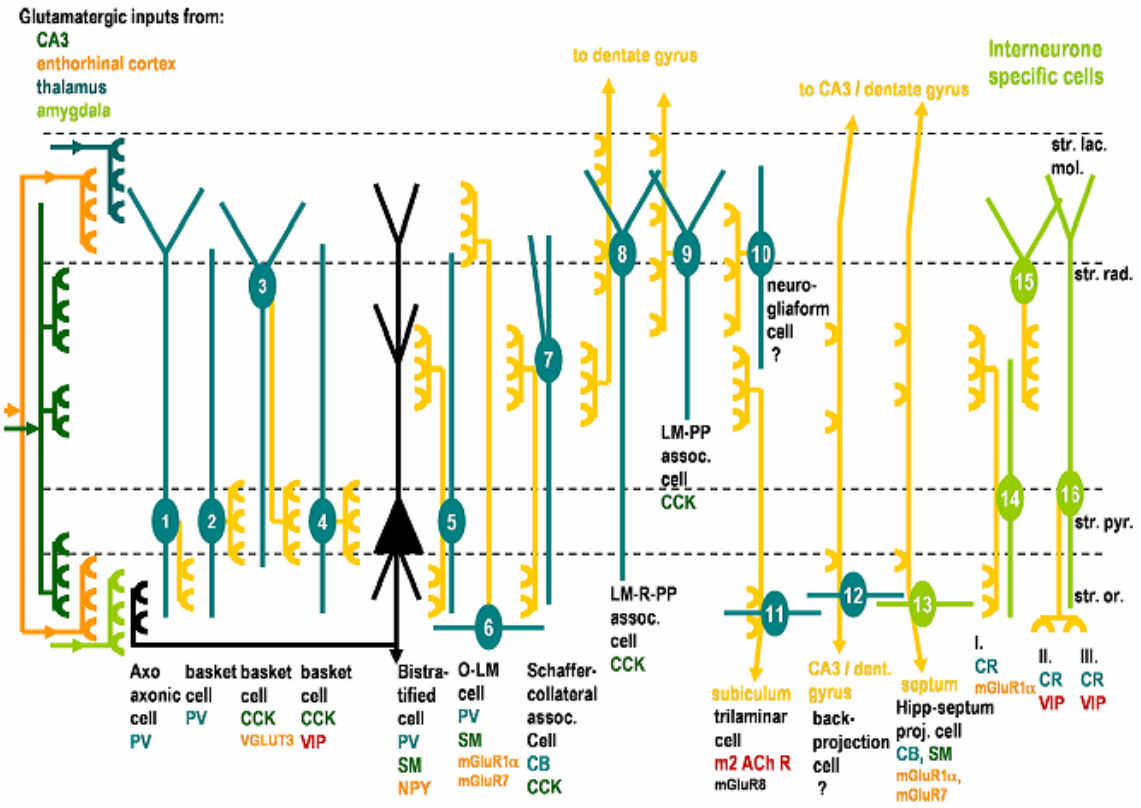

Figure 3. Hippocampal interneurone subtypes in the CA1 area of the hippocampus. Interneurones 1-12 innervate pyramidal cells whereas interneurones 13-16 innervate selectively interneurones. Somata and dendrites of interneurones are shown in blue or green and axon projections are shown in yellow. The main layers receiving glutamatergic input are shown, as is domain-specific innervation of pyramidal cells/interneurones by interneurone axons. The name and main neurochemical markers of each interneurone subtype is indicated bellow each cell. CB, calbindin; CCK, cholecystokinin; CR, calretinin; LM-PP, lacunosum-moleculare perforant path; LM-R-PP, lacunosum-moleculare radiatum perforant path; m2; muscarinic receptor type 2; mGluR7, 8, metabotropic glutamate receptor 7, 8; NPY, neuropeptide tyrosine; PV, parvalbumin; SM, somatostatin; VGLUT3, vesicular glutamate transporter 3; VIP, vasoactive intestinal peptide (Adapted by Cunha-Reis, 2006 from Somogyi and Klausberger, 2005).

Other type of interneurones relevant for this thesis, are the basket cells. The basket cells play an important role in perisomatic inhibition. These interneurones can be subdivided into three groups using neurochemical criteria. One of basket cells groups express VIP and cholecystokinin (CCK) but do not express parvalbumin, another interneurone marker, as do most basket cells in the hippocampus. VIP/CCK basket cells in the CA1 area receive afferents from CA3 Schaffer collaterals (Acsády et al 1996), suggesting that they can be activated in a feed-forward manner and these basket cells have serotonergic innervations unlike the other groups of basket cells that receive cholinergic innervations (Papp et al 1999) (See Figure 3 and Figure 8).

The electrical properties and firing pattern of interneurones in the hippocampus are different from the ones of pyramidal cells. Interneurones generate fast currents, due to the expression of glutamate receptors of specific subunit composition (Per Andersen 2006). They also generate brief action potentials in response to transient synaptic activation and discharge repetitively at very high frequencies during sustained stimulation. Factors facilitating fast

action potential initiation following synaptic excitation include depolarized interneurone resting potential, sub-threshold conductances and active dendrites. GABA release at interneurone output synapses is rapid and highly synchronized, leading to a faster inhibition in postsynaptic interneurones than in principal cells (Jonas et al 2004). Interneurones also have electrical synapses using gap junctions (Katsumaru et al 1988). These types of synapse allow a synchronization of specific groups of interneurones and work with high speed and temporal precision (Per Andersen 2006).

Hippocampal Rhythms

The theta rhythm is one of the most studied rhythms using electroencephalography and magnetoencephalography. The brain wave can be divided in five rhythms: Delta (4 Hz); Theta (4-8 Hz); Alpha (8 – 12 Hz); Beta (12 – 30 Hz); and Gamma (30 – 140 Hz). The theta rhythm is believed to play a mechanistic role in various aspects of memory (Düzel et al 2010). The loss of hippocampal theta rhythm leads to spatial memory deficits in rats (Winson 1978). In 1997, In 1997, Vertes and Kocsis proposed that ''theta cells'', cells that fire with the theta rhythm, are responsible for theta rhythms in the hippocampus (Vertes & Kocsis 1997).

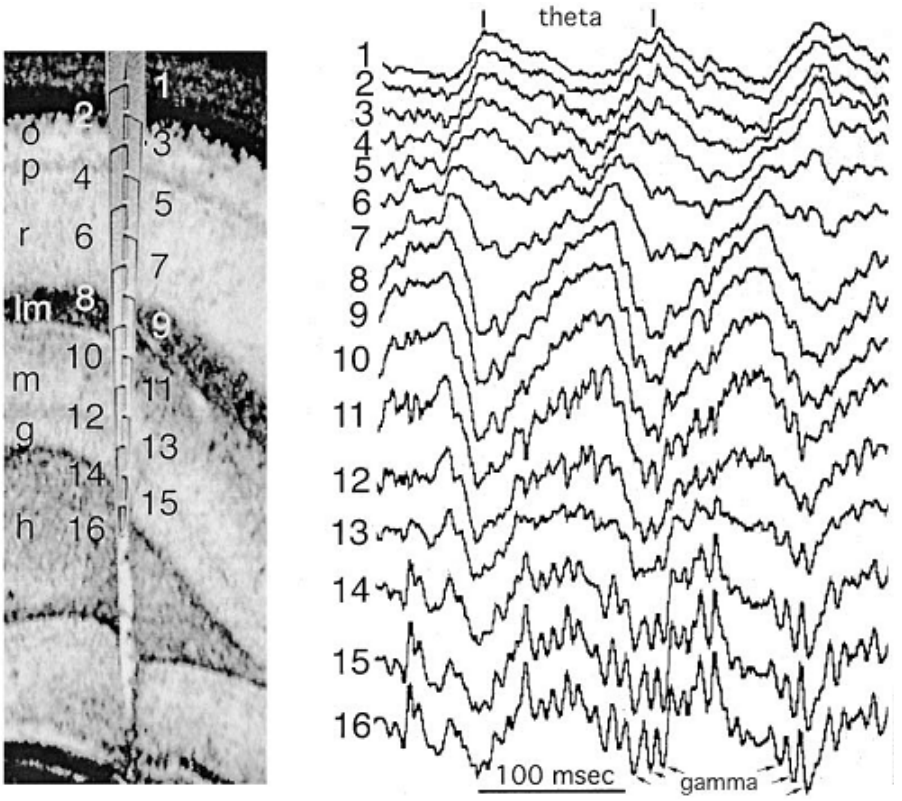

Figure 4. Depth Profile of Theta Oscillation in the rat hippocampus. (Left) o - stratum oriens, p - pyramidal layer, r - stratum. Radiatum, lm - stratum lacunosum-moleculare, g - granule cell layer, h - hilus. (Right) Theta waves recorded during exploration. Note gradual shift of theta phase from stratum oriens to stratum lacunosum-moleculare. Gamma waves superimposed on theta oscillation are marked by arrows (Adapted by (Buzsáki 2002) from (Bragin et al 1995).

Theta oscillations are most regular in frequency and with larger amplitude in the stratum lacunosum-moleculare of the hippocampal CA1 region. Both the amplitude and phase of theta waves change as a function of depth, whereas in the same layers they are robustly similar along the long axis of the hippocampus (Bullock et al 1990) (Figure 4).

Vertes et al. (2004) argued that the theta rhythm represents a strong depolarizing drive to the hippocampus, while other inputs (mainly cortical) constitute ''information-bearing'' inputs to the hippocampus that when coupled to theta rhythm produce lasting changes in hippocampal function (Vertes et al 2004). During theta states, theta oscillations would drive large populations of hippocampal neurons to threshold for the activation of N-methyl D-aspartate –sensitive (NMDA) receptor channels, and when combined with the release of glutamate from other afferents to these cells, would result in the opening of the NMDA receptor channels and consequent cellular changes. Accordingly, events occurring together with theta oscillations would have greater access to (and impact on) the hippocampus. In effect, theta oscillations serve as a ''significance signal'' to the hippocampus, that is, information arriving with theta oscillations would be stored (at least temporarily) in the hippocampus, whereas information arriving in the absence of theta oscillations would not be encoded, or not encoded to the same degree, as that reaching the hippocampus concurrently with theta oscillations (Vertes 2005).

Synaptic Plasticity in Hippocampus

One of the most important and fascinating properties of nervous system is its synaptic plasticity. Synaptic plasticity refers to the activity-dependent modification of the strength or efficacy of synaptic transmission at pre-existing synapses (Citri & Malenka 2008), and since proposed by Cajal and later by Hebb plays a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces (Cajal 1913; Citri & Malenka 2008; Lynch 2004).

Synaptic transmission can be either enhanced or depressed by activity, and these changes span temporal domains ranging from milliseconds to hours, days, and presumably even longer. Furthermore, virtually all excitatory synapses in brain simultaneously express a number of different forms of synaptic plasticity (Zucker & Regehr 2002).

Most forms of short-term synaptic plasticity are triggered by short bursts of activity causing a transient accumulation of calcium in presynaptic nerve terminals. This increase in presynaptic calcium in turn causes changes in the probability of neurotransmitter release by directly modifying the biochemical processes that underlie the exocytosis of synaptic vesicles (Citri & Malenka 2008). Long-term synaptic plasticity can be expressed as Long-term Potentiation (LTP) or long-term depression. As memories are thought to be encoded by modification of synaptic strength, LTP is widely considered one of the major cellular mechanisms that underlies learning and memory (Lynch 2004). LTP has several properties such as input specificity – only occurs in activated synapses , associativity – weak stimulation alone does not potentiate communication but if a neighbouring pathway is strongly stimulated this pathway is reinforced, cooperativity - concurrent weak stimulations of converging afferent

fibers can cooperate to induce LTP in the postsynaptic cell, and durability. Those characteristics are the same that are essential for memory acquiring according Hebb's postulate (Hebb 1949). Further evidence has been supporting the hypothesis that synaptic plasticity is the cellular mechanism supporting learning and memory. In particular, in the hippocampus, LTP inhibitors also block hippocampal-dependent learning tasks; biochemical changes that occur after LTP induction and memory acquisition are similar; and rhythmic bursts of activity that induce LTP mimic activity naturally occurring during theta rhythm recorded in the hippocampus during spacial memory acquisition (Lynch 2004).

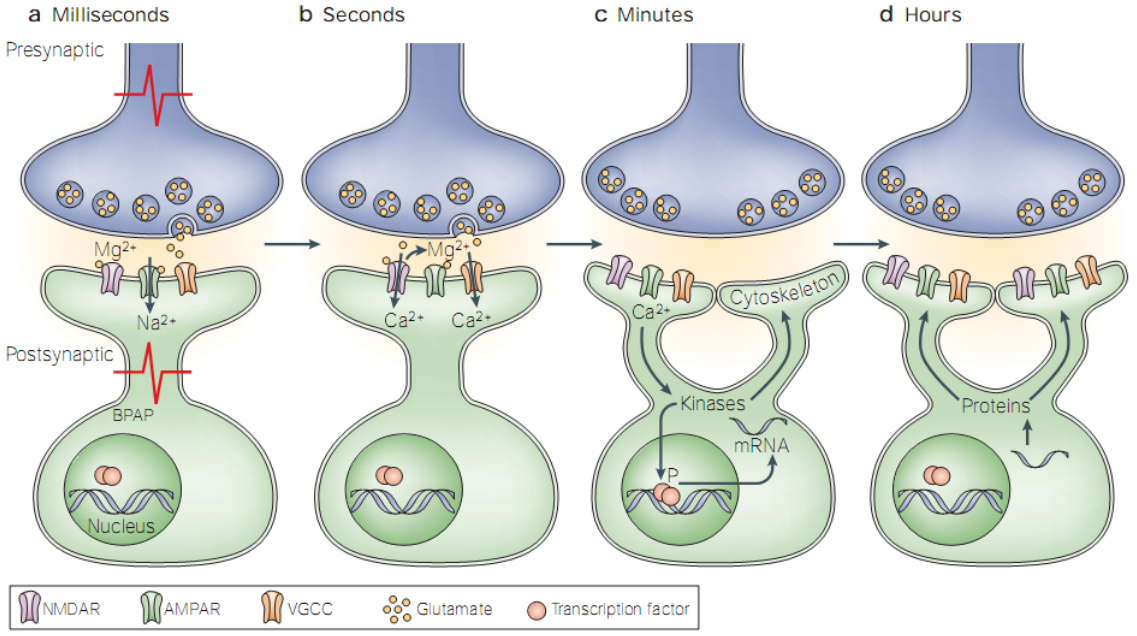

Figure 5. Molecular mechanisms involved in the initiation and maintenance of synaptic plasticity. (A) Activity-dependent release of glutamate from presynaptic neurons leads to the activation of AMPA receptors and to the depolarization of the postsynaptic neuron. Depolarization occurs locally at the synapse and/or by back-propagating action potentials (BPAP). (B) Depolarization of the postsynaptic neuron leads to removal of NMDA receptor inhibition, by Mg^{2+}, and to Ca^{2+} influx through the receptor. Depolarization also activates voltage-gated calcium channels (VGCCs), another source of synaptic calcium. (C) Calcium influx into the synapse activates kinases which, in turn, modulate the activity of their substrates. These substrates contribute to local changes at the synapse, such as morphological alteration through cytoskeletal regulation, or induce the transcription of RNA in the nucleus by regulating transcription factors (TFs). (D) Transcribed mRNA is translated into proteins that are captured by activated synapses and contribute to stabilization of synaptic changes (Adapted from Lamprecht & LeDoux 2004).

LTP was reported for the first time in 70´s by Bliss and Lomo (Bliss & Gardner-Medwin 1973). They delivered a brief period of high-frequency stimulation (HFS) to the rabbit perforant path causing a sustained increase of synaptic transmission to the granule cells of the dentate gyrus. They termed this phenomenon Long-term potentiation of synaptic transmission. The HFS triggers a depolarization that allows the activation of both α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid – sensitive (AMPA) and NMDA ionotropic glutamate receptors (See Figure 5). AMPA receptors are channels permeable to monovalent cations (Na^+ and K^+) that provide the majority of inward current for generating synaptic responses when the cell is close to its resting membrane potential. The NMDA receptors, exhibit profound voltage dependence

because of the blockade of the channel by extracellular Mg^{2+} and contribute little to the basal postsynaptic response during low-frequency synaptic transmission. However, when the postsynaptic cell is depolarized during the induction of LTP, Mg^{2+} dissociates from its binding site within the NMDA receptor channel, allowing Ca^{2+} as well as Na^{+} influx into the dendrites. The NMDA receptor activation and Ca^{2+} influx are crucial for LTP induction. This phase is usually called early-LTP (E-LTP) and is dependent on the concentration of intracellular Ca^{2+} (See Figure 6).

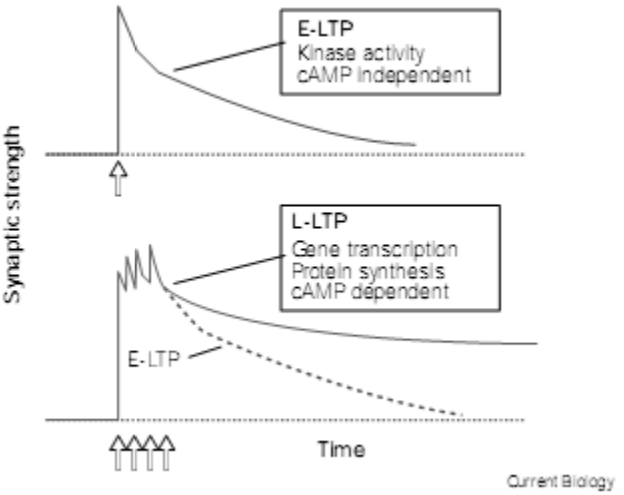

Figure 6. Early and late phases of long-term potentiation (LTP). A train of repetitive stimuli (e.g. HFS, arrows) induces an increase in synaptic strength known as LTP. A single train of stimuli induces E-LTP (red), which decays over the course of a few hours. Multiple trains induce L-LTP (blue), which remains stable for many hours (Adapted from Huang 1998).

For maintenance of LTP it is necessary to trigger numerous transduction pathways in cell, involving first the activation of various proteins/messengers such as calmodulin (CaM), calmodulin kinase II (CaMKII), and at a latter step adenylyl cyclase (AC), protein kinase A (PKA), CREB, an isoform of PKM (PKMζ) and mitogen-activated protein kinases /extracellular signal-regulated kinases (MAPK-ERK) (See Figure 7). When these latter pathways are activated LTP lasts for hours and this phase is called late-LTP (L-LTP) (Figure 6).

The activation of these transduction pathways ultimately leads to an increased number of AMPA receptors in the synaptic cleft and an increased efficiency of existing receptors, thus providing greater sensitivity to the postsynaptic neuron, thereby suggesting that long-term potentiation is a fundamental molecular process associated with learning and memory formation (Citri & Malenka 2008; Kovács et al 2007; Kullmann & Lamsa 2007; Lynch 2004; Serrano et al 2005).

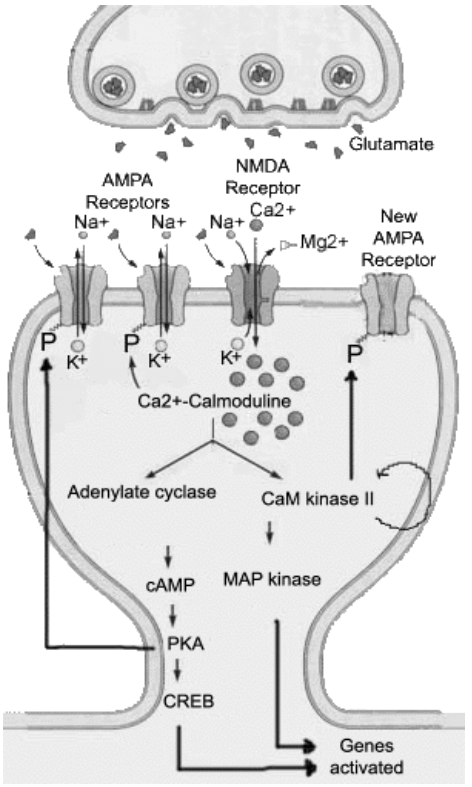

Figure 7. Schematic representation of the molecular pathways activated during the induction and maintenance of NMDA-dependent LTP. The increase $[Ca^{2+}]i$ is essential to the induction phase. The phosphorylation of MAPK and CREB are essential for the long-term maintenance of LTP (late-LTP) (Adapted from http://thebrain.mcgill.ca).

Vasoactive intestinal peptide (VIP)

Vasoactive intestinal peptide is a neuromodulator peptide that was initially isolated from porcine intestine is a 28-amino acid peptide (Harmar et al 1998; Said & Mutt 1970). VIP is member of the superfamily of structurally related peptide hormones that includes glucagon, glucagon-like peptide, secretin, and growth hormone-releasing factor. This family also includes pituitary adenylate cyclise activating polypeptide (PACAP). PACAP shares 68% sequence similarity to VIP in their N-terminal residues from 1 to 28 (Delgado & Ganea 2001; Laburthe & Couvineau 2002). It was shown to serve potentially as a neuromodulator, a neurotrophic factor and/or a neurotransmitter. Furthermore, it is neuroprotective and enhances cell proliferation in both the peripheral and central nervous systems (Harmar et al 1998). VIP and PACAP also play a neuromodulator role on cardiovascular, circulatory and respiratory systems and on metabolic function, immune system and nervous system (Dickson & Finlayson 2009).

VIP is widely distributed throughout the brain, with considerable expression in the cerebral cortex, hippocampus, amygdala, suprachiasmatic nucleus and hypothalamus (Dickson & Finlayson 2009). In the hippocampus, VIP is expressed exclusively in three types of interneurones (basket cells and Interneurone specific cells II and III), as previously mentioned (See Figure 8). The first study of the role VIP on synaptic transmission on the CA1 area was in 1992 by Haas and Gähwiler and they observed VIP modulates neuronal excitability in hippocampal slices of the rat (Haas & Gähwiler 1992). The enhancement of synaptic

transmission to CA1 pyramidal cells by VIP is also dependent on GABAergic transmission. This action occurs both through presynaptic enhancement of GABA release and postsynaptic facilitation of GABAergic currents in interneurones (Cunha-Reis et al 2004).

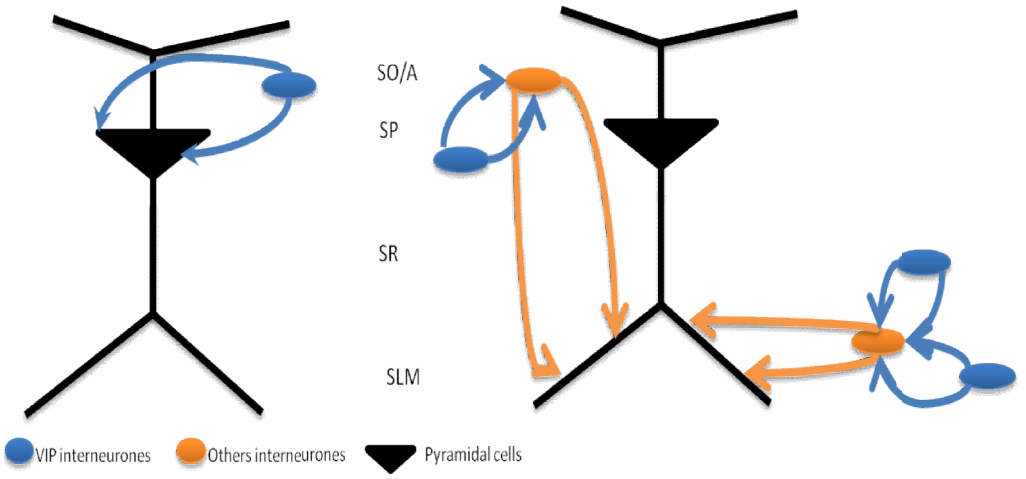

Figure 8. Schematic representation of VIP interneurones in CA1 area. SO/A - Stratum Oriens/Stratum Alveus, SP - Stratum piramidale; SLM - Stratum lacunosum-molecular (Adapted from Cunha-Reis, 2006).

VIP receptors

At least three receptors for VIP-PACAP have been identified and they all belong to the B family of G-protein-coupled receptors. VIP receptors, Vasoactive intestinal peptide receptor 1 ($VPAC_1$ receptor), and ($VPAC_2$ receptor), have similar affinities for PACAP and VIP whereas the PACAP-specific receptor (PAC_1R) exhibits a much higher (Harmar et al 1998) affinity for PACAP than VIP .

VIP receptors trigger mainly adenylyl cyclase activation through Gs proteins and cAMP production (See Figure 9). A few other signaling pathways have been described depending on the preparations and species, in particular, activation of phospholipase C through G proteins of the Gq and/or Gi/Go families in various types of cells and species (Dickson & Finlayson 2009). All of VIP receptors couple strongly to the Gαs and stimulate the cyclic AMP (cAMP)/PKA signaling pathway. Shreeve (2002) reported that the $VPAC_1$ receptor can couple to Gi/o protein in the hippocampus.

The $VPAC_1$ receptor is widely distributed in the central nervous system (CNS), most abundantly in the cerebral cortex and hippocampus, in peripheral tissues including liver, lung, and intestine and in T lymphocytes. In the CNS, the highest $VPAC_2$ receptor expression are found in the thalamus and suprachiasmatic nucleus and lower levels in the hippocampus, brainstem, spinal cord, and dorsal root ganglia. The receptor is also present in several peripheral tissues, including pancreas, skeletal muscle, heart, kidney, adipose tissue, testis, and stomach (Dickson & Finlayson 2009).

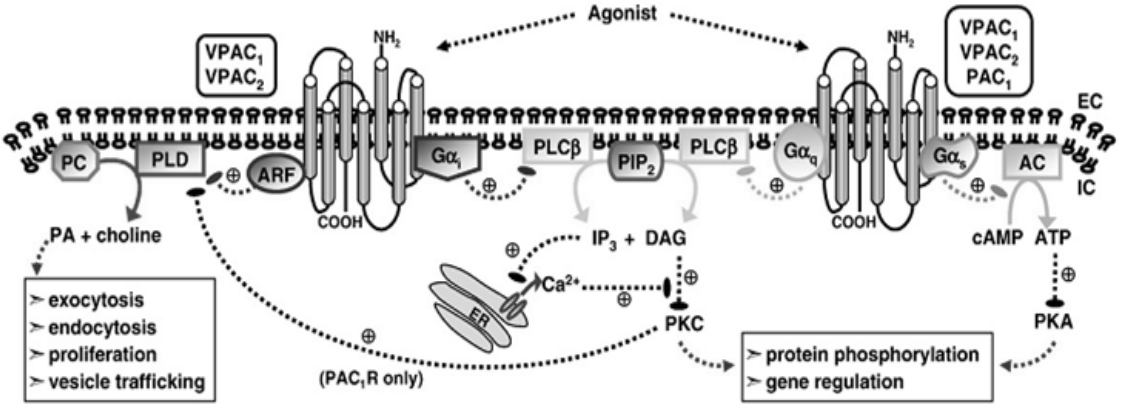

Figure 9. Intracellular signalling pathways stimulated by VPAC/PAC_1R activation. The figure highlights the principal transduction pathways activated by $VPAC_1$, $VPAC_2$ and PAC_1 receptor coupling to heterotrimeric G-proteins. Upon activation, all three receptors are capable of coupling to Gαs leading to downstream production of cAMP. In addition, the three receptors can also activate PLC leading to an increase in $[Ca^{2+}]i$, via coupling to Gαq (all three receptors) and Gαi ($VPAC_1$ and $VPAC_2$ only). PLD activity can also be stimulated by the three receptor subtypes via ARF (VPACR) and PKC (PAC_1R) sensitive pathways (Adapted from Dickson & Finlayson, 2009).

Immunohistochemical and autoradiography studies suggest that, in the hippocampus, $VPAC_1$ receptor is expressed in stratum oriens and stratum radiatum on Cornu Ammonis and it is co-localized with glial cells (Joo et al 2004; Vertongen et al 1997). However, functional evidence for the presence of $VPAC_1$ receptors in interneurones has been obtained (Cunha-Reis et al 2006). The same studies show that $VPAC_2$ receptors are majorly expressed in stratum pyramidale in the CA1 area (Joo et al 2004) (See Figure 10). The distribution of VIP receptors in the hippocampus suggests an important role of VIP in the control of pyramidal cells function.

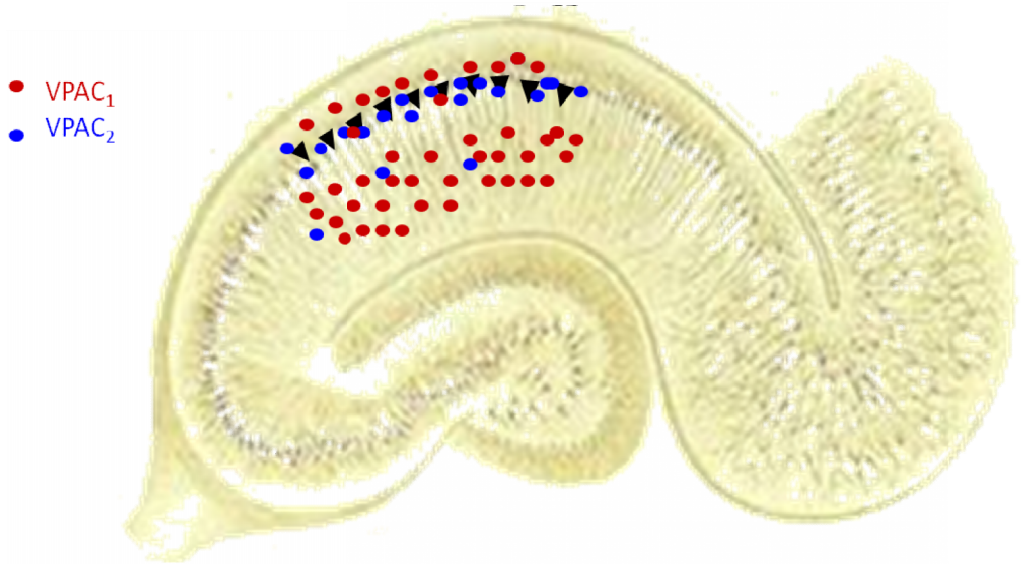

Figure 10. Distribution of VIP receptors in the Ammon's horn (Adapted from Cunha-Reis, 2006, based on the data obtained by Joo et al 2004 and Vertongen et al 1997).

Recent studies show that VIP enhancement of synaptic transmission to CA1 pyramidal cells is mostly mediated by $VPAC_1$ receptor activation and is totally dependent on PKC activity

(Cunha-Reis et al 2005). $VPAC_2$ receptor activation also contributes to that enhancement through a PKA-dependent mechanism (Ciranna & Cavallaro 2003; Cunha-Reis et al 2005).

VIP also increases NMDA-evoked currents in CA1 pyramidal cells (Yang et al 2009). This increase can be totally mimicked by simultaneous application of $VPAC_1$ and $VPAC_2$ selective agonists and was blocked by specific PKA inhibitor such as PKI 14–22 amide and Rp-cAMPS (See Figure 11), but not by PKC inhibitor bisindolylamaleimide I (Yang et al 2009). These facts suggest an involvement of both $VPAC_1$ and $VPAC_2$ receptors in the regulation of NMDA receptors in pyramidal cells (Yang et al 2009).

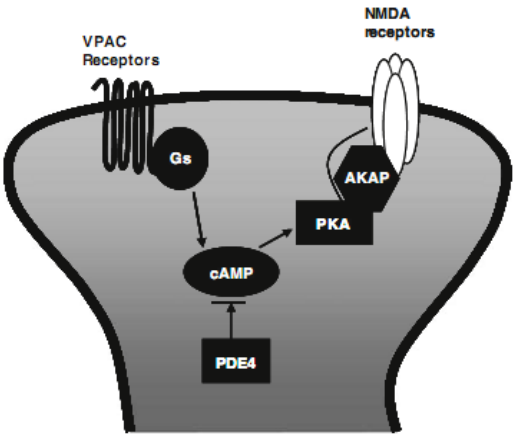

Figure 11. VIP modulation of NMDA receptors in CA1 pyramidal cells via cAMP/PKA pathway (Adapted from Yang et al 2010).

It was recently discovered in our lab that endogenous VIP has a restraining effect on LTP elicited in vitro by theta-burst stimulation through a mechanism dependent on $VPAC_1$ receptor activation (See Figure 12) and GABAergic transmission or $GABA_A$ receptors (Cunha-Reis D. 2008; Rodrigues 2009).

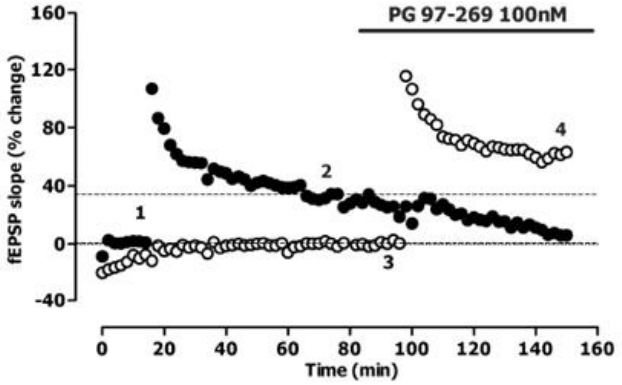

Figure 12. Influence of $VPAC_1$ receptor blockade on LTP induced by weak θ-burst stimulation, 5 bursts of four pulses at 100 Hz with an interburst interval of 200 msec. Temporal evolution of the slopes of evoked field excitatory postsynaptic potentials (fEPSP) obtained from a representative experiment in which we evaluated the LTP induced by theta-burst stimulation in the absence and presence of the drug PG 97-269 (red bar). All experiments were performed in two pathways on the same slice (S1 and S2). The independence of the two pathways was tested by studying Paired-pulse Facilitation (PPF) across both pathways, less than 14% facilitation being usually observed independent (Rodrigues 2009).

A deficit in VIP in brain is related with learning impairments in mice (Glowa et al 1992) and intracerebral administration of a VIP receptor antagonist to adult rats resulted in deficits in learning and memory in the Morris water maze task (Glowa et al 1992).

Temporal lobe epilepsy

Epilepsy is the most common severe neurological condition (Duncan et al 2006). It is characterized by spontaneous recurrent seizures, caused by focal or generalized paroxysmal changes in neurological functions triggered by abnormal electrical activity in brain (Dichter 1994). As it involves hyperexcitable neurons, a basic statement links the pathogenesis of epilepsy and the generation of synchronized neuronal activity with an imbalance between inhibitory (GABA-mediated) and excitatory (glutamate-mediated) neurotransmission, in favour of the latter (Dalby & Mody 2001).

Temporal lobe epilepsy (TLE) is the most common form of epilepsy in humans. Individuals affected with TLE typically have comparable clinical description, including an initial precipitating injuring such as status epilepticus, head trauma, encephalitis or childhood febrile seizures (Cendes 2004; Fisher et al 1998; Harvey et al 1997). There is usually a latent period of several years between this injury and the appearance of the chronic TLE characterized by spontaneous recurrent seizures originating from temporal lobe foci that lead to learning and memory impairments (Detour et al 2005; Dütsch et al 2004). In TLE, acute cell death occurs in various limbic regions, including the hippocampal formation. Gliosis and reorganization of axons of the granule cells (mossy fiber sprouting) and reorganization of excitatory and inhibitory circuits in the hippocampal formation following seizure induced neuronal loss are also involved in the development of chronic seizures in TLE (Mello et al 1993).

VIP and its receptors are increased in the human hippocampus of patients with TLE (de Lanerolle et al 1995). It is suggested that the elevated levels of receptor binding in the hippocampal seizure focus may indicate a mechanism for greater excitability of neurons and/or their survival facing the increased excitation and potential for injury in a seizure focus (de Lanerolle et al 1995). In an animal model of TLE, it was also reported an increase of VIP in hippocampus (Marksteiner et al 1989).

Aim

This study aimed to evaluate the influence of $VPAC_1$ receptor activation on a Long-term potentiation in 12-week-old rats, using a LTP induction protocol that result in a late-LTP, characterized by its several hour persistency and PKA-dependence, for this would be a valid in vitro model to study memory.

The study also aimed to elucidate the influence of $VPAC_1$ receptors on synaptic plasticity following ictal activity in in vitro models of epilepsy.

Methods

Animals

All experiments were performed with males young-adult or adult Wistar rats (6-7 weeks old or 12-13 weeks old) from Harlan Interfauna Iberica (Spain). The rats were housed in the local Animal House until use. Animal housing and handling was according to the Portuguese law and European Union guidelines (86/609/EEC).

Brain dissection and tissue preparation

The animals were anesthetized under Isoflurane atmosphere and decapitated with the aid of a guillotine. The brain was rapidly removed and the hippocampus was separated from other brain structures in ice-cold artificial Cerebrospinal Fluid (aCSF) solution of the following composition (mM): NaCl 124; KCl 3; $MgSO_4$ 1, $CaCl_2$ 2; glucose 10; NaH_2PO_4 1.25; $NaHCO_3$ 26, gassed with 95% O2 / 5% $C0_2$.

The hippocampi were cut in slices (400 µm thick) with a McIlwain tissue chopper (McIlwain Tissue Chopper, Mickle Laboratory Engineering Co. LTD) perpendicular to the long axis of the hippocampus and allowed to recover for at least 1 h in gassed aCSF solution at room temperature.

Evoked field excitatory postsynaptic potentials recordings

The chamber and perfusion system were previously superfused with water and the flux was adjusted to 3 ml/min. Chamber temperature was brought to 30.5 ºC ± 0.2 ºC using a thermostat (Harvard Apparatus, TC-202A). When a stable temperature was obtained, the chamber was superfused with aCSF and an individual slice was transferred to the chamber. Evoked field excitatory postsynaptic potentials (fEPSP) were recorded extracellularly through a microelectrode filled with 4 M NaCl (2-6 MΩ resistance) placed in the stratum radiatum of the CA1 area. fEPSP were evoked by stimulation of two independent pathways of the Schaffer collateral/commissural fibers using two bipolar concentric wire electrodes placed in the stratum radiatum. Stimulation (rectangular pulses of 0.1 ms duration) was delivered alternately to each pathway once every 10 s (one pathway being stimulated once every 20s). The initial intensity of the stimulus (90-240 µA) was adjusted to obtain a sub maximal fEPSP slope with a minimum population spike contamination, near 50% of the fEPSP slope obtained with supramaximal stimulation, and of similar magnitude in both pathways. The averages of six consecutive fEPSP from each pathway were obtained, recorded and quantified as the slope of the initial phase of the potential. Recordings were amplified with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), digitized and continuously stored on a personal computer using the LTP program (Anderson & Collingridge 2001). The independence of the two pathways was tested by studying paired-pulse facilitation (PPF) (50 ms interval) across both pathways, less than 12% facilitation being usually observed, and only in this case were the experiments considered for further analysis.

LTP induction

LTP was induced by theta burst stimulation (θ-burst) only after obtaining a stable recording of fEPSP slope in the two pathways for at least 20 minutes. θ-burst stimulation consisted always of bursts of four pulses delivered at 100Hz with an interbust interval of 200ms. In this work three different strengths θ-burst stimulation paradigms were used: weak θ-burst consisted of 5 bursts delivered during 1s; strong θ-burst (See Figure 13), consisted of 15 bursts delivered during 3 seconds and late-LTP θ-burst inducing paradigm consisted of three strong θ-burst stimulations delivered with a 6 min interval. The intensity of the stimulus was never changed during these induction protocols. For late-LTP experiments, potentiation was induced in only one pathway for each slice, control and test conditions were thus performed in independent slices.

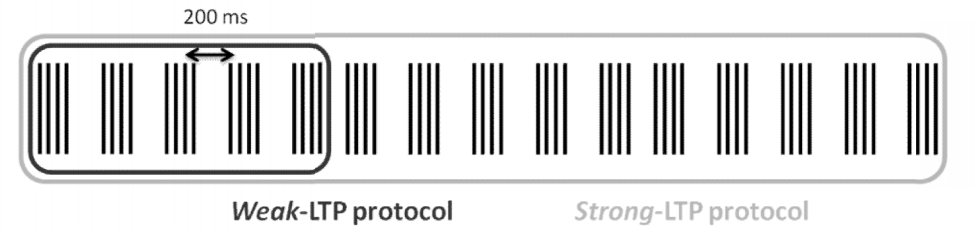

Figure 13.Theta-burst stimulation protocols. The 200 ms interval indicated in the figure refers to the time period from the onset of the first pulse in the first burst to the onset of the first pulse in the second burst. Pulses within each burst were delivered at 100Hz.

LTP was quantified as the % change in the average slope of the fEPSP taken from 50 to 60 minutes after LTP induction in relation to the average slope of the fEPSP measured during the 10 minutes that have preceded the induction of LTP (Figure 14). For analyses of late-LTP, the degree of potentiation was evaluated at two time points: 50 to 60 minutes and 110 to 120 minutes after LTP induction (Figure 15). In each individual experiment the same LTP-inducing paradigm was delivered to each pathway.

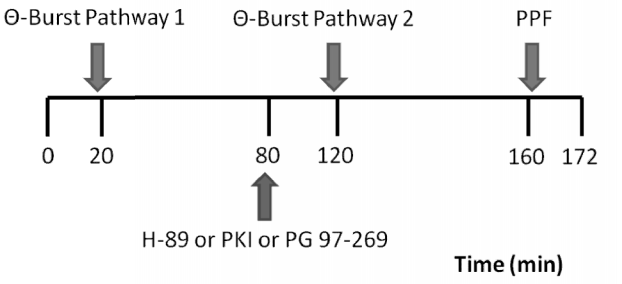

Figure 14. Schematic representation of electrophysiological experiments designed to study LTP induced by weak θ-burst or strong-θ-burst protocols.

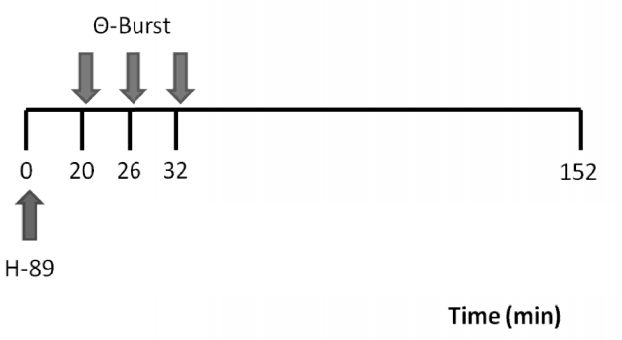

Figure 15. Schematic representation of electrophysiological experiments designed to study LTP induced by late-LTP-θ-burst protocol. H-89 was present only in test slices.

In vitro epileptiform activity

Strictly speaking, behavioural seizures (the defining feature of clinical epilepsy) cannot occur in a brain slice (Clark & Wilson 1999). Here we used the terminology outlined in comprehensive literature reviews, interictal-like discharges as epileptiform discharges or epileptiform bursts, and longer (several seconds) ictal seizure-like discharges as electrographic seizures.

After baseline recording, ictal seizure-like discharges were induced by perfusing the slices with aCSF containing Bicuculline 50 µM. During the perfusion of modified aCSF solution, the electrical stimulation was off, in order to record only the spontaneous activity evoked by the modified aCSF solution. The chamber temperature was 35ºC for all experiments because high temperatures increase synaptic transmission and increase seizure activity (Motamedi et al 2006). All experiments with epileptiform activity were performed in 6 week-old rats.

After 30 minutes of perfusion with modified aCSF solution, the slices were returned to normal aCSF solution during 1h. The effect of seizures on θ-burst LTP was tested using a strong θ-burst protocol (See Figure 16).

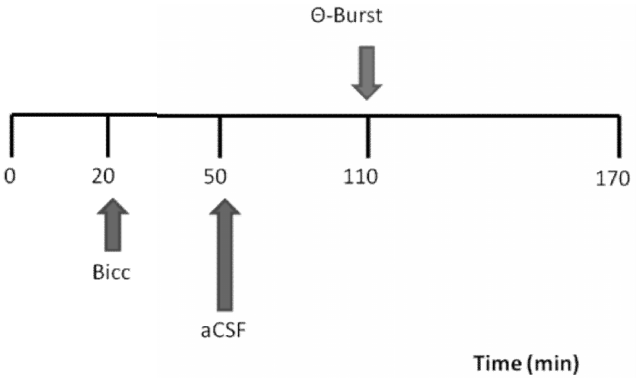

Figure 16. Schematic representation of electrophysiology experiments that study how in vitro ictal-like activity may affect LTP. Induction of ictal-like activity through perfusion with aCSF modified solution during 30 minutes. Theta-burst stimulation was performed one hour after Bicuculline washout. Evaluation of potentiation induced by the strong-LTP protocol was performed 50 to 60 minutes after theta-burst.

Pharmacological Tools

All reagents used are trademarks of Sigma or Merck, unless otherwise indicated.

The stock solution of H-89 (5 mM, PKA inhibitor, SIGMA, Portugal) was made in DMSO. The percentage of DMSO in each experiment did not exceed 0.01%. The stock solution of Bicuculline methochloride (100 mM, $GABA_A$ receptor antagonist, Tocris, Portugal) was made in water. PG 97-269 (1 mM, VPAC1 antagonist, Phoenix Peptides, USA) and PKI (5mM, PKA inhibitor, TOCRIS, UK) were prepared in 1% acetic acid. Stock solutions were stored in aliquots at -20°C until use. Dilutions of these stock solutions in aCSF to the final concentration were made freshly before slice perfusion. Isoflurane was from Abbot Laboratories (Barcelona, Spain).

Data analysis

Results are presented as mean ± Standard Error of the Mean (S.E.M.) from n experiments.

Statistical significance of the effects of drugs on LTP was assessed by a paired two-tailed Student's t test for experiments using control and test pathways in the same slice.

When comparing between experiments in which control and test conditions were observed in independent slices, statistical significance of the effects of drugs on LTP was assessed by Student's t test.

When comparing the potentiations obtained with different stimulation protocols in 6 and 12 week-old animals we used one-way ANOVA followed by Tukey's Multiple Comparison test. A p value of 0.05 or less was considered to represent significant differences. Statistical analyses were conducted with the Prism Version 5.00 for Windows (GraphPad Software).

Results

Influence of PKA on LTP induced by a θ-burst protocol

Memory formation is believed to require a stable LTP, lasting at least 2-3 hours. Previous results show that a weak θ-burst protocol, five bursts of four pulses at 100 Hz with an interburst interval of 200 msec, generates a LTP lasting for about two hours (Rodrigues 2009), which corresponds to an early-LTP (Figure 12). Since this early-LTP is not stable enough to be associated to formation of stable memories, we used a strong θ-burst protocol capable of inducing late-LTP.

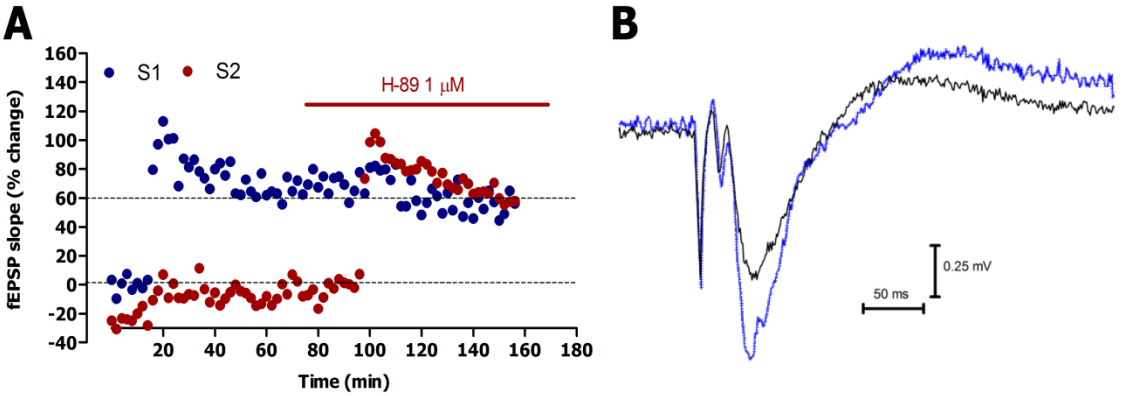

Figure 17. Influence of PKA inhibition LTP induced by strong θ-burst stimulation. Time-course of changes in fEPSP slope obtained from a representative experiment in which we evaluated the LTP induced by strong theta-burst stimulation (15 bursts of four pulses at 100 Hz with an interburst interval of 200 msec) in the absence (●) and presence of the H-89 (1µM, ●). Control and test experiments were performed in two independent pathways on the same slice (S1 and S2). The independence of the two pathways was tested by studying Paired-pulse Facilitation (PPF) across both pathways, less than 12% facilitation being usually observed. H-89 was present for at least 20 minutes before LTP induction. (B) Representative recordings of field excitatory postsynaptic potentials (fEPSPs) obtained in an individual experiment before (black) and 50 minutes after (Blue) LTP induction in the control pathway (S1 in A).

To generate a late-LTP we used a strong θ-burst protocol, 15 bursts of four pulses at 100 Hz with an interburst interval of 200 msec (See Methods), similar to the one described by Nguyen and Kandel (1997). Application of a strong θ-burst protocol caused a long lasting enhancement of fEPSP slope. The potentiation evaluated 50-60 minutes after theta-burst stimulation was 65.2 ± 10% (n = 4, p <0.05, Figure 18) and lasted above 50% two hours after burst application (Figure 17.A). It was shown previously that late-LTP induced by this stimulation pattern in the CA1 area of the hippocampus requires activation of PKA in mice (Nguyen & Kandel 1997). To confirm if our late-LTP was PKA dependent we tested the influence of a selective PKA inhibitor, H-89 (1 µM) (Chijiwa et al 1990) on late-LTP expression in our experimental model. H-89 1 µM, when applied from 20 minutes before induction of LTP in the second pathway (S2), did not change the potentiation observed 50-60 min after the strong θ-burst protocol (64.4 ± 1.5%, n =4, p <0.05, Figure 18), as compared with the LTP observed in control conditions (see above). The addition of H-89 (1 µM) did not significantly alter synaptic

transmission (fEPSPs slope). These results suggest that LTP induced, by strong θ-burst in the CA1 area of rat hippocampus, is PKA independent contrary to what was previously described by Kandel and co-workers in mice (Nguyen & Kandel 1997).

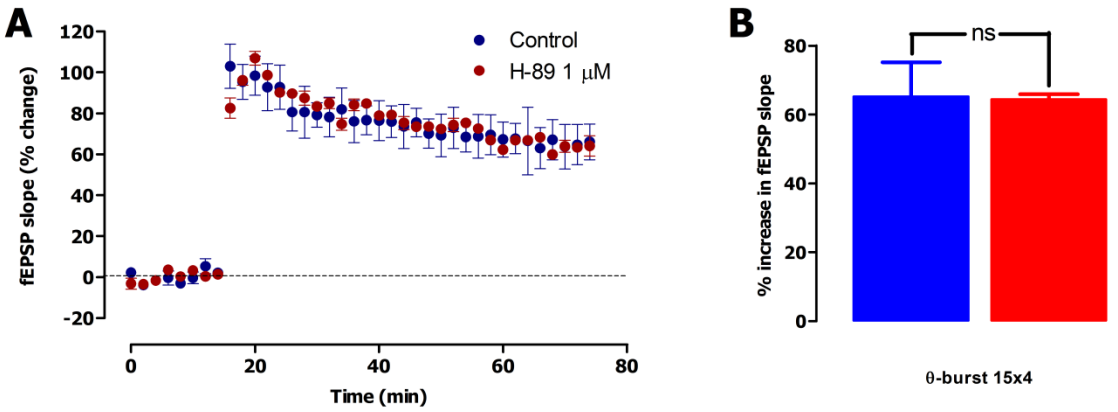

Figure 18. Inhibition of PKA with H-89 1 µM did not affect LTP induced by strong θ-burst stimulation. (A) Averaged time-course of changes in fEPSP slope obtained during late-LTP experiments performed in absence (●) and in the presence of H-89 (1 µM, ●). Each point represents the mean ± S.E.M. of four experiments (n = 4) (B) Values of potentiation obtained 50-60 min after θ-burst stimulation in the absence of drugs (■) and in presence of the PKA inhibitor H-89 (1 µM, ■). All values are mean ± S.E.M. of four experiments (n=4). ns – non-significant difference as compared with control in the same slices (paired Student's t test).

The difference between these results and the ones described by (Nguyen & Kandel 1997) could be explained by differences in the ionic composition of the aCSF, (the concentrations of Mg^{2+}, Ca^{2+} and K^{+} are different) or by the different species used (rat vs. mice). In particular, the higher K^{+} concentration could provide a larger/longer depolarization upon θ-burst stimulation. To account for these differences we performed the same experiments but using that aCSF composition (mM): 125 NaCl, 1.5 $MgSO_4$, 4.5 KCl, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1 NaH_2PO_4, and 10 glucose. Under these conditions strong theta-burst stimulation caused a potentiation of 63.7 ± 0.5% (n = 2) similar to the one described above. Application of H-89 (1 µM) 20 min before the induction of LTP decreased the potentiation to 53.4 ± 0.4 % (n =2; Figure 19). These results suggest a small influence of PKA on late-LTP induced under these experimental conditions, which is still very different from what was described by (Nguyen & Kandel 1997) in mice. The reduced number of experiments performed under these conditions does not allow drawing better conclusions on the influence of aCSF composition in late-LTP.

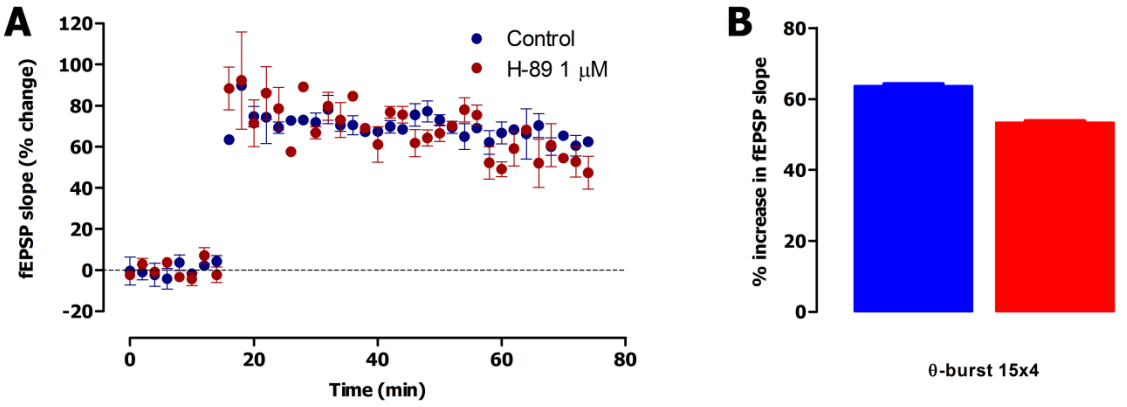

Figure 19. Inhibition of PKA with H-89 1 µM did not affect LTP induced by strong θ-burst stimulation (aCSF according to Nguyen and Kandel, 1997). (A) Averaged time-course of changes in fEPSP slope obtained during late-LTP experiments performed in absence (●) and in the presence of H-89 (1 µM, ●). Each point represents the mean ± S.E.M. of two experiments (n=2) (B) Values of potentiation obtained 50-60 min after θ-burst stimulation in the absence of drugs (■) and in presence of PKA inhibitor H-89 (1 µM, ■). All values are mean ± S.E.M. All values are mean ± S.E.M. of two experiments (n=2).

Another possibility for the absence of PKA dependence of late-LTP could be that the concentration of H-89 used was not effectively inhibiting PKA. H-89 is a competitive antagonist at the ATP binding site of the PKA catalytic subunit and its EC50 is known to depend strongly on intracellular ATP concentrations. For this reason we used one inhibitor acting through a different mechanism. We selected PKI 14-22 amide, a PKA inhibitor that binds the catalytic subunit of PKA in a way similar to the binding of the regulatory subunit and exerting a similar effect, i. e., suppressing the kinase activity (Glass et al 1989).

In the presence of PKI 14-22 amide (1 µM) the potentiation obtained upon strong θ-burst stimulation 61.8 ± 4.4 % (n =3, Figure 20) was not changed as compared to control conditions 69.8 ± 15.5 % (n =3, Figure 20). On its own, application of PKI 14-22 amide (1 µM) did not change synaptic transmission (fEPSPs slope).

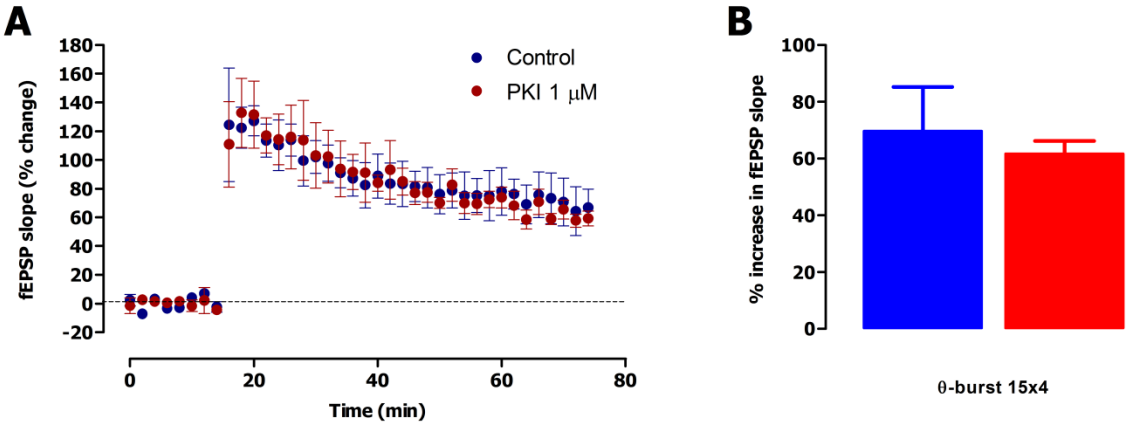

Figure 20. Inhibition of PKA with PKI 14-22 amide 1 µM did not affect LTP induced by strong θ-burst stimulation. (A) Averaged time-course of changes in fEPSP slope obtained during late-LTP experiments performed in absence (●) and in the presence of PKI 14-22 amide (1 µM, ●). Each point represents the mean ± S.E.M. of three experiments (n = 3) (B) Values of potentiation obtained 50-60 min after θ-burst stimulation in the absence of drugs (■) and in presence of the PKA inhibitor PKI 14-22 amide (1 µM, ■). All values are mean ± S.E.M. of three experiments (n=3).

Altogether, the results described above suggest that LTP induced by strong θ-burst stimulation in the CA1 area of rat hippocampal slices is PKA independent.

To investigate if late-LTP is truly PKA independent in this animal model we used a stronger θ-burst protocol (Late-LTP θ-burst protocol) consisting of three strong θ-burst stimulations delivered with a 6 minutes interval (see Methods). With this protocol we expected to obtain a strong late-phase LTP by delivering a stronger initial depolarization. The degree of potentiation induced by this stimulation protocol was assessed both at 50-60 min and 110-120 minutes after the last strong θ-burst stimulation and different slices were used for control and test experiments, to ensure that the slice would be healthy throughout the experiment. (Otherwise the experiment would last 4-5 hours). The potentiation obtained in control conditions one hour after Late-LTP θ-burst stimulation was of 93.9 ± 7.8% and was of 80.5 ± 8.3% after two hours (n = 7, Figure 21). This potentiation was larger than the one obtained with the strong θ-burst protocol (after 1h: 65.2 ± 10%, n = 4, Figure 20). When Late-LTP θ-burst stimulation was performed in the presence of H-89 (1 µM) the potentiation was 79.5 ± 22.5% one hour after the theta-burst stimulation, and 74.2 ± 4.5% after two hours (n = 2, Figure 21). Since this was only a mild decrease in the potentiation, we decided to increase the concentration of H-89 to 3 µM (limit concentration for a selective effect), to exclude the possibility of any drug efficacy problem. Furthermore we used again the PKI 14-22 amide (1 µM). When H-89 (3 µM) was present the potentiation induced by Late-LTP θ-burst stimulation was 90.3 ± 10.1% one hour after the last θ-burst stimulation and was of 76.2 ± 9.7% two hours after (n = 5, Figure 21). These potentiations were not significantly different from the ones obtained in control slices. In the presence of PKI 14-22 amide (1 µM), the potentiation one hour after the last θ-burst stimulation was 96.5 ± 2.5 and was of 75.0 ± 1.5 after two hours (n = 2, Figure 21). These results with the Late-LTP θ-burst stimulation further demonstrate that in this animal model late-LTP in the CA1 area of rat hippocampal slices does not require PKA activity.

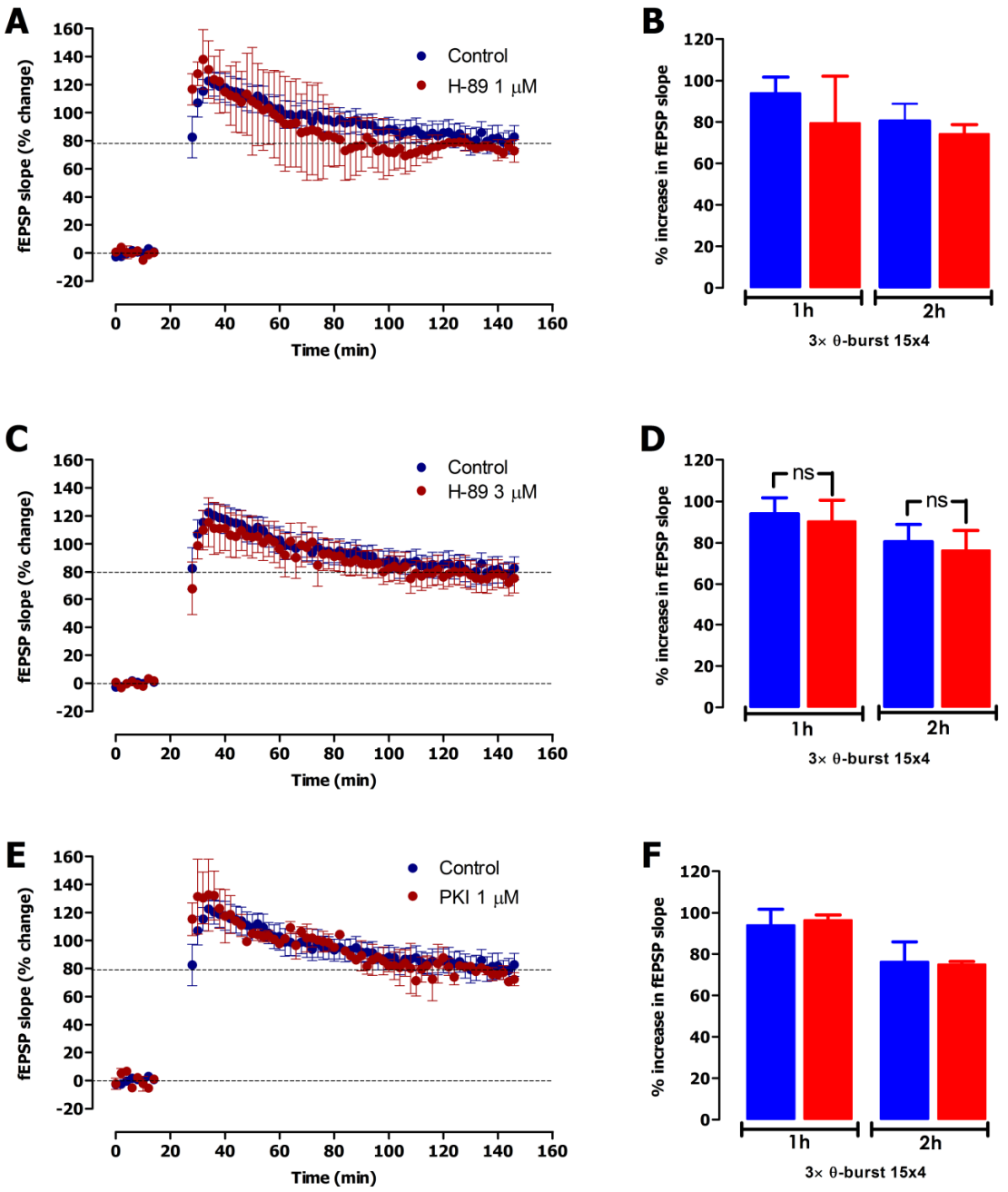

Figure 21. Inhibition of PKA does not change LTP induced by Late-LTP θ-burst stimulation in the CA1 area of the hippocampus. (A) Averaged time-course of changes in fEPSP slope obtained during late-LTP experiments performed in absence (●, n=7, A, C, E) and in the presence of H-89 (1 µM, n=2, ●, A), H-89 (3 µM, n=5, ●, C) and PKI 14-22 amide (1 µM, n=2, ●, E). Late-LTP was induced by three strong θ-burst stimulations delivered with a 6 min interval. Each point represents the mean ± S.E.M. of n experiments. (B) Values of potentiation obtained 50-60 min and 110-120 min after Late-LTP θ-burst stimulation in the absence of drugs (■, n=2, B, D, F) and in presence of the PKA inhibitors H-89 (1 µM, n=2, ■, B), H-89 (3 µM, n=5, ■, D) or PKI 14-22 amide (1 µM, n=2, ■, F). All values are mean ± S.E.M. of n experiments. ns – non-significant difference as compared with control in independent slices (Student's t test).

The role of VIP on LTP induced by a θ-burst protocol

Since it has previously been observed in our laboratory that endogenous VIP inhibits LTP induced by θ-burst stimulation through $VPAC_1$ receptor activation (Cunha-Reis D. 2008), we investigated if this modulation was still present in 12-week-old rats using a strong θ-burst stimulation protocol.

To test this hypothesis we studied LTP induced by strong θ-burst stimulation in the presence of the $VPAC_1$ antagonist, PG 97-269 (100 nM) (Cunha-Reis et al 2005). Application of PG 97-269 (100 nM) to hippocampal slices did not change basal synaptic transmission (fEPSP slope). The presence of PG 97-269 20 min before the induction of LTP decreased ($p < 0.05$) the potentiation to 53.4 ± 4.8 % (n =5, Figure 22.B) when compared to the control pathways (67.1 ± 5.6 %, n = 5; Figure 22.B). These results suggest that $VPAC_1$ receptor activation enhances Late-LTP induced by strong θ-burst stimulation in 12 weeks-old rats. These results are the opposite of what was previously observed by Cunha-Reis et al. (Cunha-Reis D. 2008) in 6 weeks-old rats using weak θ-burst stimulation. The different effects of endogenous VIP in the modulation of LTP could be explained by differences in the stimulation protocol because strong θ-burst stimulation activates pathways (and likely receptors) different from those activated by the weak θ-burst stimulation. Another explanation would be that the age could be affecting the mechanisms by which the $VPAC_1$ receptor could modulate LTP.

To test the first hypothesis we studied the influence of PG 97-269 on LTP induced with a weak θ-burst protocol in 12 week-old rats. The value of potentiation obtained after weak theta-burst stimulation, was 33.0 ± 4.9% (n=6, Figure 24) in the control conditions (absence of added drugs).This was not significantly different from the potentiation obtained with weak θ-burst stimulation in 6 week-old rats (29.6 ± 2.6%, n=8, Figure 24). In the presence of PG 97-269 (100nM) the potentiation was enhanced to 46.4 ± 4.5 % (n =6, $p < 0.05$, Figure 22.D), a nearly 24% enhancement of the potentiation. To test the influence of age in the potentiation induced by strong θ-burst stimulation we tested the strong θ-burst protocol in 6 weak-old rats. The potentiation obtained using this protocol was of 44.0 ± 0.2 %, (n = 3). This was significantly smaller than the one obtained in 12 week-old rats (Figure 24). Application of PG 97-269 20 min before the induction of LTP increased the potentiation (around 26% when compared to the control) to 59.7 ± 11.5 % (n =3, Figure 22.F). Thus, LTP induced by strong θ-burst stimulation in 6 week-old rats is inhibited by endogenous VIP acting on $VPAC_1$ receptors. This is similar to what is observed for weak θ-burst stimulation in both 6 and 12 week-old rats and the opposite of what it is observed using a strong θ-burst protocol in 12 week-old rats.

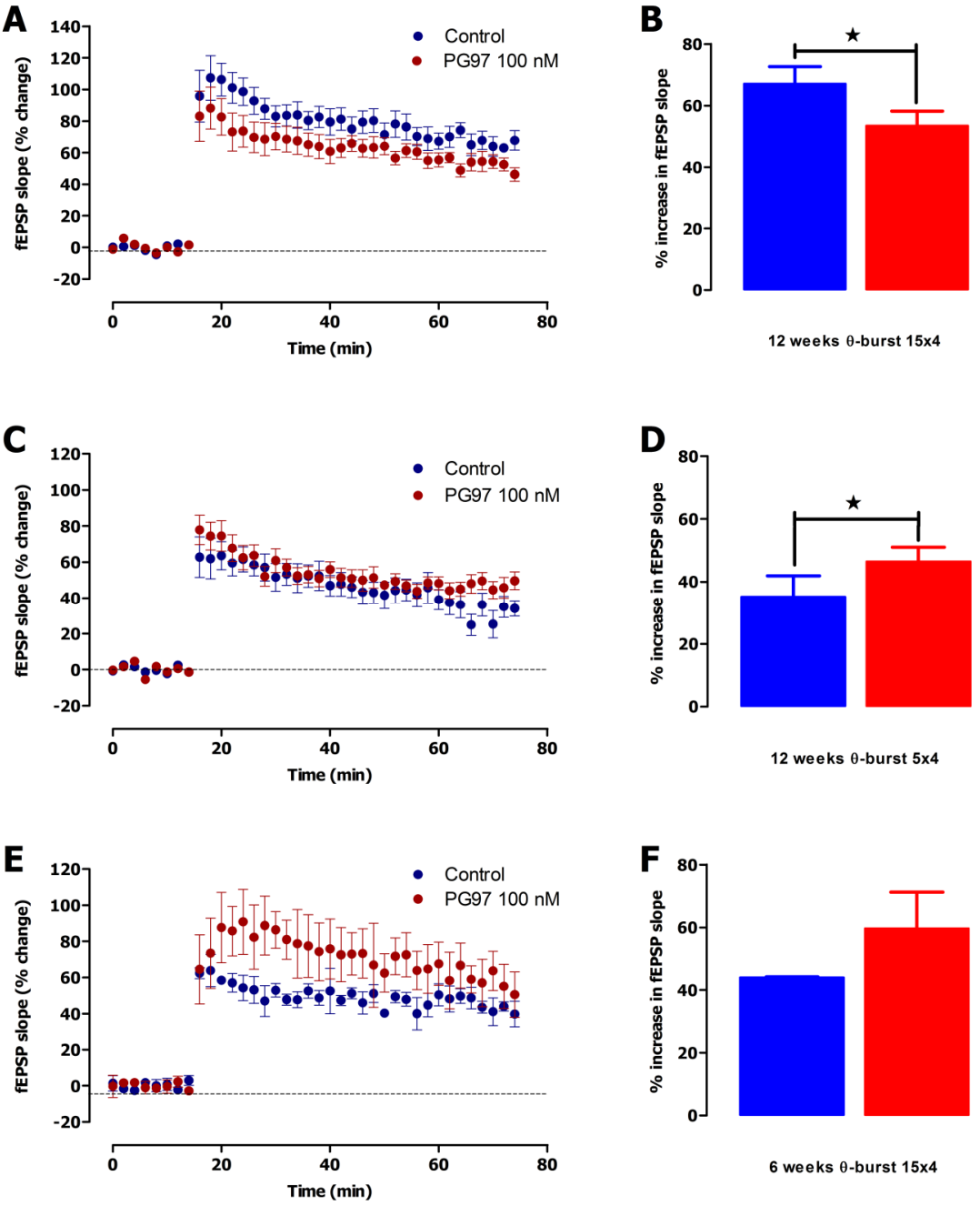

Figure 22. Blockade of VIP VPAC1 receptors as opposing effects on LTP induced by strong θ-burst stimulation in the CA1 area of the hippocampus. (A) Averaged time-course of changes in fEPSP slope obtained during late-LTP experiments performed in absence (●, n=3-6, A, C, E) and in the presence of PG 97-269 (100 nM, n=3-6, ●, A, C, E). Late-LTP was induced by strong θ-burst stimulation. Each point represents the mean ± S.E.M. of n experiments. (B) Values of potentiation obtained 50-60 min after strong θ-burst stimulation in the absence of drugs (■, n=3-6, B, D, F) and in presence of the VPAC1 antagonist PG 97-269 (100 nM, n=3-6, ■, B, D, F). All values are mean ± S.E.M. of n experiments. ★ – significant difference as compared with control in the same slices (paired Student's t test).

To control for the possibility that a larger release of VIP caused by the strong θ-burst protocol could not be overcome by the $VPAC_1$ antagonist, we tested also a larger concentration (300nM) of $VPAC_1$ receptor antagonist. The addition of the PG 97-269 300 nM did not change basal synaptic transmission (fEPSP slope). In the presence of the $VPAC_1$

antagonist the potentiation induced by strong θ-burst stimulation (37.1 ± 13.4%, n =2) decreased about 17% when compared to the control pathways (44.9 ± 8.2%, n = 2, Figure 23.F). These results suggest that LTP enhances $VPAC_1$ receptor activation after a strong θ-burst stimulation in 12 weeks-old rat and raised others questions such as the possibility that $VPAC_1$ receptor inhibits LTP after late-LTP induced by Late-LTP θ-burst protocol.

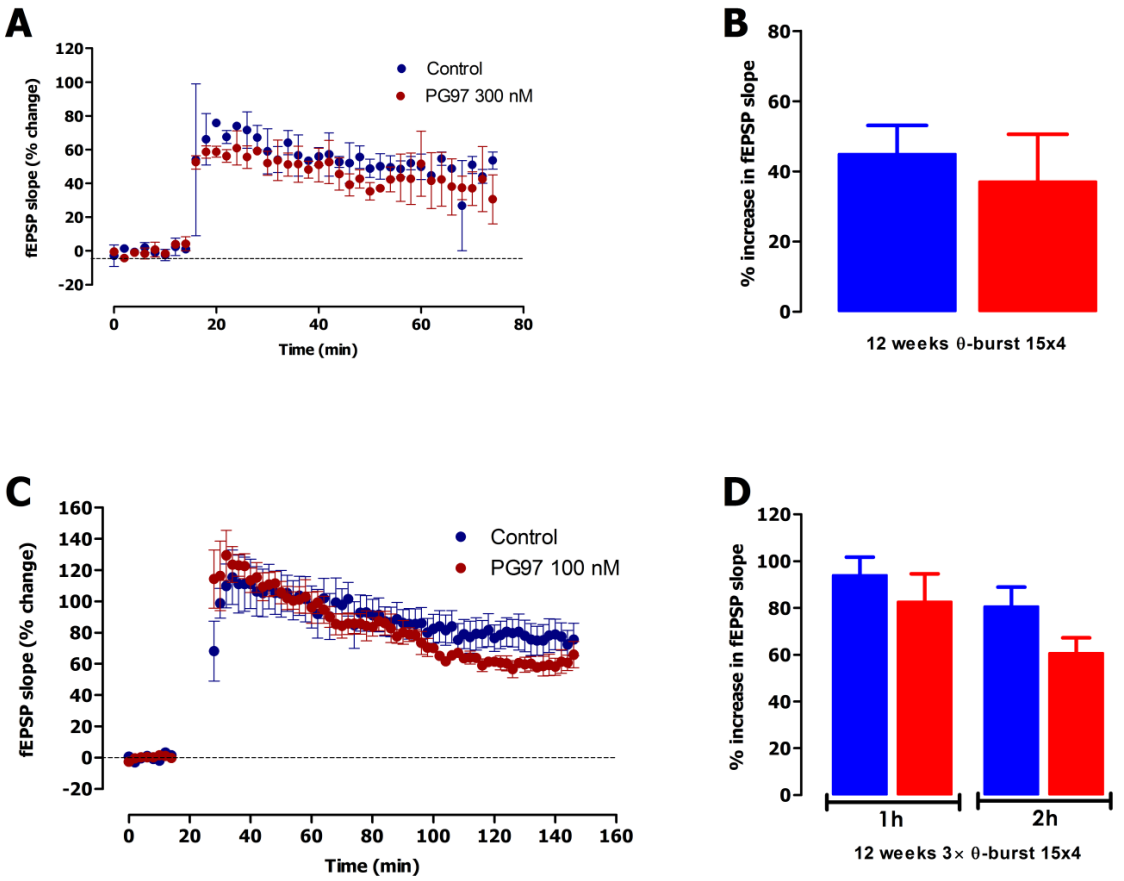

Figure 23. The $VPAC_1$ blockade decreased LTP induced by strong and late-LTP θ-burst stimulation in 12 week-old rats. (A) Averaged time-course of changes in fEPSP slope obtained during late-LTP experiments performed in absence (●, n=2-7, A, C) and in the presence of PG 97-269 (100 nM, n=2, ●, A, C). Late-LTP was induced by strong θ-burst stimulation (A, B) in the same slice or Late-LTP θ-burst stimulation in independent slices (C, D). Each point represents the mean ± S.E.M. of n experiments. (B) Values of potentiation obtained 50-60 min after strong θ-burst stimulation in the absence of drugs (■, n=2) and in presence of the $VPAC_1$ antagonist PG 97-269 (100 nM, n=2, ■). (B) Values of potentiation obtained 50-60 min and 110-120 min after strong θ-burst stimulation in the absence of drugs (■, n=7) and in presence of the $VPAC_1$ antagonist PG 97-269 (100 nM, n=2, ■). All values are mean ± S.E.M. of n experiments.

We investigated also the effect of the $VPAC_1$ antagonist (PG 97-269) on late-LTP using the Late-LTP θ-burst protocol. The enhancement of fEPSP slope was assessed 50-60 min and 110-120 minutes after θ-burst stimulation and again different slices were used for control and test experiments. The value of potentiation obtained 1 hour after theta-burst stimulation, was of 82.6 ± 11.9% and of 60.7 ± 6.6% after two hours (n = 7, Figure 23). When the $VPAC_1$ receptor was blocked with PG 97-269 (100nM) we observed a small increase of potentiation to 93.9 ± 7.8% one hour after the Late-LTP θ-burst stimulus and 80.5 ± 8.3% after 2 hours (n = 2, Figure 23).

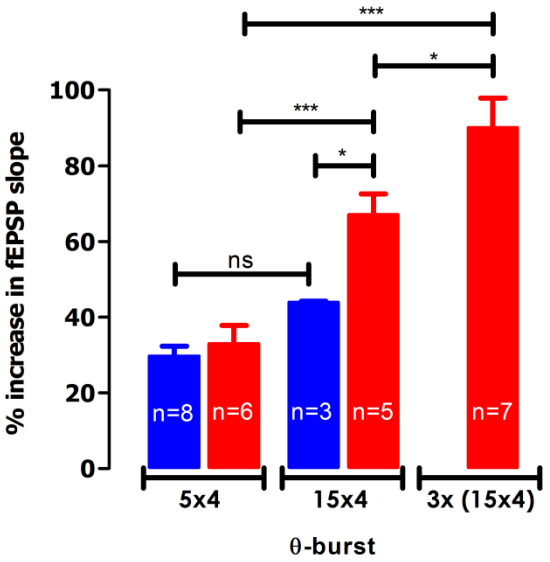

Figure 24. Influence of protocol strength and of age on the LTP induced by theta-burst stimulation. Values of potentiation obtained 50-60 min after θ-burst protocols using rats with 6 weeks (blue) and 12 weeks (red). weak θ-burst protocols – 5x4 , strong θ-burst protocols – 15x4, late-LTP θ-burst protocols –3x (15x4). (ns – no significance, ★ - p<0,05, ★★★ - p<0,001, one-way ANOVA followed by Tukey's Multiple Comparison test) . All values are mean ± S.E.M..

In vitro model of epilepsy in slices

Since TLE leads to learning and memory impairments (Detour et al 2005; Dütsch et al 2004), we decided to study if spontaneous epileptiform activity elicited in vitro leads to changes in Late-LTP in 6 week-old rats. For this purpose, we tested several methods described in the literature for in vitro induction of epileptiform activity such as perfusion with pilocarpine (muscarinic receptor agonist) (Nagao et al 1996), a Mg^{2+}-free aCSF (inhibitor of NMDA receptor, (Tancredi et al 1988) or bicuculline ($GABA_A$ receptor antagonist, (Avoli et al 1996; Debanne et al 2006). Both perfusion with pilocarpine (1µM-1mM) and Mg^{2+}-free aCSF failed to induce ictal-like epileptiform activity. Perfusion with bicuculline (50 µM, 30 min), in a higher concentration than the ones described in the literature by Avoli et al. (1996) and Debanne et al. (2006), induced reproducible spontaneous ictal-like epileptiform activity (Figure 25). During the bicuculline perfusion, the spontaneous activity was recorded during 30 seconds. Due to limitations of the acquisition system after the 30 seconds there was a gap of approximately 12 seconds during which no acquisition was performed. The spontaneous activity, number of spontaneous events (fEPSP) and the maximum fEPSP amplitude per wave, was quantified manually using WinLTP program© (Anderson & Collingridge 2001) (Figure 25.D).

After 30 minutes of bicuculline perfusion the slices were washed with aCSF solution during 1 hour. After 1 hour of wash it is possible to see a LTP-like effect with an increase of 20% in the fEPSP slope (n =2, Figure 26). When a strong θ-burst stimulation was applied 60 minutes after bicuculline washout the potentiation of fEPSP slope that was assessed 50-60 minutes after theta-burst stimulation was of 46.4 ± 8.9 % (n =2, Figure 26). This value is similar

to the potentiation obtained in the absence of bicuculline treatment for 6 week-old animals (Figure 24).

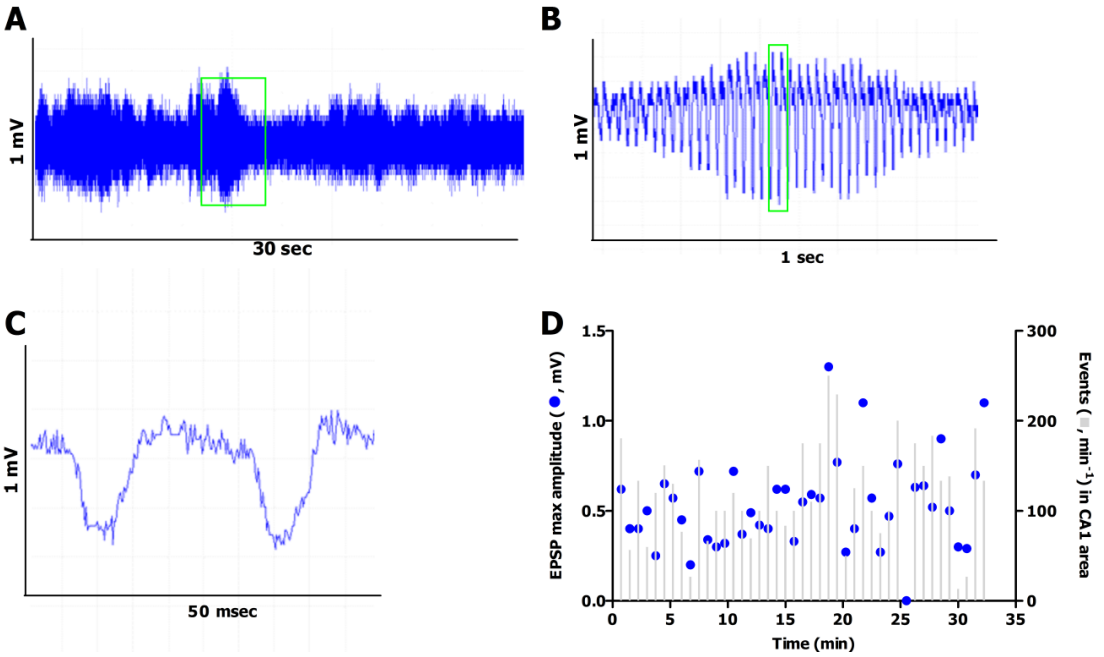

Figure 25. Ictal-like epileptiform activity observed in the presence of the $GABA_A$ receptor antagonist, bicuculline 50 µM. (A) Spontaneous activity of the field potential recorded in stratum radiatum in CA1 area of the rat hippocampus. (B) Representative Ictal-like event (expansion of the area delimited in A with a green square). (C) Representative fEPSP recording from the CA1 pyramidal layer (expansion of the area delimited in B with a green square). (D) Changes in fEPSP maximum amplitude (●) and frequency of spontaneous fEPSP activity ( ) in a representative experiment.

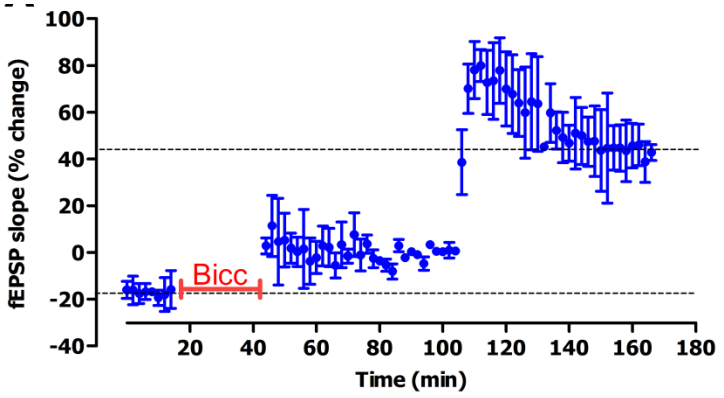

Figure 26. Potentiation of excitatory synaptic transmission in CA1 area after epileptiform activity induced with $GABA_A$ receptor antagonist, bicuculline 50 µM, and influence on Late-LTP induced by strong θ-burst stimulation 1h after Bicuculline perfusion. (A) Averaged time-course of changes in fEPSP slope of 6 week-old rat slices subjected to bicuculline treatment followed by LTP induction with a strong θ-burst protocol. Potentiation was evaluated 50-60 min after θ-burst application. Each point/bar represents the mean ± S.E.M. of two experiments (n = 2). Bicc – bicuculline.

Discussion

Long-term potentiation of synaptic transmission in the hippocampus is the leading experimental model for studying synaptic plasticity phenomena that may underlie learning and memory (Linden & Routtenberg 1989). It was recently discovered in our lab that endogenous VIP has a restraining effect on LTP, elicited in vitro by theta-burst stimulation. This effect is mediated by $VPAC_1$ receptor activation and is dependent on GABAergic transmission or $GABA_A$ receptors (Cunha-Reis D. 2008). These studies were performed in young adult rats (6-7 weeks-old), that could not provide an adequate age-matched control for epileptic rats. Furthermore it was used a theta-burst stimulation pattern that did not elicit a late-LTP, a better cellular model for the molecular alterations leading to the formation of stable memories. In this work we tried to establish the experimental conditions to study the influence of $VPAC_1$ receptors on late-LTP in epileptic rats, by using 12 week-old rats and stronger theta-burst stimulation paradigms. Our goal was to elicit a robust LTP lasting for more than two hours, and occurring through a mechanism dependent on PKA activation (the trigger for downstream phosphorylation of CREB).

To elucidate the role played by PKA on LTP induced by several θ-burst stimulation protocols in the CA1 area of hippocampus, we tested the effect of two different PKA inhibitors, H-89 and PKI 14-22 amide, on LTP induction and expression. In all experimental conditions tested, LTP was found to be independent of PKA activation, since it was unaltered when induced in the presence of H-89 (1 µM). This may result from inefficient PKA inhibition due to the concentration of H-89 used, since the IC_{50} for PKA inhibition by H-89 is highly dependent on the intracellular ATP concentration (Murray 2008). In fact, H-89 is a competitive antagonist of ATP at its binding site for the PKA catalytic subunit. This hypothesis was excluded when the concentration of H-89 was increased to 3 µM, which is the limit concentration for selectivity (Chijiwa et al 1990), and still no effect upon LTP was observed. However, the addition of H-89 did decrease LTP when the aCSF solution was switched to one that matched the ionic composition of that used by Kandel and co-workers (Nguyen & Kandel 1997), but not to the same extent. Our aCSF solution was closer to the ionic concentrations observed in physiological conditions than used by Kandel and co-workers so it was decided, that the remains study would continue with our aCSF solution.

Due to the absence of effect observed with H-89, which opposed that previously observed with other PKA inhibitors (KT-5720 1 µM and RpcAMPs 100 µM) that shared the same mechanism of action (Nguyen and Kandel, 1997), we then tested another type of PKA inhibitor (PKI 14-22 amide), under the same experimental conditions. Unlike H-89, PKI 14-22 amide selectively inhibits the free catalytic subunit of PKA, PKI is a competitive inhibitor with respect to phosphoryl-accepting peptide and protein substrates, although it is not phosphorylated itself (Glass et al 1989; Howard et al 1994). The results obtained with either of the inhibitors, acting at different PKA sites, confirmed that PKA is not involved on late-LTP induced by a strong θ-burst protocol. It should be noted that the requirement for PKA recruitment in early and late LTP is still a controversial subject. In fact, while Kandel and co-workers argue that PKA only affects the late-LTP (Nguyen & Kandel 1996), other groups

suggest there is a role for PKA in early LTP (Otmakhova et al 2000), but the first used high-frequency stimulation protocols to induce LTP and the later used unusually high concentrations of the PKA inhibitors, that are likely outside its selectivity range. The differences observed in our experimental conditions, compared with the literature on the subject may be explained by differences in protocol and concentration range of inhibitors. For these reason we will compare our results only with the ones in Nguyen and Kandel (1997). Clearly PKA plays a critical role in the consolidation of spatial memory using a transgenic mice in which PKA activity is decreased, for which late phase LTP induced by HFS appears to provide an important neurophysiologic contribution (Abel et al 1997). Since it appears clear from our results that we were successful inducing a late-LTP under our experimental conditions, and Nguyen and Kandel (1997) showed that PKA should be activated early in the process of LTP induction (Nguyen & Kandel 1997), we decided to test a stronger θ-burst stimulation protocol (late-LTP θ-burst) consisting of three strong θ-burst trains, that was expected to induce the expression of a clear late phase LTP (Morgan & Teyler 2001). This would likely overcome the differences in the extent of depolarization caused by the different ionic composition of the aCSF used by Nguyen and Kandel (1997) that favours a stronger depolarization during LTP induction. Our results with this stimulation pattern confirmed that the inhibition of PKA did not affect LTP thus confirming the data previously obtained.

Previous results suggest that hippocampal CA1 LTP is strongly dependent on CaMKII activity, which is consistent with previous observations on different stages of LTP requiring distinct signalling pathways (Huang & Kandel 1994). Previous results obtained by our group confirm the importance of CaMKII in the induction of LTP by a weak θ-burst protocol in 6 week-old rats (Rodrigues 2009). Further studies are needed to investigate the signalling cascades activated using a strong θ-burst protocol. One way to investigate this hypothesis would be applying pharmacological tools that simultaneously interfere with the CaMKII signalling pathway, while using the same strong θ-burst stimulation protocol. An alternative strategy would be to reproduce the experimental conditions and the PKA inhibitors so as to exactly match previous work by Kandel and colleagues (Nguyen & Kandel 1997). However, they used mice as their animal model, which was not so convenient for our work and it could explained the differences.

To elucidate the influence of $VPAC_1$ receptor activation on LTP induced by strong θ-burst protocols we used a $VPAC_1$ receptor antagonist, PG 97-269 100nM (Cunha-Reis et al 2005). The results obtained in this thesis suggest that $VPAC_1$ receptor activation enhances LTP induced by strong θ-burst in 12 week-old rats. These results are the opposite of previous data obtained in our lab, showing that activation of $VPAC_1$ receptors decreased LTP expression in 6 weeks-old Wistar rats (Cunha-Reis D. 2008). The discrepancy of results could be explained by differences in stimulation protocol or it could be due to the use of animals in different developmental stages. To try to understand the differences observed between the two experimental conditions we tested the weak θ-burst protocol in 12 week-old rats and the strong θ-burst protocol 6 week-old rats, and compared the data with that previously obtained in the lab for the weak θ-burst protocol, in 6 week-old rats. The data obtained suggest that $VPAC_1$ receptor activation inhibited LTP only when using the weak θ-burst protocol in 12 and 6 week-old rats. Enhancement of LTP by $VPAC_1$ receptor activation was only observed when using a strong θ-burst protocol 12 week-old rats. $VPAC_1$ receptor activation inhibited LTP,

although to a minor extent, with the strong θ-burst protocol in 6 week-old rats. According to these observations, three hypotheses may explain the observed discrepant results: 1) that VIP activates different cellular mechanisms according to the strength of the stimulation protocol used; 2) that $VPAC_1$ receptors levels suffer a developmental regulation between 6 and 12 weeks; 3) that the cellular/transduction mechanisms involved in LTP expression change from 6 to 12 weeks.

The first hypothesis alone would not explain the shift of $VPAC_1$ receptor function when using the same induction protocol, in slices prepared from animals with different ages (strong protocol, 6 vs. 12 weeks). The second hypothesis implies that developmental change would require not only a change in the receptor levels but also a spatial redistribution of these because the effect of $VPAC_1$ receptors on LTP changes from an inhibition to an enhancement of LTP. This hypothesis is supported by previous evidence of a decrease in $VPAC_1$ receptor levels in the hippocampus, during aging (Joo et al 2005). More studies concerning changes in $VPAC_1$ receptor expression and function during aging are required, starting with newborn to old rats. In fact, previous studies only focus on effects observed in adult (4–6 months old) and aged (24–29 months old) animals (Joo et al 2005). However, it is possible to speculate that a decreased density of $VPAC_1$ receptors in the synaptic cleft would lead to a smaller impact of their function in LTP modulation by VIP. The decrease of $VPAC_1$ receptor expression could be selective to certain types of interneurones, namely, in VIP expressing interneurones that project to the alveus innervating interneurones that are responsible for feedback inhibition, for this is the only VIP-containing neuronal pathway that has been demonstrated to provide inhibition to pyramidal cell dendrites (Yanovsky et al 1997). Such a selective decrease in $VPAC_1$ receptor expression would lead to a decreased inhibition of pyramidal cell dendrites, allowing for a larger LTP. This together with an increase in the weight of VIP-mediated disinhibition (Cunha-Reis et al 2004) could account for a reversal of VIP effects on LTP. By decreasing VIP influence on feedback GABAergic inhibition, glutamate-mediated synaptic transmission to pyramidal cell dendrites in the CA1 area of the hippocampus would be expected to increase by a mechanism dependent on GABAergic transmission. The third hypothesis is based on the influence of protocol strength and of age on the LTP induced by theta-burst stimulation because when it was applied a weak theta burst stimul the potentiation was similar in both ages but when we increased the intensity of theta burst stimulation protocol we observed differences in LTP between 6 and 12 weeks-old rats (Figure 24).

Another fact to take into account, is that VIP can enhance NMDA currents via $VPAC_1$ and $VPAC_2$ receptor activation, when applied in low concentration (1 nM), while in higher VIP concentrations, the effect is due to PAC_1 receptor activation (Yang et al 2009). This could suggest a role for VIP basket cells in the modulation of LTP because these interneurones have projection to soma of pyramidal cells. Such a strong influence of VIP on the soma of pyramidal cells could also provide a strong influence on the outcome of synaptic plasticity at pyramidal cell dendrites. In our experiments, only antagonists were used.

Future studies will aim at elucidating how $VPAC_1$ receptor modulates LTP in different developmental stages, using these same protocols, and how the levels and subcellular receptor distribution of these receptors changes between 6 and 12 weeks of age should prove useful in answering the questions raised in this discussion. Further studies are needed to

investigate which interneurone subtype(s) can modulate LTP via $VPAC_1$ receptor activation. These studies would give us a more complete picture of how hippocampal long-term potentiation is controlled by $VPAC_1$ receptor activation in the CA1 area of the hippocampus. One way to investigate this would be by using patch-clamp recordings to intracellularly apply pharmacological tools that interfere with $VPAC_1$ receptor function on different VIP interneurones, while registering single-cell currents and how they are affected when LTP is induced, in pyramidal cells of CA1 area.

Altough it was initiallyproposed to perform LTP experiments in epileptic rats, the time taken to study and establish the mechanism involved in late-LTP induced by strong θ-burst stimulation protocol was long. We decided then to use an in vitro model of seizures to investigate the role of $VPAC_1$ receptors on LTP following seizures. To elucidate the influence of in vitro model of TLE in LTP, we tested the strong θ-burst protocol on slices from 12 week-old rats subjected to an in vitro treatment generating ictal like activity. The value of potentiation in fEPSP slope (46.4% ± 8.9 %, n =2; 35ºC), obtained when LTP was induced one hour after epileptiform activity, was similar to that observed in control conditions (44.0 ± 0.2 %, n = 3; 30.5ºC). The reduced number of experiments further limits interpretation of these results. Before it is possible to suggest that the in vitro model of TLE has no effect upon LTP, it is necessary to make some experiments to control for the fact that both experimental conditions were performed at different temperatures. In fact, LTP experiments in "epileptic" slices were performed at a higher temperature, which is known to increase synaptic activity (Bittar & Muller 1993). Restrictions in the time available to perform the experimental work conducting to the present thesis made it impossible to investigate further this subject and to conclude on the influence of $VPAC_1$ receptors on the expression of LTP post-seizures.

Further studies are thus needed to clarify the role of VIP on epilepsy. Some insight on these subjects might be gained by performing studies to address several key questions, such as: 1) How are $VPAC_1$ receptor mediated effects on synaptic transmission changed after ictal-like discharges (Figure 27)? 2) How is $VPAC_1$ receptor-mediated modulation of LTP affected, after the induction of ictal-like discharges (Figure 28)? 3) Can $VPAC_1$ activation prevent or attenuate ictal-like discharges (Figure 29)? Several protocols are proposed to address each of these questions at the Figures mentioned at the end of the respective question.

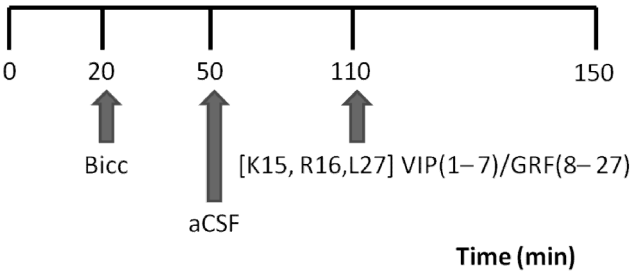

Figure 27. Schematic representation of an electrophysiology experiment designed to study of the effect of $VPAC_1$ receptor activation on synaptic transmission, after an ictal-like epileptiform event in vitro. Bicuculline – $GABA_A$ antagonist, [K15, R16,L27] VIP(1–7)/GRF(8–27) – $VPAC_1$ agonist, aCSF - Artificial cerebro spinal fluid.

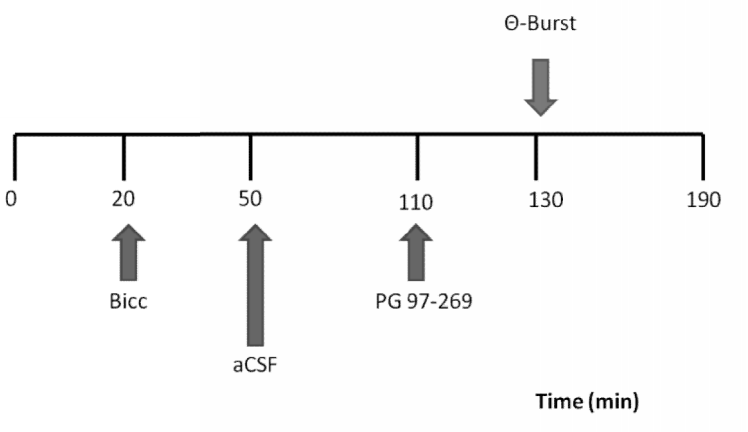

Figure 28. Schematic representation of an electrophysiology experiment designed to study how VPAC1 receptor activation may affect LTP after an ictal-like epileptiform event in vitro. Bicuculline – $GABA_A$ antagonist, PG 97-269 – $VPAC_1$ antagonist, aCSF - Artificial cerebro spinal fluid.

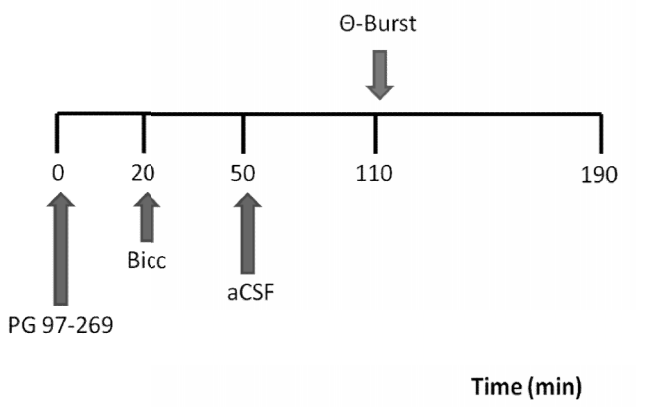

Figure 29. Schematic representation of an electrophysiology experiment designed to study how $VPAC_1$ receptor activation may affect in vitro seizure-like activity and LTP expression after an ictal-like epileptiform event. Bicuculline – $GABA_A$ antagonist, PG 97-269 – $VPAC_1$ antagonist, aCSF - Artificial cerebro spinal fluid.

Conclusion

In this master thesis it was possible to conclude that LTP was PKA independent when induced by a strong θ-burst protocol, in 12-week-old rats. These experiments were performed as a way to validate the protocol as a suitable in vitro model to study memory. The role of $VPAC_1$ receptor on LTP induced by a θ-burst protocol varied with the experimental conditions. The activation of $VPAC_1$ receptors led to a decrease in LTP except when a strong stimulation protocol was used in slices prepared from 12 week-old rats, for which $VPAC_1$ receptor activation exerted the opposite effect on LTP, i.e. the activation of $VPAC_1$ receptor enhanced LTP.

It was not possible for now to draw clear conclusions on the role of $VPAC_1$ receptors in epilepsy because the time to conclude the master thesis was limited. Nevertheless, the results obtained will allow a better approach of epilepsy models taking into account variables such as the animal age.

Further studies on the modulation of LTP by endogenous VIP are required to fully understand how $VPAC_1$ receptors influence LTP in the hippocampus because 40 years after the discovery of VIP by Mutt and Said, the role of VIP on synaptic transmission in the hippocampus remains unclear. Still, the results described in this thesis clearly suggest that modulation by VIP may function as a filter for LTP expression.

Acknowledgements

Ao finalizar esta importante fase da minha vida é inevitável agradecer a todos os que contribuíram, de alguma forma, para a realização desta dissertação.

Em primeiro lugar agradeço ao professor Alexandre Ribeiro e à professora Ana Sebastião por me terem recebido no laboratório de neurociências do Instituto de Medicina Molecular proporcionando-me assim a oportunidade de trabalhar com esta equipa fantástica.

Desejo também deixar um agradecimento especial aos meus orientadores prof. Doutor Rodrigo Almeida e doutora Diana Cunha Reis por me terem aceitado. Um particular obrigado à Diana Cunha Reis por me permitir viver durante este estágio no mundo fascinante da investigação em neurociências e por me ter transmitido valiosos ensinamentos.

Agradeço igualmente a todos os membros do laboratório e em especial à ala de electrofisiologia, por tantas situações partilhadas. Obrigada Nádia Rodrigues e Vânia Batalha pelo apoio e ajuda ao longo do estagio. Agradeço à Maria José Diógenes e à Luísa Lopes pelas ajudas nos momentos de aflição. Ao "Mestre" Diogo Rombo e à Raquel Dias um sincero e gigante obrigado pelo apoio, momentos de reflexão e muito mais…

Gostaria ainda de agradecer à Elvira, ao Sr. João, à Cristina e à Alexandra pelo apoio técnico e administrativo essencial para o desenvolvimento desta tese.

Aos alunos de doutoramento e de mestrado: Ana Filipa, André Santos, Daniela Calçada, Jorge Valadas, Rita Aroeira, um muito obrigado pelo companheirismo de bancada e também por estarem sempre disponíveis para ajudar e debater o trabalho. Tornaram o laboratório um óptimo local de convívio e bem-estar.

Fica também uma palavra de agradecimento a todos os meus amigos, pelo apoio em todos os momentos que levaram a realização deste trabalho e não só:

Ana, Catarina, Daniel e Sara, meus amigos desde do básico agradeço-vos todo o suporte emocional e mesmo intelectual.

Um enorme obrigado ao meu grande amigo Hugo pelas horas perdidas nos momentos de aflição e alegria.

À Mariana por ser a irmã de vida e mesmo que pudesse descrever todo o seu significado não haveria espaço suficiente para escrever, à Sara por seres assim, à Ana Filipa e à Soraia pelo despertar para realidade, ao Daniel e ao Miguel pelos momentos divertidos principalmente no andebol e ténis de forma a libertar o stress do dia-a-dia, ao Bruno pela sua peculiaridade, ao Ivan pela amizade e pela similaridade, à Maria João e ao Carlos que mesmo

distante estão sempre presentes, fica aqui profundo e sincero obrigado por terem participado neste 5 longos anos de tristezas e alegrias extremas.

A realização desta tese, assim como toda a minha formação académica, não seria possível sem o suporte da minha família tanto de sangue como afectiva por, isto tudo e muito mais, fica aqui um eterno obrigado. Deixo inevitavelmente um especialíssimo obrigado à minha mãe e irmã pelas ausências, tristezas e alegrias.

Um agradecimento final à Direcção Principal de Preparação de Tropas e Ensino do Estado Maior General das Forças Armadas Angolanas pelo financiamento dos meus estudos superiores, que me foram tão importantes. Fica aqui também um desejo de estar a altura das obrigações da DPPTE e do meu país.

Mungo ngondoia, mukuanhi uegia kissua ngondo kuisa. Qui bangue kima se usuku ucala dikanga. Ò luanha ondó kwissa.

Nga sakidila

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