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Participación de ERK en la reactivación del ciclo celular en neuronas hipocampales secundaria a daño sináptico

T E S I S

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ÍNDICE

Lista de Abreviaturas

AK Ácido Kaínico

ßA ß-amiloide

CE Corteza entorrinal

EA Enfermedad de Alzheimer

ERK Proteína cinasa regulada por señales extracelulares (por sus siglas en inglés)

GD Giro dentado

i.c.v. intra-cerebroventricular

LD Lámina dorsal del giro dentado

LV Lámina ventral del giro dentado

MAPKs Proteínas cinasas activadas por mitógenos (por sus siglas en inglés)

MEK Proteína cinasa activada por mitógenos extracelulares (por sus siglas en inglés)

PHF-1 Paired helical filaments (por sus siglas en inglés)

PPA Proteína Precursora del amiloide

I. ORGANIZACIÓN DE LA TESIS

Dentro del presente trabajo de tesis en el apartado correspondiente a la introducción, se presenta un artículo de revisión en el que se describen las evidencias sobre la reactivación del ciclo celular en neuronas post-mitóticas y su participación en algunas condiciones neurodegenerativas, en particular la EA. Así mismo, se destacan los hallazgos que relacionan los mecanismos celulares implicados en la plasticidad cerebral con aquellos involucrados en la activación del ciclo celular. Artículo de revisión: Hernández-Ortega K, Quiroz-Baez R, Arias C. Cell cycle reactivation in mature neurons: a link between brain plasticity, neuronal injury and neurodegenerative diseases? Neurosci Bull 2011, 27:185-96. Además, dentro de la introducción se incluye una sección sobre la cinasa ERK, su papel en la neuroplasticidad y en la activación del ciclo celular neuronal. Además, se adjunta un apartado acerca de la relevancia del circuito CE-hipocampo en la EA y su organización anatómica.

Como antecedente directo de este estudio se cita un estudio previo que corresponde a mi trabajo de tesis de licenciatura, mismo que se presenta para su consulta en el apéndice. Artículo (2): Hernández-Ortega K, Ferrera P, and Arias C. Sequential expression of cell-cycle regulators and Alzheimer's disease–related proteins in entorhinal cortex after hippocampal excitotoxic damage. J Neurosci Res 2007, 85:1744–51.

Dentro de la sección de materiales y métodos, solo se presenta un diagrama del diseño experimental seguido en este trabajo, ya que las especificaciones del mismo se describen en el artículo incluido en la sección de resultados.

En los resultados, se muestra la publicación correspondiente al presente trabajo de investigación. Artículo (1): Hernández-Ortega K, Arias C. ERK activation and expression of neuronal cell cycle markers in the

hippocampus after entorhinal cortex lesion. J Neurosci Res 2012, (en prensa). Los resultados de los experimentos referentes al efecto de la administración del inhibidor de MEK 1/2 sobre la expresión de la ciclina D en el GD no están incluidos en el artículo, por lo que se presentan en la sección de resultados adicionales.

Por último, se presenta una sección de discusión global e integración de los resultados reportados en este trabajo.

II. RESUMEN

Evidencias recientes sugieren que de manera frecuente, concomitante a la muerte neuronal ocurre una expresión aberrante de proteínas reguladoras del ciclo celular en neuronas post-mitóticas. En la enfermedad de Alzheimer (EA), además de la degeneración sináptica, uno de los eventos iniciales es la activación aberrante del ciclo celular en neuronas postmitóticas, la cual, puede contribuir al proceso neurodegenerativo. Inicialmente, los cambios patológicos se presentan en la alocorteza transentorrinal y se acentúan en la vía perforante que conecta la corteza entorrinal (CE) con el giro dentado (GD) y el área de CA3 del hipocampo. Nosotros describimos previamente que la expresión de dos proteínas implicadas en la EA: la proteína precursora del amiloide (PPA) y la tau hiperfosforilada puede estar asociada a la activación del ciclo celular en neuronas de la CE posterior a un daño excitótoxico hipocampal. En este sentido surgió la interrogante sobre cuales vías de señalización podrían estar implicadas en esta reactivación del ciclo celular. En las neuronas diferenciadas, los mecanismos moleculares inicialmente encargados del control de la proliferación celular como la vía Ras-ERK1/2, participan en la modulación de la plasticidad sináptica. Una posibilidad es que la pérdida de aferentes sinápticas genere señales celulares capaces de activar el ciclo celular en neuronas post-mitóticas.

Considerando la hipótesis anterior, en este trabajo utilizamos un modelo de desaferentación inducido por una lesión en la CE y evaluamos la participación de las cinasas ERK 1/2 en la reactivación del ciclo celular de neuronas maduras conectadas con el sitio de la lesión. Nuestros resultados mostraron que después del daño en la CE, un gran número de neuronas del GD y CA3 expresa la forma activa de estas cinasas, fosfo-ERK1/2, asociada con la expresión de ciclina D1 (marcador de la fase G1) y ciclina B (marcador de transición G2-M). La administración

intra-cerebroventricular (i.c.v.) de U0126, un inhibidor de MEK1/2 disminuye significativamente la activación de ERK1/2, así como la expresión de los marcadores de ciclo celular. Estos resultados sugieren que ERK 1/2 puede participar en la activación del ciclo celular de neuronas que perdieron aferentes sinápticos por una lesión excitotóxica. En general, estos resultados sugieren que las señales que regulan la plasticidad sináptica también pueden participar en la reactivación del ciclo celular en neuronas maduras durante la neurodegeneración.

III. ABSTRACT

Current findings suggest that neuronal cell death is frequently concomitant with the aberrant expression of cell cycle-regulatory proteins in post-mitotic neurons. In Alzheimer's disease (AD), the early synaptic loss is accompanied of expression of cell cycle regulators in post-mitotic hippocampal neurons, which can contribute to the neurodegenerative cascade. Previously, we have reported that the appearance of cell cycle markers in post-mitotic neurons of the entorhinal cortex (EC) after excitotoxic hippocampal damage is associated with the expression of phospho-tau and amyloid precursor protein (APP). However, the signaling pathways involved in the cell cycle reentry remains unresolved. Differentiated neurons use the molecular mechanisms initially acquired to direct cell proliferation such as the Ras-ERK 1/2 pathway to regulate synaptic plasticity. In this work we explored if ERK 1/2 (extracellular signal-regulated kinase) related signaling may contribute to the cell cycle reentry in hippocampal neurons after a unilateral EC lesion. We showed that within the first 24 h after hippocampal deafferentation, numerous neurons expressed phospho-ERK 1/2, conconmitant to the gradual increases in cyclin D1 and cyclin B immunoreactivity in the dentate gyrus, and CA3 hippocampal regions. Several of these immuno-positive cells to phospho-ERK 1/2 and cyclin B in hippocampus are post-mitotic neurons as they are positive to NeuN. The intracisternal administration of U0126 (a MEK inhibitor), previous to the excitotoxic lesion, decreased the activation of ERK 1/2, and the expression of cyclin D1 and cyclin B in the hippocampus. The present findings support the notion that ERK1/2 plays a role in cell cycle reactivation in mature neurons after the loss of synaptic input.

IV. Introducción

El incremento substancial en el número de casos de enfermedades neurodegenerativas alrededor del mundo se asocia con el aumento significativo en la expectativa de vida. La EA es el tipo de demencia más frecuente en las personas mayores de 60 años y se caracteriza por una pérdida progresiva de las funciones cognitivas que conduce a una completa dependencia del paciente y culmina con la muerte. En la mayoría de los casos, la EA es de tipo esporádico y tiene como principales factores de riesgo el envejecimiento y el alelo ε4 del gen polimórfico de la lipoproteína ApoE. Sólo en un porcentaje menor al 5% de los casos existe alguna de las varias mutaciones en los genes de la proteína precursora de la βA (PPA, cromosoma 21) o de las presenilinas 1 y 2 (PS-1 y PS-2, cromosomas 14 y 1, respectivamente) (para revisión ver Arias, 1999; Huang y Mucke, 2012).

Histopatologicamente la EA, se caracteriza por una pérdida sináptica selectiva y por la presencia de dos tipos de agregados proteicos, las marañas neurofibrilares y las placas seniles o amiloideas. Las marañas neurofibrilares son inclusiones intraneuronales compuestas principalmente de la proteína asociada a microtúbulos axonales, Tau en estado hiperfosforilado, ensamblada en una conformación de filamentos helicoidales apareados (PHF) (Grundke-Iqbal y col., 1986). Las placas seniles se componen de un depósito extracelular de βA, denominado núcleo de la placa, rodeado por neuritas distróficas y microglía reactiva (Lichtenthaler y col., 1999).

Se han propuesto varias hipótesis para entender la fisiopatología de la EA incluyendo la participación de la proteína βA, la hiperfosforilación de tau, la inflamación y el estrés oxidante. Sin embargo, ninguna de estas considera la vulnerabilidad selectiva a la neurodegeneración en áreas

cerebrales comprendidas dentro de la formación hipocampal (Hyman y col., 1984; Braak y Braak, 1997). Entre las que destacan, la pérdida significativa de contactos sinápticos en el hipocampo (Dekosky y col., 1996; Gómez–Isla y col., 1996), así como la expresión neuronal de proteínas reguladoras del ciclo celular previo a la aparición de las marañas neurofibrilares, las placas amiloideas y la muerte neuronal en el hipocampo y la CE de cerebros con EA (Busser y col., 1998; Nagy y col., 1997; Nagy, 2000). Una característica de estas regiones cerebrales es que mantienen un alto grado de plasticidad a lo largo de la vida, la cual permite relacionar la susceptibilidad temprana a la neurodegeneración con alteraciones de la neuroplasticidad en la EA (Arendt, 2000; 2003; 2012).

Una hipótesis centrada en la relación entre vulnerabilidad selectiva y neuroplasticidad plantea que las señales celulares involucradas en la modulación de la conectividad y la plasticidad sináptica también permiten la permanencia de las neuronas en estado diferenciado. Así mismo, impiden la migración neuronal y la reactivación de mecanismos que lleven a la proliferación. Por lo tanto, el papel dual de las señales intracelulares inducidas por factores de crecimiento involucradas en morforegulación, representan un riesgo mayor para aquellas neuronas que retienen amplia capacidad plástica. Bajo condiciones patológicas, estas señales morforeguladoras podrían conducir a la pérdida del estado diferenciado y a la reactivación del ciclo celular en neuronas maduras. Este proceso involucra eventos moleculares que en células capaces de dividirse podrían llevarlas a la transformación celular, mientras que en células post-mitóticas como las neuronas, puede conducirlas a la muerte celular programada o a la neurodegeneración de tipo Alzheimer (Arendt, 2000; Nagy, 2000; Arendt, 2012).

En este sentido a continuación se resumen las evidencias relacionadas con la reactivación del ciclo celular en neuronas post-mitóticas y su participación en algunas patologías neurodegenerativas, en particular en la EA.

IV.1 ARTÍCULO DE REVISIÓN

Cell cycle reactivation in mature neurons: a link between brain plasticity, neuronal injury and neurodegenerative diseases?

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Abstract: Although the cell cycle machinery is essentially linked to cellular proliferation, recent findings suggest that neuronal cell death is frequently concurrent with the aberrant expression of cell cycle-regulated proteins in post-mitotic neurons. The present work reviews the evidence of cell cycle re-entry and the expression of cell cycle-associated proteins as a complex response of neurons to contend with insults in the adult brain but also as a mechanism underlying brain plasticity. The basic aspects of cell cycle mechanisms, as well as the evidence for cell cycle protein expression in the injured brain, are reviewed. The discussion includes recent experimental work attempting to establish a correlation between altered brain plasticity and neuronal death and an analysis of fresh evidence on how neural cell cycle dysregulation is related to neurodegenerative diseases especially the Alzheimer's disease. Understanding the mechanisms that control re-expression of proteins required for cell cycle progression which is involved in brain remodeling, may shed new light into the mechanisms involved in neuronal demise under diverse pathological circumstances. This would provide valuable clues about the possible therapeutic targets, leading to potential treatment of presently challenging neurodegenerative diseases.

1 Cell cycle machinery and neuronal function

Fine regulation of cell cycle is critical for a wide range of cellular processes such as proliferation, differentiation, apoptosis and cancer. It is now accepted that in mature neurons, many signaling pathways, transcriptional programs and an elaborate orchestration of cell cycle exit are required for a variety of brain functions. Quiescent neurons reside in the G0 phase until mitogenic signals activate the cell cycle machinery. In general, the cell cycle involves 4 phases: G1 (first gap), S (DNA synthesis), G2 (second gap) and M (mitosis) phases. Additionally, cells that exit the cell cycle may remain in a quiescent G1 state for a long period. In fact, during the G1 gap, cells may face many metabolic, environmental or stress signals, which may influence their future fate: proliferation, differentiation or death.

Progression along the cell cycle phases is regulated by the sequential expression, activation and inhibition of cell cycle proteins including cyclins, cyclin-dependent kinases (Cdks), and cyclin-dependent kinase inhibitors (Cdkis)[1,2]. The binding of cyclins to CDKs allows them to become catalytically competent. The transition between one cell cycle phase and another is regulated by check-points, better described as molecular switches which ensure that essential events of each phase are completed before entering the next phase.

During the G1 stage, cells incorporate environmental signals that lead them to progress through the first restriction point, after which cells are committed to divide. G1 progression is promoted by D-type cyclins, which bind and activate Cdk4 and Cdk6. Phosphorylation of the retinoblastoma protein (Rb) by Cdk4, Cdk6 and cyclin E/Cdk2 along G1 progression inhibits its affinity to the E2F transcription factors. Derepressed E2F proteins can induce the expression of downstream target genes necessary for subsequent cell cycle progression, including those encoding cyclins D, E and A, DNA polymerase, Cdc6, mini-chromosome maintenance (MCM) proteins and origin recognition complex (ORC) proteins[3].

The activity of the cyclin E/Cdk2 complex allows the G1/S transition, and DNA duplication begins. In the early S phase, cyclin E is degraded concomitantly with the binding of Cdk2 to cyclin A. At the end of DNA duplication, cyclin A forms a complex with Cdk1 (or Cdc2) and leads the cell cycle to the G2 phase, during which cells verify the fidelity of DNA replication prior to mitosis. Once DNA duplication fidelity is confirmed, cells undergo mitosis, which implies division of the genomic material into 2 daughter cells. During the G2/M transition, cyclin A is degraded, and now Cdk1 is joined to cyclin B. The activity of cyclin B/Cdk1 is essential for progression along mitosis. Towards the end of the M phase, cyclin B is degraded and thus the cyclin B/Cdk1 complex is inactivated[1].

In fact, although the sequential activation of different cyclin-dependent kinases (Cdk4, Cdk6, Cdk2 and Cdk1) in mammalian cell cycle progression is well established, a recent report indicates that Cdk1 is the only essential factor for cell cycle progression.

Moreover, in embryos lacking Ckd2, Cdk3, Cdk4 and Cdk6, Cdk1 can still bind to all cyclins and execute all the events necessary for cell division during organogenesis[4].

The activity of the cyclin/Cdk complexes is inhibited by 2 families of proteins, the Kip/Cip inhibitors and the INK4 inhibitors. The members of the Kip/Cip family (p21, p27 y p57) mainly regulate the activity of the G1 cyclin/Cdk complexes and, to a lesser extent, the activity of the cyclin B/Cdk1 complex. In contrast, the INK4 family (p16, p15, p19 y p18) specifically inhibits Cdk4 and Cdk6. The expression and content of cell cycle proteins are regulated by transcription, translation and degradation, specifically by ubiquitination in proteasomes[1,5,6]. Progression along the cell cycle phases can be prevented by pharmacological inhibitors such as flavopiridol which inhibits all Cdks and arrests cells at the G1/S or G2/M transition, and roscovitine which displays selectivity toward Cdks1, 2, 5, 7 and 9, competing for the ATP-binding site[7-9].

2 Significance of cell cycle marker expression in mature neurons

Numerous studies have provided evidence that cell cycle-associated mechanisms contribute to neuronal death in various pathological conditions including target deprivation[10,11], DNA damage[12], excitotoxicity[13] and exposure to amyloid β protein (Aβ)[14-16]. Furthermore, aberrant cell cycle re-entry has been implicated in neuronal death during stroke and trauma[17,18], epilepsy[19], amyotrophic lateral sclerosis (ALS)[20], Parkinson's disease (PD)[21] and Alzheimer´s disease (AD)[22-30].

For a long time it was thought that neurons cease going through the cell cycle process lastingly once they have differentiated. However, certain evidence has suggested the neuronal ability of re-activatinng the cell cycle machinery along cellular life[10-30]p, although some studies have shown that in post-mitotic neurons, experimentally driving the cell cycle results in cell death rather than cell proliferation.[10, 12-15].This late attempt to bring about cell cycle re-entry has frequently been found to be associated with neuronal death. Rb, a tumor suppressor, typically arrests the cell cycle at the G1 phase, is expressed during pathological neuronal death. Studies in transgenic $Rb^{-/-}$ mice have

shown that DNA synthesis is accompanied by neuronal apoptosis in the central nervous system (CNS) and the peripheral nervous system (PNS)[31-33]. In fact, Rb is proposed as an essential gatekeeper of cell cycle re-activation and possibly neuronal apoptosis in the CNS[34]. Cell cycle-related proteins are expressed in neurons exposed to repairable DNA damage, implying a relationship between the cell cycle machinery and DNA repair processes in post-mitotic neurons[35]. Terminally differentiated neurons are highly susceptible to oxidative DNA damage, and repair of double-strand breaks in neurons exposed to H_2O_2 is accompanied by phosphorylation of Rb protein by cyclin C-associated kinase, which induces the transition from G0 to G1. In other words, cell cycle re-entry represents the need of resting cells to activate DNA repair mechanisms[36].

Although in most cases, cell cycle reactivation in mature neurons leads to apoptosis, recently differentiated neurons have been revealed to be capable of undergoing sustained cell division while maintaining typical features of mature neurons including synaptic contacts. This particular process seems to be involved in the origin of highly aggressive metastatic retinoblastoma in mice and is achieved in horizontal interneurons from a $Rb^{-/-}$; $p107^{+/-}$; $p130^{-/-}$ background[37].

Another attractive hypothesis concerning an alternative role of cell cycle machinery links cell cycle protein expression with brain plasticity. In this regard, formation and continuous reshaping of synapses are necessary events in neurons that have withdrawn from the cell cycle. Expression of cell cycle-related proteins in terminally differentiated neurons has been associated with physiological functions beyond cell cycle particularly in the control of microtubule-based mechanisms of neuroplasticity[38]. Schmetsdorf *et al.*[39] have demonstrated constitutive neuronal expression of Cdks 1, 2, and 4, as well as their activators (cyclins D, A, B, and E) and inhibitors within the neocortex of adult mice. More recently, the same research group has shown an association of cyclins and Cdkss with the microtubule network. Cyclins D, E, A and B as well as Cdks1, 2 and 4 are associated with the axonal microtubule-associated protein tau, and these cyclin/Cdk complexes exhibit kinase activity towards tau[38]. These findings suggest that the presence of cyclin complexes with CDKs in mature neurons may reflect their role in network stabilization

and regulation of cytoskeleton dynamics during morphogenetic adaptation, and that tau, in addition, may act as an important phosphorylation target. Moreover, it has been proposed that to maintain synaptic connections, neurons must permanently withdraw from the cell cycle to activate molecular mechanisms primarily developed to control proliferation, in order to influence synaptic plasticity[40]. Thus, many external signals that influence concentrations of cyclins and their inhibitors are mitogenic signals involved in brain plasticity. The mitogenic-activated protein kinase (MAPK) signaling pathway plays a key role in promoting Cdk activation. The classical cascade involves the sequential activation of Ras, Raf-1, MEK and the extracellular-signal-regulated kinase (ERK)[41]. ERK phosphorylates and stabilizes c-Myc, a transcription factor that induces the expression of cyclin D1 and suppresses expressions of Cdk inhibitors[42]. Overexpression of Ras in mature cortical pyramidal cells results in a significant enlargement of pyramidal neuron size and an increase in the complexity and spine density of dendritic trees leading to cortical expansion[43]. These results suggest that the mitogenic Ras pathway not only controls proliferation in cycling cells but also regulates neuronal plasticity in differentiated neurons.

Cell cycle progression depends on a precise control of the oscillatory expression of cyclins and cyclin inhibitors. This temporal precision of protein expression also depends on the proteasomal machinery and its associated accessory and regulatory proteins involved in protein degradation. Two ubiquitin ligase complexes are responsible for controlling protein degradation during the cell cycle: the SCF family of ubiquitin ligases and the anaphase-promoting complex/cyclosome (APC/C)[6,44]. Interestingly, APC/CCdh1 and APC/CCdc20 are recently implicated in axonal growth and dendrite morphogenesis, respectively. Since plasticity events in CNS depend on *de novo* protein synthesis and protein degradation, APC/C complex is also shown to be involved in learning and memory function[45]. Other pioneering studies have shown that knock-in *Cdh1* in cultured cerebellar granule neurons causes a specific increase of axonal growth without affecting the size of dendrites[46].

Thus, novel and unusual biological functions of proteins in cell cycle regulation and

progression in post-mitotic neurons have been described. These essential cell cycle regulators participate at different developmental stages, including neuronal migration, axonal elongation, dendrite morphogenesis, synaptic maturation and adaptation of neuronal networks.

Long-term changes of synaptic strength in response to neuronal activity require active transcription and protein synthesis, and may be controlled by Cdh1, Cdc20, ORC, and the Polo-like kinases (PLKs) that participate in cell cycle progression [3]. Moreover, Cdk5, a kinase highly active in post-mitotic neurons, seems to be involved in cell cycle suppression when it is located in nucleus[47], and control a variety of complex neuronal functions such as learning and memory-associated synaptic plasticity[48].

Taken together, the present evidence indicates that the main molecular components that control the orderly progression along the cell cycle may participate not only in the dynamic organization of neuronal networks and in DNA repair mechanisms of damaged neurons, but also in the erroneous conversion of plasticity and repair signals into pathological cell cycle re-activation processes and neuronal death.

3 Mechanism of cell death during cell cycle activation

As mature neurons do not divide, activation of a neuronal cell cycle seems to be abortive in many cases, with a final outcome being the initiation of apoptosis or neuronal dysfunction. The association between an apoptotic stimulus and the reactivation of cell cycle machinery in mature neurons is not well understood, although the activation of Cdk4, a Cdk involved in the G1 stage, seems to be essential for neuronal death[49]. Recently, 3 different models of apoptotic neuronal death showed the involvement of the transcriptional co-regulator Sertad 1 in Cdk4 activation to initiate the cascade that causes Rb hyperphosphorylation and of proapoptotic genes[50]. On the other hand, cytoplasmic Cdk5 appears to retard neuronal death by a mechanism probably involving the anti-apoptotic protein Bcl2. Cdk5 may act in 2 ways depending on its location within neurons:

in the nucleus it suppresses the cell cycle, and in the cytoplasm it transiently delays cell death[51]. As has been mentioned, high expression levels of cell cycle Cdks, their cyclin partners and Cdkis have been detected in several neurodegenerative conditions. Furthermore, expression of these cell cycle proteins is not a direct consequence of death because the specific inhibition of Cdks can prevent death of cultured neurons induced by DNA-damaging agents exposure, trophic factor deprivation[52] and ischemic damage *in vivo*[53].

At the G1/S transition, for instance, E2F1 is able to initiate the apoptotic mechanism of cell death, directly activating the expression of the human tumor-suppressor protein p14ARF, which binds to the Mouse double minute-2 (MDM2)–p53 complex and prevents p53 degradation, leading to cell cycle arrest or apoptosis[54]. In another case, unrestrained E2F1 activity forces cell entry to the S phase and promotes apoptosis upregulating death effectors such as the apoptotic protease-activating factor (Apaf) [55] and several caspases[56]. Contrariwise, a dominant negative form of E2F1 can block the activity deprivation-induced *cdc2* transcription and thus prevent apoptosis in cerebellar granule cells[57].

Recently, the mitotic kinase Cdk1, has been implicated in the G2/M transition and mitosis progression in neuronal apoptosis in postmitotic neurons through the phosphorylation of the forkhead transcription factor (FOXO1), a protein involved in cell death and DNA repair[58]. Moreover, the proapoptotic protein BAD has been identified as a direct target of Cdk1-mediated phosphorylation and activation[59].

The aforesaid evidence supports the proposition that neuronal cell cycle re-entry is also a component of a well-regulated response to stress signals, which may ultimately lead to neuronal death.

4 Activation of cell cycle regulators in apoptosis mediated by the lack of neuronal activity and/or deprivation of neurotrophic support

During normal brain development, the absence of trophic signals derived from either lack of neuronal activity or deprivation of neurotrophic support is a major cause of neuronal apoptosis. In cultured neurons, apoptosis induced by nerve growth factor (NGF) deprivation is accompanied by changes in cyclin expression and Cdk activity[60,61]. Besides, the use of cell cycle inhibitors promotes cell survival in sympathetic neurons deprived of serum and in PC12 cells lacking NGF but only before the G1/S transition point[62].

The *staggerer* mouse is a useful model for the study of neuronal death induced by lack of trophic signals. The *staggerer* mutation consists of a deletion in *ROR*α gene, which encodes the retinoic acid–related orphan receptor alpha. As a result, Purkinje cells do not develop completely, nor do they form synapses with cerebellar granule neuron axons[63]. In consequence, the inferior olive population of cerebellar granule cells and neurons die, almost certainly resulting from the loss of target-dependent trophic signals from the Purkinje cells [64]. Apoptosis of the inferior olive granule neurons is preceded by increased expression of cyclin D and the proliferating cell nuclear antigen (PCNA), cell cycle markers of the G1 and S phases, respectively[10].

In relation to activity deprivation, it is known that cerebellar granule neurons undergo apoptosis when they lack membrane-depolarizing concentrations of KCl. In 2003, Konishi *et al.*[57] defined a novel apoptotic mechanism whereby E2F1 selectively couples an activity deprivation-induced signal to Cdk1 transcription in the absence of stimulating DNA synthesis, thus culminating in Cdk1-mediated apoptosis of post-mitotic granule neurons in the developing cerebellum. These findings suggest that the lack of neuronal activity or deprivation of neurotrophic support leads to the activation of cell cycle regulators and thus culminates in apoptosis of post-mitotic neurons (Fig. 1).

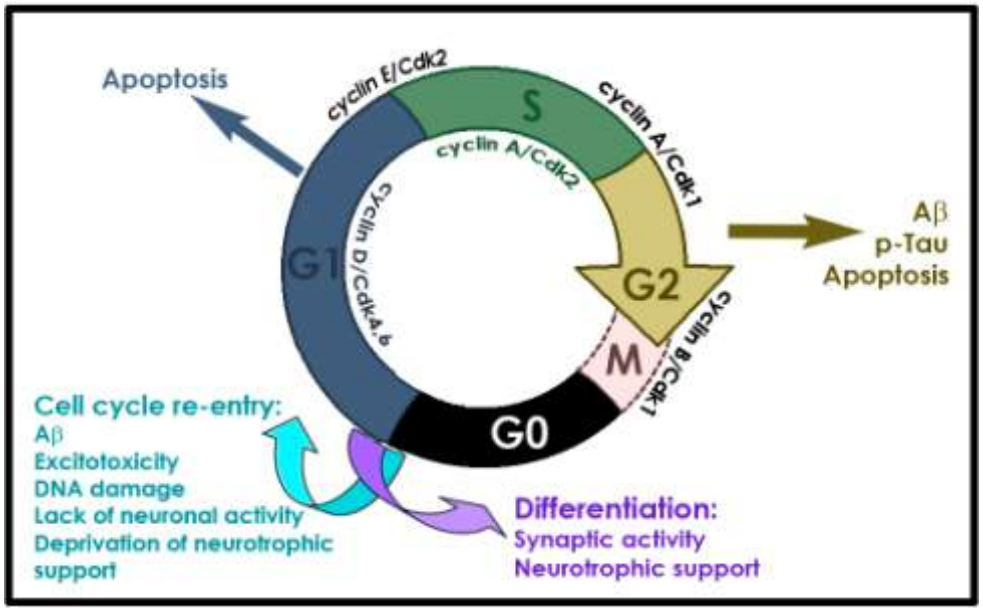

Fig. 1 Schematic representation of neuronal cell cycle progression. During G1 phase, many metabolic, environmental or stress signals represent mitotic stimuli for mature neurons. At G1 phase, neurons can re-differentiate, progressing into the cell cycle or undergoing apoptosis. The G1/S arrest may be a state to develop age-related neurodegenerative pathology. If the neuronal cell cycle progress to G2 phase, many components of the Alzheimer's-related pathology (Aβ and phosphorylated tau) may be expressed. Oxidative or mitotic insult may alter the outcome of cell cycle progression and induce neuronal apoptosis.

5 Cell cycle reactivation in AD

AD is the most frequent dementia in the old and is characterized by a progressive cognitive deficit. The main neuropathological markers include synaptic loss and neuronal death, with extracellular accumulation of β-amyloid protein (Aβ) and intraneuronal presence of neurofibrillary tangles composed by a biochemically modified tau protein. Several causes have been implicated in the pathological mechanism of AD, including Aβ cascade, tau phosphorylation, oxidative stress, and most recently, cell cycle reactivation. However, the initial events that precipitate in neurodegeneration during AD are still under constant debate.

The first reports implicating the reactivation of the cell cycle in AD were obtained from the analysis of AD brain tissues, showing expression and activation of cyclin B and

Cdc2 in differentiated neurons[24,25]. It has been proposed that Cdc2 activity contributes to the formation of amyloid plaques and neurofibrillary tangles. The cyclin B/Cdc2 complex derived from the AD brain can phosphorylate tau[24], which is essential for neurofibrillary tangle formation. Moreover, it is known that amyloid precursor protein (APP) is phosphorylated *in vitro* by Cdc2, favoring the production of amyloid fragments[65]. These data strongly support a direct link between cell cycle reactivation and the 2 major histopathological markers of AD.

In 1997, Nagy *et al.*[23,66] described the expression of Ki-67 (a protein present in dividing cells along the G1, S and G2/M phases), cyclin E and cyclin B in vulnerable regions of the AD brain, particularly in the hippocampus. Although cyclin E expression was also detected in some neurons from matched-age control subjects (suggesting that ectopic cell cycle markers occur even in neurons from healthy aged subjects), its increase was significantly accentuated in patients with AD. Moreover, cyclin B-reactive nuclei in neurons from AD were also positive for hyperphosphorylated tau in AD-related epitopes[23]. Later, another study reported the presence of the cyclin D/Cdk4 complex, PCNA and cyclin B expression in regions with abundant neuronal loss such as the hippocampus and the entorhinal cortex[25]. Thus, the lack of control of cell cycle regulators is postulated as a source of regionally specific neuronal death in AD.

Considering that Aβ seems to play an important role in AD, whether Aβ is involved in the abnormal cell cycle re-entry observed in AD, and if the Aβ-induced oxidative stress and the cell cycle activation are related to each other are interesting questions to be investigated. Recent evidence suggests that DNA oxidative damage induced by Aβ can lead to cell cycle re-entry. Cultured neurons exposed to $A\beta_{1-42}$ or its active fragment $A\beta_{25-35}$ express G_1 markers: phospho-Rb and the complex Cdk4/6, which is accompanied by neuronal death[15]. Another study reported that blocking the G_1/S transition by a cyclin D antisense or dominant-negative mutant of Cdk2 prevented Aβ-induced apoptosis[14].

However, controversies exist on the significance of Aβ-induced cell cycle reactivation and whether cell cycle reactivation is a cause or a consequence of neuronal

damage. A recent study in the triple transgenic mice model of AD, reported no cell cycle reactivation induced by Aβ or tau pathology whereas in a model of neuronal death, cell cycle reactivation was linked to acute neuronal loss[67]. On the other hand, intracerebroventricular injection of $A\beta_{1-40}$ induced phospho-Rb and PCNA expression mediated by Cdk5 overactivation[68].

Using the fluorescent *in situ* hybridization (FISH) technique, Yang *et al.*[69] described that in brains of AD patients, hippocampal and basal forebrain neurons have 4 separate genetic loci replicated on 3 different chromosomes. They concluded that in AD neurons, mitosis is not initiated and cells remain aneuploid before they die, suggesting that this imbalance contributes to the neuronal loss in AD. This is the first report of DNA replication in a neurodegenerative condition. Recently, increased aneuploidy of chromosomes 17 and 21 has been also observed in buccal cells of AD patients, as well as in aged subjects over 64 years[70].

Further evidence supports that neurons in AD re-enter the cell cycle and progress through the S phase activating a specific component of the DNA replication machinery (MCM2 protein), producing cell cycle stasis with the consequent expression of AD markers and the subsequent neuronal degeneration[71]. In AD, accumulating evidence indicates that although vulnerable neurons proceed as far as DNA replication and entry into the S phase can be demonstrated in dying neurons, progression through the M phase has never been reported. This incomplete process results usually in stasis, dysfunction and death, and has been called "abortosis"[72]. Although the presence of binucleated neurons has been reported[73], no cytokinesis has ever been described, consistent with the idea that susceptible neurons may be arrested at the G2/M transition before they die. At this point, activated Cdk1 can phosphorylate tau[24,74]. Therefore, abnormally increased level of phosphorylated tau may be explained in the perspective of an effort to modulate G2 neuronal architecture and prepare it for mitosis[75]. In addition, Cdk1 can phosphorylate and activate the pro-apoptotic BAD protein, linking the cell cycle to the programmed cell death mechanisms[59].

Interestingly, the brain regions with a high vulnerability to neuronal loss during AD and with marked re-expression of several cell cycle proteins are those with a high degree of plasticity, supporting a link between altered brain plasticity and neurodegeneration[76].

Although significant progress has been made to understand the ethiopathological mechanisms of AD, there is still a lack of a comprehensive theory, and thus research has been conducted to explore alternative hypotheses to gain insight into the mechanisms underlying neuronal differentiation and plasticity. As brain plasticity depends on continuous synaptic reshaping, plasticity molecules involved in this process are constantly secreted and may contribute to synaptic modifications and neuronal cell cycle reactivation[77]. Loss of synaptic input reduces the activation of post-synaptic neurons which could be interpreted as a lack of mitogenic support and drives neurons to exit from the G0 phase. This pathological cell cycle reactivation would be particularly frequent in circuits submitted to high rates of synaptic adjustments such as the perforant pathway that connects the entorhinal cortex with the dentate gyrus of the hippocampus, and which is particularly vulnerable in AD.

However, the role of mitogenic stimulation may be interpreted in 2 ways: a transient lack of stimulation could activate cell cycle and induce its progression to the G1 phase, while on the other hand, permanent mitogenic stimulation could induce cells to arrest the cell cycle in advanced phases such as G2 and S, and induce a hypermitogenic state in which AD markers (Aβ and phosphorylated tau) may appear and disseminate (Fig1).

Consistent with this hypothesis, our research demonstrated that after loss of synaptic contact between 2 interconnected areas, the entorhinal cortex (EC) and the dentate gyrus (DG), by excitotoxic damage of the hippocampus, several cell cycle proteins of the G1, S and even G2 phases were found to be up-regulated in the EC. In addition, there we described the progressive expression of 2 AD-related proteins, phospho-Tau identified by PHF-1 and APP, which reached higher levels immediately after the increase in G1/S-phase markers[11]. Similarly, we have found that after deafferentation of DG neurons by excitotoxic damage into the EC, numerous neurons expressed p-ERK 1/2,

formerly to a gradual increase of cyclin D1 and cyclin B immunoreactivity in the DG [unpublished data]. These suggest that the signals participating in the establishment of synaptic plasticity may also have a role in cell cycle reactivation in mature neurons (Fig. 2).

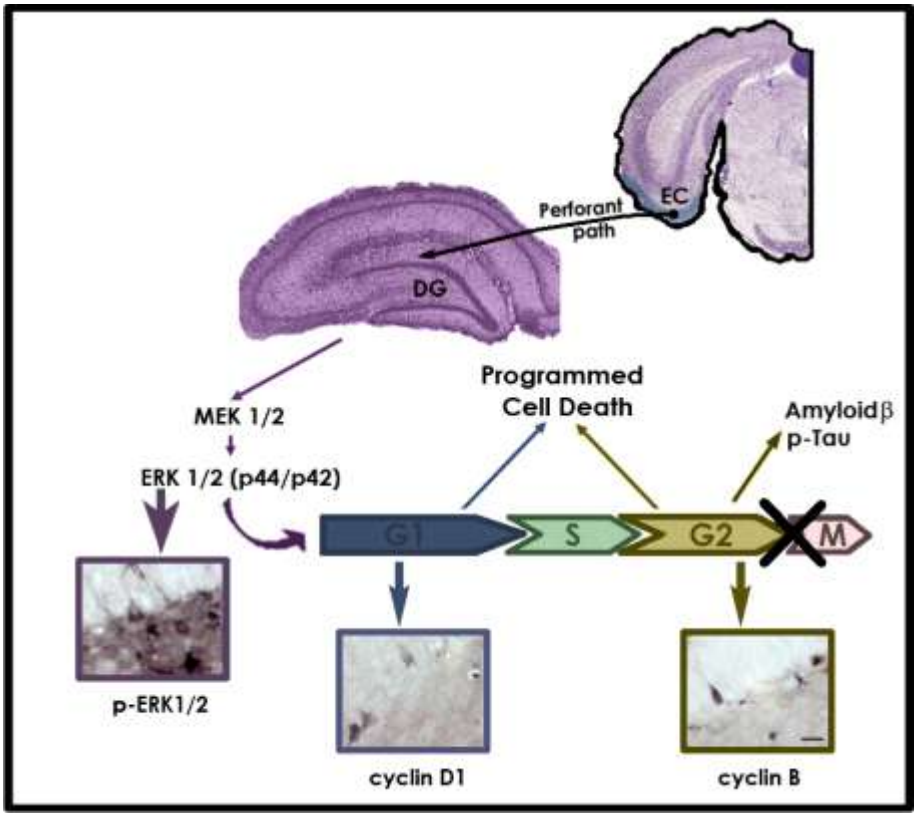

Fig. 2 Expression of several markers of mitogenic pathway and cell cycle machinery after hippocampal deafferentation. Reduced activation of dentate gyrus neurons after damage to the entorhinal cortex could be interpreted as a lack of mitogenic support and drive neurons to exit from the G0 phase. Previous to cell cycle reactivation a rise in p-ERK 1/2 expression in large number of granule neurons is observed. The progressive expression of cell cycle markers from G1 (cyclin D) and G2/M (cyclin B) phases, reached higher levels immediately after the increase in p-ERK 1/2. [Unpublised data]

6 Cell cycle reactivation in other neurodegenerative conditions

Aberrant neuronal expression of numerous cell cycle markers has been associated

with many other pathological conditions and is inextricably linked to neuronal death. This is the reason why cell cycle re-entry has been proposed to represent a general form by which post-mitotic neurons die during pathological aging. In fact, as neurons age, their ability to maintain the regulation of the G0 quiescent phase is compromised, probably due to inefficient energy metabolism, accumulative oxidative DNA damage and an imbalance in neurotrophic support. Mature neurons re-entering the cell cycle are neither able to advance to a new G0 quiescent state nor to revert to their earlier G0 state. Particularly in several stroke models, there is evidence that Cdks are activated and required to initiate the apoptotic cascade[78].

Available data suggest that cell cycle-associated mechanisms are linked to the process of neurodegeneration in PD. Expression of cell cycle proteins such as phosphorylated Rb protein and E2F1, as well as DNA synthesis, have been observed in postmortem samples of PD patients[21,79]. A mutation in the ubiquitin ligase Parkin is an identifiable cause of familial PD. Interestingly, Parkin deficiency increases the levels of cyclin E and promotes apoptosis during kainate-mediated excitotoxicity in post-mitotic neurons[80]. Other genes that underlie familial forms of PD have also been linked to cancer or implicated in cell cycle regulation, such as ubiquitin C-terminal hydrolase (UCHL1) which presumably contributes to the degradation of Kip1, also known as cyclin-dependent kinase inhibitor 1 B (CDKN1B)[81]. In a rat model of PD induced by 6-hydroxydopamine (6-OHDA), overexpression of Cdc2 was found in dopaminergic neurons of the *substantia nigra*[82] and in PC12 cells, 6-OHDA-induced oxidative stress is accompanied by expression of several markers of cell cycle reactivation[83]. In addition, in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, the use of flavopiridol, a general Cdk inhibitor or the expression of a dominant-negative of Cdk5 can provide neuroprotection to some extent[84].

Also worth mention is the finding of dysregulation of the cyclin system in postmortem human brain and spinal cord in ALS[85]. More recently, using microarray analysis in single motor neurons in the SOD1 transgenic murine model of familial ALS, researchers found a significant increase in the expression of cyclins D2, E2, and I[86].

Finally, overexpression of cyclin D1 and the transcription factor E2F1 in striatal neurons have also been described in Huntington's disease[87].

In order to determine the exact role of cell cycle re-entry in neuronal dysfunction and death observed in many neurodegenerative diseases, Lee *et al.*[88] developed a new transgenic mouse model in which forebrain neurons were induced to re-enter the cell cycle by over-expression of the proto-oncogene *MYC* under the control of the *CAMKII* promoter. Research using this model has shown that the induction of ectopic cell cycle re-entry (determined by the expression of PCNA, Ki-67 and cyclin D1, and BrdU incorporation) results in neuronal cell death, gliosis and cognitive deficit. These findings provide compelling evidence that *in vivo*, dysregulation of cell cycle re-entry results in neurodegeneration.

7 Conclusion

Expression of diverse cyclins, cyclin activators and inhibitors could constitute a final convergence point for all neurodegenerative diseases. This is based on the fact that several markers of the G1 phase and even the G2 and the S phases are present in adult neurons, in both human brain and in experimental models that exhibit neurodegeneration. Mitotic activation in mature neurons may be the result of extensive DNA damage, which ultimately triggers apoptotic mechanisms of cell death. Given that neurons are terminally differentiated, the possible involvement of cell cycle machinery along neuronal life is striking. Although the re-expression of cell cycle markers has been most frequently associated with extensive neuronal damage which ultimately leads to apoptosis, constitutive but subtle expression of Cdks and their respective cyclin activators and Cdkis has also been demonstrated under normal conditions. These facts support the idea that the transient expression of cell cycle proteins may be related to mechanisms of neuroplasticity and/or the processes which maintain neuronal stability. In brain diseases associated with abnormal cell cycle reactivation, the mechanism of neuronal death may be linked to aberrant plasticity events. These findings provide new insights into

therapeutic intervention to control the neuronal death that occurs in neurodegenerative conditions.

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IV.2. ANATOMÍA DEL CIRCUITO CE-HIPOCAMPO DE LA RATA

La formación hipocampal incluye a seis estructuras: la CE, el GD, el hipocampo, el presubículo, el subículo y el parasubículo (Fig. 3). La CE presenta seis capas. En la capa I se hallan neuronas GABAérgicas, estrelladas y horizontales, las cuales proyectan a las células granulares del GD y al hipocampo. En la capa II, las células estelares proyectan hacia el GD y el área de CA3, contribuyendo a la vía perforante (Germonth y col., 1989). En la capa III, las células piramidales proyectan sus axones bilateralmente hacia CA1, CA3 y al subiculum. Además, se presentan células multipolares, estrelladas y fusiformes cuyos axones contribuyen también a la vía perforante. La capa IV es mayormente acelular. Por su parte, la capa V tiene una división estructural; la lámina Va es una banda densa de neuronas piramidales, mientras que en la lámina Vb, la densidad de las células piramidales es menor. Por último, la capa VI presenta una población celular heterogénea organizada en columnas. Algunas de

estas células aportan proyecciones hacia el GD e hipocampo (Kohler, 1986; Lingenhohl y Finch, 1991).

El hipocampo se divide en las regiones de: CA1, CA2 y CA3, las cuales presentan una organización laminar similar, primordialmente constituida por una capa de neuronas piramidales (Fig. 3). Abajo de esta capa, se encuentra el stratum oriens, área donde las células piramidales extienden sus dendritas. En CA3, sobre la capa de células piramidales se presenta el estrato lucidum, una zona ocupada por las fibras axonales provenientes del GD (fibras Mossy o musgosas). Sobre el estrato lucidum en CA3 y las células piramidales en CA2 y CA1, se localiza el estrato radiatum. Esta región contiene conexiones asociativas de CA3 a CA3 y fibras de las colaterales de Schaffer que van de CA3 a CA1. La parte más superficial del hipocampo es el estrato lacunoso molecular. En la práctica, el término hipocampo comprende anatómicamente al GD además de las regiones CA1-CA3.

El GD está conformado por: la capa molecular, la capa granular y la capa polimórfica también llamada hilus (Fig. 3). La capa molecular concentra a las dendritas de células granulares, células en canasta, polimórficas y terminales axónicas diversas. La capa granular tiene un arreglo denso de células granulares glutamatérgicas; en su parte profunda se localizan algunas células en canasta GABAérgicas. En la capa polimórfica existen varios tipos celulares: musgosas glutamatérgicas, fusiformes y multipolares GABAérgicas (Amaral y Witter, 1995).

La vía perforante comprende a las proyecciones de la CE hacia el GD, CA3 y el subículo. Sin embargo, en el uso cotidiano dicho término se refiere a las aferencias más prominentes de la CE, es decir, las que llegan al GD. Las fibras de este circuito provienen, como ya se mencionó, principalmente de neuronas de la capa II y de algunas de la capa III de la

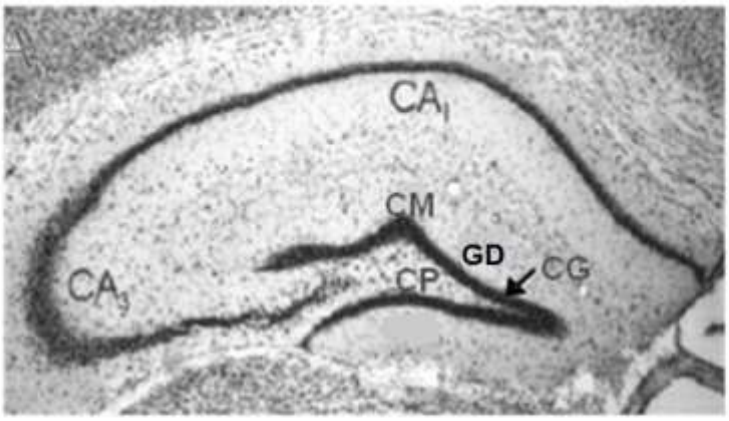
CE, una pequeña fracción de esta proyección se origina en las capas profundas (IV-VI). Esta vía inerva primordialmente a neuronas granulares glutamatérgicas y en menor proporción a neuronas GABAégicas (Amaral y Witter, 1995).

IV.2 VULNERABILIDAD DEL CIRCUITO CE-HIPOCAMPO EN LA EA

La corteza entorrinal (CE) y el hipocampo son componentes esenciales del sistema de memoria del lóbulo temporal medial. La CE conecta a la neocorteza con el hipocampo, conduce información sensorial multimodal primordialmente hacia el GD y la región CA3 (Squire y Morgan, 1991). Debido a que la pérdida de memoria es un síntoma inicial y prominente en la EA, diversos estudios histopatológicos se han enfocado a la búsqueda de alteraciones tempranas en ambas estructuras (Hyman y col., 1984; Braak y Braak, 1997; Van Hoesen y col., 1991).

Considerando que las marañas neurofibrilares no se distribuyen homogéneamente a lo largo del cerebro, se estableció una escala temporal de degeneración neurofibrilar que comprende de la etapa I-VI (Braak y Braak, 1997). De acuerdo con esta escala, en las etapas iniciales I y II, las alteraciones neurofibrilares se concentran en la corteza transentorrinal y la CE. Posteriormente, el proceso patológico se extiende hacia el hipocampo en las etapas III y IV. Finalmente, en las etapas V y VI la mayoría de las áreas de asociación neocortical se ven afectadas, incluso en fases avanzadas de la etapa VI algunas áreas sensoriales primarias se afectan. Por otro lado, las placas seniles se presentan inicialmente en la región perirrinal, desde donde se extienden hacia regiones adyacentes y hacia el hipocampo, cabe mencionar que la vía perforante es ampliamente afectada por estos depósitos. Eventualmente, los depósitos de βA alcanzan todas las áreas corticales (Braak y Braak,

A)



B)

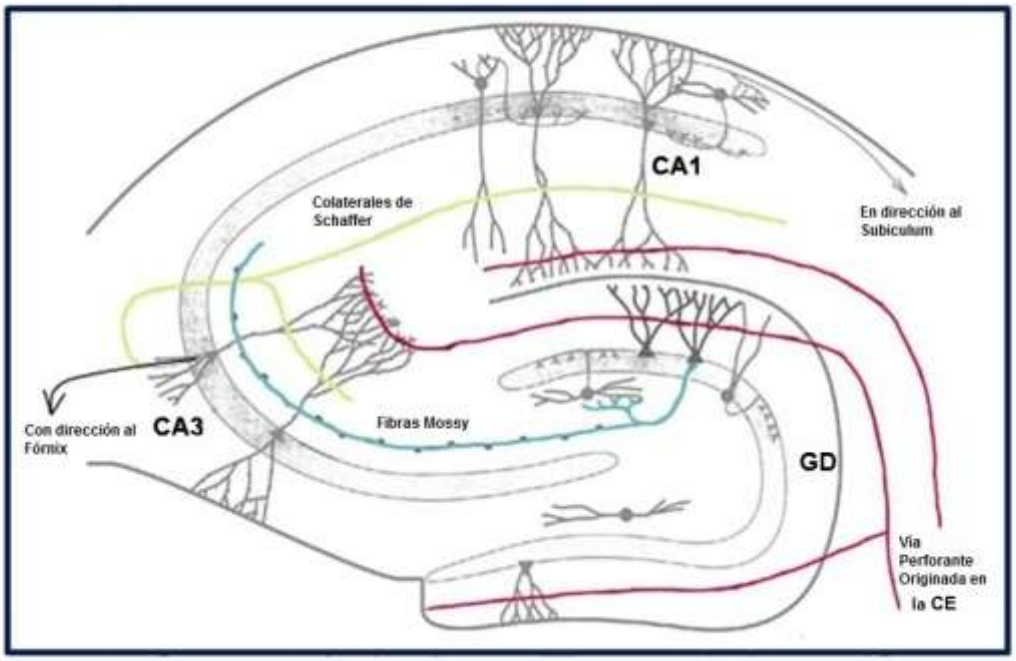


Fig. 3. Circuito CE-Hipocampo. A) Fotomicrografía de una sección coronal del hipocampo teñida con violeta de cresilo donde se señalan las regiones de CA1, CA3 y GD. En el GD se señalan la capa polimórfica (CP), la capa de células granulares (CG) y la capa molecular (CM). B) Esquema de un corte horizontal de la formación hipocampal mostrando sus principales circuitos neuronales: la vía perforante, las fibras Mossy y las colaterales de Schaffer.

1997). Otro dato que señala la vulnerabilidad del circuito CE-GD es la pérdida significativa de contactos sinápticos observada en la capa molecular del GD (Dekosky y col., 1996), la cual correlaciona con la conocida pérdida celular en la lámina II de la CE, la principal proyección excitadora a esta área (Hyman y col., 1984; Gómez–Isla y col., 1996).

Es en el hipocampo y la CE donde se ha detectado la expresión aberrante y prominente de proteínas reguladoras del ciclo celular (Nagy y col., 1997; Busser y col., 1998), lo que sugiere una asociación entre la expresión de estas proteínas y la aparición de marcadores relacionados con la EA (Nagy, 2000). En conjunto, estos hallazgos sustentan el planteamiento acerca de que el circuito más vulnerable en el cerebro de individuos con EA es la proyección que se origina en la CE y termina en el GD (Mann y Esiri, 1988).

IV.3. LA VÍA RAS/ERK COMO UNA POSIBLE VÍA DE SEÑALIZACIÓN IMPLICADA EN LA REACTIVACIÓN DEL CICLO CELULAR EN NEURONAS POSTMITÓTICAS

Con base en los reportes sobre la expresión temprana, en la EA, de diversos marcadores de ciclo celular en regiones de la formación hipocampal susceptibles a pérdida sináptica, se planteó la hipótesis de que la pérdida de conectividad sináptica podría alternativamente promover la reentrada al ciclo celular (Arendt, 2000; 2003; 2012). En un estudio previo reportamos que la expresión de reguladores del ciclo celular en la CE después de un daño excitotóxico en el hipocampo, se acompaña con la inducción de marcadores característicos de la EA. A partir de estos resultados, se derivó la interrogante sobre que vías de señalización podrían estar involucradas en esta reentrada a ciclo celular.

Una cascada de señalización potencial es la vía Ras/ERK, ya que además de su clásico en la proliferación celular, en neuronas diferenciadas modula procesos de plasticidad sináptica. En las siguientes secciones se describirán las características generales de esta vía de señalización y los datos que sugieren su participación en la reactivación del ciclo celular en neuronas maduras.

IV.3.1. LAS MAPKS

Las proteínas cinasas activadas por mitógenos (MAPKs) fosforilan residuos de serinas (Ser) y treoninas (Thr) en todos los organismos eucariontes. Las MAPKs son activadas por diversos estímulos extracelulares, lo que resulta en una variedad de respuestas como proliferación, apoptosis, sobrevivencia y diferenciación, entre otras. Estas proteínas cinasas se dividen en convencionales y no convencionales. En el primer grupo se encuentran las cinasas regulada por señales extracelulares 1/2 (ERK 1/2), las cinasas amino N-terminal de c-Jun 1/2/3 (JNK 1/2/3), la cinasa p38 (isoformas α, β, γ, y δ) y la cinasa ERK5 (Cargnello y Roux, 2011).

La cascada de activación de las MAPKs convencionales incluye a tres cinasas activadas secuencialmente: una MAPK, una cinasa de MAPK (MAPKK) y una cinasa de MAPKK (MAPKKK). Las MAPKKKs son cinasas de Ser y Thr activadas por fosforilación y/o por interacción con proteínas G pequeñas de la familia Ras/Rho. La activación de la MAPKKK lleva a la activación y fosforilación de la MAPKK, la cual, a su vez activa a la MAPK a través de una fosforilación dual dentro de la secuencia motivo Thr-X-Tyr (Cargnello y Roux, 2011).

Por otro lado, el grupo de las MAPKs no convencionales incluye a las ERK3/4, la ERK 7 y la cinasa similar a NEMO (NLK). La activación de las MAPKs atípicas no incluye a la cascada de tres cinasas. Sin embargo,

ambos tipos de MAPKs se consideran cinasas dirigidas por prolina (Cargnello y Roux, 2011).

IV.3. 2. LA VÍA RAS/ERK

Las cinasas de Ser/Thr ERK 1 y ERK 2, también conocidas como p44/p42 MAPK, son activadas principalmente por factores tróficos, incluyendo al factor de crecimiento epidérmico (EGF), al factor de crecimiento derivado de plaquetas (PDGF), al factor de crecimiento neuronal (NGF), y a la insulina, entre otros. También, pueden ser activadas por ligandos de receptores acoplados a proteínas G como citocinas, o por estrés osmótico. La ruta de activación de ERK mejor caracterizada es la correspondiente a la cascada de señalización de Ras/RAF/MEK/ERK. Esta cascada participa en la regulación de una diversidad de procesos celulares como adhesión, proliferación, migración, sobrevivencia, diferenciación, neurodegeneración, metabolismo, transcripción génica y plasticidad sináptica (Impey y col., 1999; Roskoski Jr, 2012).

El esquema básico de la vía Ras/ERK involucra la activación de receptores con actividad de cinasa de Tyr promovida por factores tróficos. La dimerización del receptor inducida por el ligando estimula la autofosforilación del receptor en residuos de Tyr. Estos residuos fosforilados sirven como sitios de unión para proteínas como Grb2, la cual interactúa con SOS, un factor intercambiador de nucleótidos, y lo conduce a la membrana plasmática. SOS estimula el intercambio de GDP por GTP en Ras, necesario para la activación de esta GTPasa. En seguida, la cinasa RAF (Raf-1y B-Raf, en el cerebro) (Girault y col., 2007) es reclutada en la membrana plasmática por su interacción con Ras-GTP, lo que induce su activación. Posteriormente, Raf en estado activo fosforila a MEK 1/2 y la

activa. Finalmente, ERK 1/2 es activada por fosforilación de MEK 1/2 en las Tyr204/187 y en las Thr 202/185 respectivamente. Una vez activa ERK 1/2 fosforila una amplia gama de sustratos citoplásmicos o nucleares incluyendo factores de transcripción como Elk-1, c-Ets-1, c-Ets-2, ATF y AP-1(Fos/Jun) (para revisión ver Sears y Nevins, 2002).

Respecto al papel de ERK en la modulación de la proliferación celular, se ha sabe que el promotor del gen de la Ciclina D1 contiene sitios consenso para factores de transcripción como Fos/Jun (AP-1) y ATF2, ambos activados por esta cinasa (Albanese y col., 1995). Como sabemos la inducción de Ciclina D1 y su unión con Cdk4 o Cdk6 es determinante para la progresión del ciclo celular a través de la fase G1.

A lo largo de varias décadas la vía Ras/ERK ha sido ampliamente explorada dada su relevancia en procesos de cáncer. Más recientemente el estudio de la cinasa ERK 1/2 en las neuronas, ha revelado su papel en procesos como la diferenciación neuronal, procesos de aprendizaje y memoria e incluso en la muerte neuronal de tipo apoptótica (Sweat, 2001; Subramaniam y Unsicker, 2010).

IV.3.2.a PAPEL DE ERK 1/2 EN LA NEUROPLASTICIDAD Y SU POSIBLE PARTICIPACIÓN EN LA REACTIVACION DEL CICLO CELULAR NEURONAL

En humanos, las secuencias de ERK1 y ERK2 presentan alrededor de un 84% de similitud y ambas cinasas comparten muchas de sus funciones, por lo que frecuentemente son referidas como ERK1/2 (Lloyd, 2006). Estas cinasas se expresan diferencialmente en todos los tejidos, con altos niveles en el cerebro, el timo y el corazón (Carganello y Roux, 2011). En el cerebro, ERK está involucrada en la modulación de procesos relacionados con la plasticidad sináptica.

La neuroplasticidad en general comprende a los mecanismos por los cuales la información es adquirida, almacenada y modificada en las sinapsis, neuronas y circuitos neuronales para guiar el comportamiento de un organismo. Dentro de estos procesos, se incluyen aquellos involucrados en la reorganización estructural como en las modificaciones de la actividad eléctrica y neuronal (Gillick y Zirpel, 2012).

La exposición neuronal a algunas neurotrofinas puede promover la formación y crecimiento de neuritas a través de la activación de ERK 1/2. Un ejemplo claro es el de la diferenciación de las células PC12 a neuronas de fenotipo-simpático mediante el tratamiento con NGF. Dicho proceso depende de la activación de ERK, ya que el bloqueo de su activación inhibe la formación de neuritas, mientras que la activación sostenida de ERK promueve su crecimiento (Barrie y col., 1997). Este papel de ERK en el desarrollo de neuritas se ha descrito en diversas líneas y tipos neuronales (Hausott y col., 2008; Curtis y col., 2012). Como ejemplo, la actividad de esta cinasa promueve la arborización dendrítica en neuronas dopaminérgicas (Collo y col., 2012) y el aumento de espinas dendríticas en células piramidales del hipocampo (Alonso y col., 2004).

ERK además de participar en la reorganización estructural neuronal, puede regular la actividad sináptica. En neuronas, la despolarización de la membrana, el influjo de Ca^{2+} y la señalización glutamatérgica son capaces de activar directamente a ERK1/2 (para revisión Thomas y Huganir, 2004). En 1997, se publicó el primer reporte sobre la activación de ERK durante la inducción de un mecanismo de plasticidad sináptica, la potenciación a largo plazo (LTP por sus siglas en inglés) en rebanadas del hipocampo. El tratamiento con PD 098059, un inhibidor de MEK 1/2 afecto la fase de inducción de la LTP hipocampal (English y Sweatt, 1997).

Si bien los datos anteriores aluden a la participación de ERK en mecanismos de plasticidad sináptica en neuronas diferenciadas, en años recientes se reportó que productos avanzados de glucosilación (AGEs) y S100B, una proteína de unión a Ca^{2+}, pueden actuar como señales mitogénicas para la estimulación de células neuro2a en la progresión del ciclo celular. La cascada de señalización inicia con la activación del receptor a AGEs (RAGE), prosigue con la activación de p42/p44 MAPK y culmina con la inducción de ciclina D1/cdk4 (Schmidt y col., 2007). Estos datos señalan una posible contribución de la vía ras/ERK en la reactivación del ciclo celular de neuronas diferenciadas.

Por otro lado, se ha descrito que en etapas iniciales de la EA, Ras, RAF-1, MEK 1 y ERK 1/2 se encuentran altamente expresadas en áreas cerebrales susceptibles a pérdida sináptica y donde, a su vez, se ha descrito reactivación del ciclo celular, sugiriendo su posible papel en el mecanismos patológicos de esta enfermedad (Gärtner y col., 1999; McShea y col., 1999; Mei y col., 2006). Adicionalmente, la translocación nuclear de ERK en neuronas de tejido cerebral de pacientes con EA señala la activación de esta cinasa (Arendt y col., 1995). Estos datos en conjunto, sugieren la participación activa de la vía ras/ ERK en la neurodegeneración observada en la EA.

Se ha planteado la posibilidad de que la pérdida de conectividad sináptica asociada al envejecimiento patológico reduzca la activación de neuronas postmitóticas, lo cual podría alternativamente considerarse como una carencia de soporte trófico y promover la entrada al ciclo celular (Arendt, 2000; 2003; 2012). Esta reactivación del ciclo celular, puede ser particularmente frecuente en circuitos neuronales sometidos a un alto grado de reorganización sináptica como, la vía perforante. En un estudio previo, nosotros describimos la activación del ciclo celular en

neuronas de la CE posterior a un daño excitotóxico en el GD e hipocampo. Considerando el conjunto de datos anteriores, nosotros planteamos que la actividad de ERK podría contribuir a la reactivación del ciclo celular en neuronas sometidas a una pérdida significativa de aferentes en un circuito neuronal altamente vulnerable (ver Fig. 2 del artículo de revisión).

V. PLANTEAMIENTO DEL PROBLEMA

La reactivación del ciclo celular en neuronas maduras conduce a la muerte celular programada y puede contribuir a la neurodegeneración en diversas patologías como la EA. En etapas tempranas de la EA, se ha reportado la expresión de diversos marcadores de ciclo celular en regiones de la formación hipocampal que son susceptibles a una pérdida sináptica. Ya que la mayoría de los datos derivan del análisis post-mortem de cerebros y de modelos transgénicos, resulta de gran utilidad contar con un modelo que reproduzca la pérdida sináptica temprana observada en la EA y permita explorar el curso y los mecanismos moleculares de la neurodegeneración resultante. En un trabajo previo, reportamos que la reactivación del ciclo celular en la CE después de un daño excitotóxico en el GD se asocia con la expresión de fosfo-Tau y PPA, marcadores ambos de la patología de tipo Alzheimer. A partir de ese estudio, surgió la interrogante sobre que vías de señalización podrían mediar esta reentrada a ciclo celular. La cinasa ERK además de regular la proliferación celular, en neuronas diferenciadas participa en procesos de plasticidad sináptica. Por este motivo, en el presente trabajo utilizamos un modelo de desaferentación sináptica para estudiar la posible activación de ERK 1/2 y su contribución en la reactivación del ciclo celular en neuronas maduras con disminución o pérdida de contactos sinápticos.

VI.HIPÓTESIS

La pérdida de contactos sinápticos en neuronas de la CE que forman parte de la vía perforante genera señales que activan a ERK1/2 y conducen a la reactivación del ciclo celular en neuronas blanco del GD.

VII. OBJETIVOS

OBJETIVO GENERAL

Estudiar en un modelo *in vivo* de pérdida sináptica en el circuito de la vía perforante, la participación de la cinasas ERK1/2 en la reentrada al ciclo celular de neuronas del GD que perdieron contactos sinápticos provenientes de neuronas de la CE.

OBJETIVOS PARTICULARES

1.-Evaluar el efecto de una lesión excitotóxica unilateral en la CE sobre la pérdida del control del ciclo celular en neuronas conectadas con el sitio de la lesión.

2.- Estudiar el curso temporal de la expresión y activación de ERK1/2 y su asociación con la inducción de marcadores del ciclo celular en neuronas hipocampales que perdieron contactos sinápticos provenientes de la CE.

3.- Analizar el efecto de la administración i.c.v. de U0126, inhibidor de MEK1/ 2, sobre La activación de ERK1/2 y la reactivación del ciclo celular secundaria a la desaferentación en neuronas del GD y CA3.

VIII. MATERIALES Y MÉTODOS

En esta sección solo se muestra un esquema general de la estrategia experimental utilizada en el presente trabajo de investigación (Fig.4). La descripción detallada del diseño experimental y los materiales usados en este estudio se presentan en el artículo (1): "ERK activation and expression of neuronal cell cycle markers in the hippocampus after entohrinal cortex lesion".

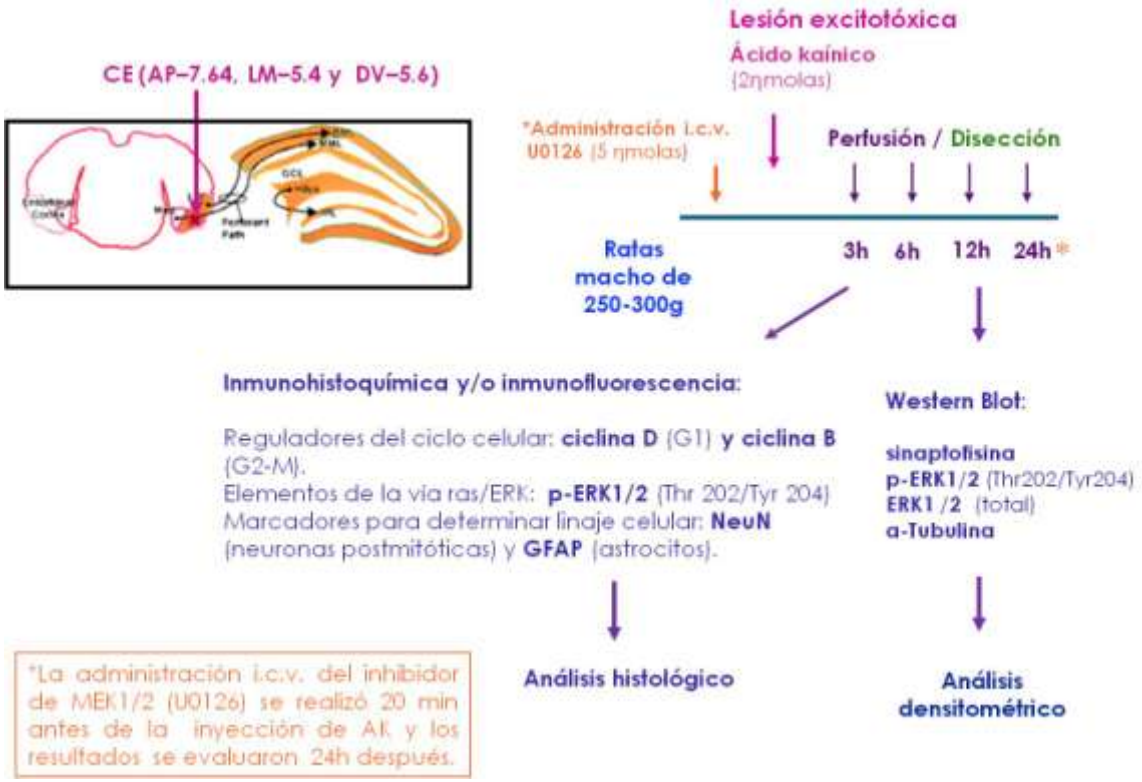


Fig. 4 . Esquema correspondiente al diseño experimental general seguido en este estudio. El modelo de desaferentación hipocampal se basa en inducir una lesión excitotóxica en el CE, la cual genera una disminución en la conectividad sináptica con los sitios de proyección, GD y CA3. Dentro de las primeras 24h posterior a la lesión, se evaluó por diferentes técnicas la presencia y activación de fosfo-ERK (p-ERK), así como la expresión de algunos marcadores de ciclo celular en las neuronas conectadas al sitio de la lesión. Con el propósito de

evaluar la relación de ERK y activación del ciclo celular en el GD, a un grupo sujetos se le administro i.c.v. un inhibidor de MEK1/2, previo a la inyección de AK.

IX. RESULTADOS

IX. 1. ARTÍCULO (1):ERK activation and expression of neuronal cell cycle markers in the hippocampus after entohrinal cortex lesion

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Keywords: Cell cycle; neurodegeneration; entorhinal cortex; ERK1/2; dendate gyrus; synaptic loss; excitotoxic damage.

Abstract

Current findings suggest that neuronal cell death is frequently associated with the aberrant expression of cell cycle-regulatory proteins in post-mitotic neurons. Aberrant cell cycle reentry has been implicated in diverse neurodegenerative conditions, including Alzheimer's disease (AD). Previously we have reported that

the appearance of cell cycle markers in post-mitotic neurons of the entorhinal cortex (EC) after excitotoxic hippocampal damage is associated with the expression of phospho-tau and amyloid precursor protein (APP). However, the question about the signaling pathway involved in this cell cycle reentry remains unresolved. Differentiated neurons use the molecular mechanisms initially acquired to direct cell proliferation such as the Ras-ERK 1/2 pathway to regulate synaptic plasticity. In this work we explored if ERK 1/2 (extracellular signal-regulated kinase) related signaling may contribute to the cell cycle reentry in hippocampal neurons after a unilateral EC lesion. We showed that within the first 24 h after hippocampal deafferentation, numerous neurons expressed phospho-ERK 1/2, concomitant to the gradual increases in cyclin D1 and cyclin B immunoreactivity in the dentate gyrus and hilus. Several of these immuno-positive cells to phospho-ERK 1/2 and cyclin B in hippocampus are post-mitotic neurons as they are positive to NeuN. The intracisternally administration of U0126 (a MEK inhibitor), previous to the excitotoxic lesion, decreased the activation of ERK 1/2, and the expression of cyclin D1 and cyclin B in the hippocampus. The present findings support the notion that ERK1/2 plays a role in cell cycle reactivation in mature neurons efferently connected to the lesion site.

Introduction

Numerous studies have provided evidence that cell cycle-associated mechanisms contribute to neuronal death in various pathological conditions including target deprivation (Hernández-Ortega et al., 2007; Herrup and Busser, 1995), DNA damage (Park et al., 1998), excitotoxicity (Verdaguer et al., 2002) and exposure to amyloid β protein (Aβ) (Bhaskar et al., 2009; Copani et al., 1999; Giovanni et al., 1999). Furthermore, aberrant cell cycle re-entry has been implicated in neuronal death during stroke and trauma (Byrnes and Faden, 2007; Hayashi et al., 2000), epilepsy (Nagy, 1998), amyotrophic lateral sclerosis (ALS) (Rangathan and Bowser, 2003), Parkinson's disease (PD) (Jordan-Sciutto et al., 2003) and Alzheimer´s disease (AD) (Bonda et al., 2009; Busser et al., 1998; Copani et al., 2007; McShea et al., 2007; Nagy et al., 1997; Vincent et al., 1997).

The first reports implicating the reactivation of the cell cycle in AD were obtained from analysis of AD brain tissue, showing expression and activation of cyclin B and Cdc2 in differentiated neurons (Nagy et al., 1997; Vincent et al., 1997). The cyclin B/Cdc2 complex derived from the AD brain can phosphorylate tau (Vincent et al., 1997), which is essential for neurofibrillary tangle formation. Further evidence supports that some neurons in AD re-enter into the cell cycle and progress through the S phase, producing cell cycle stasis with the consequent expression of AD markers (Aβ and phospho-tau) and neurodegeneration (Bonda et al., 2009). Although significant progress has been made in understanding the ethiopathological mechanisms of AD, a comprehensive theory is still lacking; thus, research has been conducted to unravel the mechanisms underlying neuronal differentiation and plasticity as events associated with neuronal damage when are altered in the mature brain. As brain plasticity depends on continuous synaptic reshaping, plasticity molecules involved in this process are constantly secreted and may contribute to synaptic modifications and neuronal cell cycle reactivation under pathological conditions, as has been previously proposed (Arendt et al., 2000; 2001; 2005). Loss of synaptic input reduces the activation of post-synaptic neurons, which might be sensed as a lack of trophic support driving neurons to exit from the G0 phase. This pathological cell cycle reactivation would be particularly frequent in circuits submitted to high rate of plastic adjustment such as the perforant path that connects the EC with the dentate gyrus (DG) (Arendt and Brückner, 2007). In this context, we showed that after loss of synaptic contact between the two interconnected areas, EC and the dentate gyrus (DG), by excitotoxic damage in the hippocampus, several cell cycle proteins of G1, S and even G2 phases are up-regulated in the EC. In addition, the progressive expression of two AD-related proteins, phospho-tau and amyloid-β protein reached higher levels immediately after the increase in G1/S-phase markers (Hernández-Ortega et al., 2007). However, the question about the signaling pathway involved in this cell cycle reentry remains unresolved. Differentiated neurons use the molecular mechanisms acquired to direct cell proliferation such as the Ras-ERK 1/2 pathway to control synaptic plasticity (Impey et al., 1999; Sweatt, 2004;

Wiegert and Bading, 2011). In this study, we explored if ERK1/2 also named p44/p42 MAPK is activated following deafferentation of DG by unilateral EC lesion and if the appearance of cell cycle markers depends on this activation. Excitotoxic damage was induced in the EC, and analyzed the participation of activated phospho-ERK1/2, in the increase of cyclin D1 and cyclin B immunoreactivity in the hippocampus.

Material and Methods

Stereotaxic Injection

Male Wistar rats (250-300g) were used throughout the study and handled with all precautions necessary to diminish their suffering, in agreement with the Regulations for Research in Health Matters (México), and with approval of the local Animal Care Committee.

Animals were anesthetized with 3-4% isoflurane (Vet One) in 95%O_2 **/**5% CO_2 mixture and placed in a stereotaxic frame with the nose bar positioned at –0.3 mm. Unilateral injection of kainic acid (KA; Sigma-Aldrich, Chemicals) was applied into the right entorhinal cortex at the following coordinates with reference to bregma: A -7.64, L -5.4 and V -5.6 (Paxinos and Watson, 1998). KA was dissolved in 1M NaOH, the pH was adjusted to 7.0–7.5 and the solution was brought to the desired volume with 10 mM phosphate buffer (pH 7.4). Two µmoles of KA were infused in a 1µl volume, using a microsyringe mounted on a microinjection pump (Stoelting, Illinois, USA). Control animals received 1µl of 10 mM phosphate buffer. After the stereotaxic injection the skin was sutured, anesthesia was discontinued, and the rats were returned to individual cages and provided with food and water *ad libitum*.

Other group of rats were injected i.c.v. with the MEK1/2 inhibitor, U0126 (Calbiochem, Merck Chemicals) or with the vehicle solution, 20 min before performing the EC lesion. One µl of 50% DMSO/sterile saline containing two µg of U0126 was injected into the right lateral ventricle at the following coordinates: A.P.-1.0, L.M -2.0 y D.V.-3-3 (Kuroki et al., 2001; Sutton et al., 2005).

Immunohistochemistry

At 3, 6, 12 and 24 h after treatment, control and injected rats were anesthetized with sodium pentobarbital and transcardially perfused with ice-cold PBS for 4 min, followed by ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min. Brains were removed and left in fixative solution for 24 h. Then, brains were successively transferred to 20% and 30% sucrose (24 h each) and 40 µm coronal sections were cut in a cryostat. Coronal sections were treated for immunohistochemistry or stained with cresyl violet to verify the damaged area induced by the KA injection. Free-floating sections were incubated at room temperature for 30 min in phosphate-buffered saline (PBS) containing 0.25% Triton X-100 and 0.3% H_2O_2 and left 2 h at 4º C in PBS/5% BSA solution. Slices were then immersed for 2 h at room temperature in a PBS/5% BSA solution containing one of the following antibodies: monoclonal anti-cyclin D1 (1:300) (Zymed Laboratories, Inc.), monoclonal anti-cyclin B (1:200) (Santa Cruz Biotechnology, Inc.) or monoclonal anti-phospho-p44/p42 MAPK (Thr 202/Tyr 204) (1:400). Following this period, the brain slices were washed three times with PBS, each for 5 min, and incubated with the secondary antibody, biotinylated mouse IgG (1:500) (Vector Laboratories) for 2 h at room temperature. Finally, the slices were, processed with the ABC-biotin-avidin-peroxidase kit (Vector Laboratories) and revealed with diaminobenzidine tetrahydrochloride as substrate for peroxidase reaction. For negative controls, primary antibodies were removed from the procedure.

For immunofluorescence studies, brain slices were maintained in antifreeze solution (PBS, 30% ethylene glycol and 30% glycerol) at 4°C until used. In brief, the slices were washed with PBS three times, each for 10 min, and then treated with TBS/0.1% Triton X-100 for 30 min. To block non-specific binding sites the sections were incubated with TBS/0.1% Triton X-100/3% BSA for 2 h at room temperature and incubated overnight at 4°C in blocking solution containing one of the following antibodies: monoclonal anti-NeuN (used as a marker of mature neuronal nuclei, 1:500) (Chemicon International), polyclonal anti-GFAP (used for labeling glial cells, 1:500) (DAKO, Denmark A/S), policlonal anti-phospho-p44/p42 MAPK

(Thr202/Tyr204) (1:300) (Cell Signaling technology), monoclonal anti-cyclin B (1:200) (Zimed Laboratories, Inc). Sections were washed three times with TBS/ 0.1% Triton X-100 and then incubated with Alexa 546 conjugated goat anti-rabbit IgG (3 µg/ml) (Sigma-Aldrich, USA) or Alexa 488 conjugated rabbit anti-mouse IgG (3.5/ µg/ml), for 2h at room temperature. Finally, the sections were washed with TBS/0.1% Triton X-100 and mounted on slides coated with silane (g-methacryloxypropyltrimethoxysilane, Sigma-Aldrich, USA) for analysis.

For double fluorescent labeling, sections were washed three times with PBS/0.1% Triton, each for 10 min, and incubated with the next primary antibody. Subsequently, sections were washed three times with TBS/0.1% Triton X-100 and then incubated for 2 h at room temperature with a mixture of fluorescence-labeled secondary antibodies. The sections were subsequently washed and mounted as described above. For negative controls, primary antibodies were removed from the procedure.

Cell Counting

Histological sections were photographed with a Zeiss CCD camera attached to a Zeiss Axioskop 40 microscope (Gottingen, Germany). The number of positive cells for different markers was estimated from 4 representative 30 µM sections located between bregma AP -3.3 and -4.16 (Paxinos and Watson, 1998) from 4-5 animals per condition. Cell counting was performed at a 40x magnification within the DG and each section analyzed was separated by 60µm from the next. Data for the estimation of the number of immunofluoresce sections where cell counting was performed were captured and analyzed using Olympus BX51 WI microscope equipped with a disk scanning unit (Tokyo, Japan) or confocal Zeiss microscope (facility from Instituto Nacional de Cancerología, Mexico City) in the same way described above. Each section analyzed contained information from a 14µm optical section in the axial plane. Cell counting was performed within the DG ipsilateral to the lesion. The number of positive cells per optical field for the different cell markers was recorded and the average was calculated.

Enriched DG fraction

Three, 6, 12 and 24 h after the KA injection, control and experimental rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and their brains were removed and immersed in cold artificial cerebrospinal fluid (aCSF: 126 mM NaCl, 3.5 mM KCl, 1.2mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, 25 mM $NaHCO_3$, pH 7.4) at 4°C during 1 min. Then, the brains were fixed on a plate and placed in a vibratome chamber (Campden Instruments, USA) with ice-cold aCSF. Three coronal hippocampal slices (600µm thick) were collected per rat. Immediately, DG was carefully dissected (see Fig. 1M) in ice-cold aCSF and homogenized (Sonics, Vibra Cell, USA) in 120µl lysis buffer containing: 50 mMTris–HCl, pH 7.5, 1% NP-40, 0.5% deoxycholate, protease and phosphatase inhibitors (Complete™, Roche Diagnostics and inhibitor Halt ™, Thermo Scientific, respectively).

Western blot

Forty µg of enriched fraction of DG proteins were loaded onto a 12% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Blots were blocked with PBS/5% non-fat dry milk for 2 h at room temperature and then incubated overnight at 4°C with the following antibodies: monoclonal p44/42 MAPK (1:800) (Cell signaling, Technology), monoclonal anti-phospho-p44/p42 MAPK (Thr202/Tyr204), (1:400) (Cell signaling, Technology), mouse monoclonal anti-synaptophysin (1:500) (Chemicon International, Temecula, CA) and mouse monoclonal α-Tubulin (1:1000) (Sigma Aldrich). After three washes with PBS/0.1%Tween 20, the membranes were incubated for 2 h at room temperature with goat anti-mouse IgG conjugated to horseradish peroxidase (1:6000) (Invitrogen) and detected by chemiluminescence (Immobilon Western, Millipore) on Kodak X-Omat film. Western blots were densitometrically analyzed by using NIH ImageJ software.

Statistical analysis

Data are presented as the mean ± SEM. Student's *t*-test was or as indicated, analysis of variance (ANOVA) were used to evaluate the statistical significance of data followed by Scheffe's *post-hoc* analysis. Differences between means were considered significant at $p < 0.05$.

Results

Damaging effects of KA injection in the EC

The time course of the EC focal lesion induced by KA and the synaptophysin content in the enriched DG fraction was presently analyzed, in order to assess the reduction in the amount of synaptic contacts (Fig. 1). Nissl staining revealed disorganized EC cellular layers II and III as early as 3 h after KA, which progressed in time until substantial neuronal death became evident at 24 h (Fig. 1A-E). While granule cells in the DG were apparently not damaged, in the hilar (Fig. 1F-J) and CA3 hippocampal regions (supplemental Fig.S.1 A-D) some cells appeared shrunken 12 h after the lesion and neuronal death was observed in the CA3 sector, 24 h after KA. GFAP immunohistochemistry revealed some gliosis mainly located in the hilar region of the hippocampus (Fig. 1K,L and high magnifications K',L'). EC neuronal death was accompanied by a significant 35% reduction in synaptophysin content in the DG, indicating a partial disconnection of this region from the lesioned EC (Fig. 1N).

Induction of cell cycle-related proteins in the hippocampus after EC lesion

After EC damage a gradual increase in cyclin D1 (involved in G1 phase) and cyclin B (involved in the G2 to M transition) (Fig. 2) was observed and was located mainly in the subgranular zone and granular layer of the DG (Fig. 2A and C), in hilar cells (Fig. 2 B and D) and in some CA3 pyramidal neurons (Fig. S. 2 I-L and Fig. S. 3I-L, respectively). Although cyclin D1 and cyclin B immunostaining was mainly observed in neuronal somata and nuclei, few neurites were also stained. The extranuclear localization of cell cycle-related proteins including cyclin D1 and B, in differentiated neurons has been reported by (Gärtner et al., 2003;

Sumrejkanchanakij et al., 2006; Schmetsdorf et al., 2009). In control saline injected animals as well as in the contralateral hippocampus of KA treated rats, the immunoreactivity for both cyclins was sparse or undetectable and never exceeded 2-5 per field (Figure 2 E-F, dotted lines). As shown in Fig. 2E, 3 h after KA sparsely stained cells were positive to cyclin D1 in the DG (1.75 ± 0.48). Although the number of cyclin D1–positive cells increased progressive over time, the rising was significant until 12 h (11 ± 0.91) after EC lesion. The largest number of cyclin D1–positive cells was observed after 24h of EC lesion (20.5 ± 0.65). Cyclin B was expressed in DG cells as early as 3 h after the EC lesion (1.75 ± 0.63; Fig. 2F). The number of cyclin B- positive cells began to increase after 6h (7.25 ± 1.11) and reached its highest levels after 24h (33.75 ± 2.1), as can be seen in Fig. 2F. Most cells expressing cyclin B were neurons as demonstrated by the absence of co labeling with the glial marker, GFAP (Fig. 6A-C).

ERK 1/2 activation in the hippocampus after EC lesion

To evaluate if the induction of G1 and G2-M markers of cell cycle was associated with ERK activation, we studied the temporal induction of phospho-ERK1/2 in the hippocampus. ERK1/2 activation occurs through the phosphorylation of Thr202/Tyr 204 and Thr185/Tyr187 residues respectively, by the action of MEK1/2 (Payne et al., 1991; Butch and Guan, 1996). A gradual rise in activated ERK1/2 was registered in the deafferentated hippocampus along the first 24 h after EC damage (Fig. 3 and Fig. S. 1). In the DG (Fig. 3A, insert) and CA3 regions (Fig. S. 1F), phospho-ERK1/2 was scarcely present 3 h after KA. However, between 6 to 24 h after the KA injection phospho-ERK1/2 immunoreactivity was clearly observed in neuronal somata and neurites in the DG (Fig. 3A, E and I; magnifications F-H) and CA3 (Fig. S.1G-I). In the contralateral hippocampus phospho-ERK1/2 immunoreactivity was scarcely distributed in the neuropil and few positive cells were detected at all analyzed times (Fig. 3B-D). As shown in Figure 3J, the number of phospho-ERK 1/2-positive cells reaching the highest levels 24 h after EC lesion in the DG (23.3 ± 2).

To further study the content of activated ERK1/2, an enriched DG fraction was obtained and analyzed by western-blot. While total ERK content (p44/p42MAPK) in the DG remained unchanged over time (Fig. 4A), a marked increase in phospho-ERK2 (phospho-p42MAPK) expression was evident after the first 3 h, and remained elevated until 24 h after the KA-induced EC lesion (Fig. 4B).

MEK inhibition decreases ERK1/2 phosphorylation and cyclin B expression in the hippocampus after EC lesion

To deepen into the molecular mechanisms that link the appearance of cell cycle markers with ERK activation, a group of animals were intracisternally injected with the upstream inhibitor, U0126 prior to KA injection, and both ERK activation and cyclin B expression were analyzed. It has been demonstrated that the U0126 inhibitor is specific to MEK1/2 kinase (Favata et al., 1998) as well as its effectiveness to induce MEK inhibition in vivo (Jaffee et al., 2000; Kuroki et al., 2001; Sutton et al., 2005). 24 h after EC lesion, double immunofluoresce for phospho-ERK1/2 and NeuN revealed that mature neurons in the DG (5A-C, and high magnification see C) and CA3 (Fig. S. 1E) expressed phospho-ERK1/2. In support for this conclusion double labeling experiments showed that the majority of cells expressing phospho-ERK 1/2 were GFAP negative, thereby excluding glial cells as the predominant cell type expressing this marker after hippocampal deafferentation (Fig. 6G-I). We also found a number of mature neurons in which phospho-ERK immunoreactivity colocalized with cyclin B (Fig. 5D-F and high magnification see insert F). The injection of the MEK inhibitor, U0126 attenuated the coexpression of phospho-ERK and cyclin B in the hippocampus (Fig. 5M). Furthermore, 24 h after EC damage, the U0126 reduced the number of cells positive for cyclin B from 33.7 $\pm$ 2.09 to 10.0 $\pm$ 0.7 (Fig. 6D and see Fig. 2F) in the hippocampus. In addition to the morphological analysis, the western-blot performed in the DG fraction showed that U0126 significantly reduced ERK activation 24 h after KA treatment, corroborating that MEK/ERK cascade is activated in the hippocampus in vivo after the EC damage (Fig. 5J-K).

Discussion

The present study reports a model of hippocampal deaferentation that generates the expression of cell cycle markers in the hippocampus associated to MEK/ERK signaling. We also demonstrated that a partial loss of EC input into the hippocampus is sufficient to promote cell cycle activity in mature neurons.

Deafferentation of the hippocampus by a stereotaxic lesion of the EC has been extensively used as a model to study the mechanisms of synaptic reorganization following brain injury (Bechmann and Nitsch, 2000; Deller et al., 1996, 2007; Fagan and Gage, 1994). Neurons of the EC receive multimodal sensory information from primary sensory and associated cortices and, through their axons that form the perforant path, relay this information to the hippocampus, particularly to DG and CA3 (Amaral et al., 1987; Dolorfo and Amaral, 1998; Germroth et al., 1989). Several studies have demonstrated the vulnerability of this circuit during healthy and pathological aging (Chao at al., 2010; Devanand et al., 2007; Stoub et al., 2011). This circuit has, in fact, been described as the initial site in which neurodegenerative changes associated with AD pathology are prominent (Braak and Braak, 1991, 1995; deToledo-Morrell et al., 2004; Devanand et al., 2007). Moreover, substantial evidence has demonstrated in AD that the loss of synaptic contacts in the GD (Dekosky et al., 1996; Scheff and Price, 2006) correlates with neuronal loss in lamina II of the EC (Gómez-Isla, 1996; Price et al., 2001).

In AD, a marked re-expression of several cell cycle proteins in the hippocampus and EC has led to postulate a link between aberrant cell cycle progression and neuronal death (Raina et al., 2000). Moreover, aberrant cell cycle activation by simian virus 40 large T antigen (Tag) expression in mice induces AD-like pathology in hippocampal postmitotic neurons (Park et al, 2007). But so far, the nature of the factors that could contribute to the loss of differentiation control and induce mature neurons to modify their quiescent state and activates the cell cycle machinery remains unknown. We previously postulated that loss of synaptic input between two interconnected areas may drive mature neurons to exit from the G0 phase and re-express different cell cycle markers and AD-related proteins

(Hernández-Ortega et al., 2007). In this work, we expanded these results and found that a reduction in synaptic input from the EC leads to the phosphorylation of ERK1/2 and to the expression of cell cycle markers in hippocampal neurons. We reported the induction of cell-cycle proteins of the G1 and G2 phases, which reached their highest levels 24 h after the excitotoxic damage to EC and consecutive to the expression of phospho-ERK. Interestingly, the sites of highest expression levels of these molecules were those innervated by axons from the lesioned neurons and placed in layer II of the EC (Dolorfo and Amaral, 1998; Germroth et al., 1989).

Growing evidence suggests that ERK1/2 plays a crucial role in promoting neuronal death, depending on the severity and duration of its expression. Hence, sustained activation of ERK1/2 has been implicated in neuronal death under long-term exposure to glutamate (Jiang et al., 2000; Hidalgo and Núñez, 2007) and to oxidative stress (Luo and DeFranco, 2006), while transient ERK1/2 activation generated by growth factor stimulation may play a protective role (Almeida et al., 2005; Subramaniam et al., 2005). In the present model we report rapid, sustained activation of ERK1/2 in the deafferenteated hippocampus, and suggest its involvement in the onset of molecular changes that lead to neuronal dysfunction and may finally produce neuronal death. At present it is not clear yet whether ERK1/2 directly activates cell death pathways or regulates the expression of pro-death genes, but the strongest evidence points to its involvement in controlling cell proliferation (Lavoie et al., 1996; Subramaniam and Unsicker, 2010). In a recent study in which the impact of ERK1/2 activity duration was systematically analyzed, it was concluded that ERK1/2 activation must be sustained until late G1 for successful S-phase entry (Yamamoto et al., 2006). Herein, we propose that a loss of synaptic connectivity contributes to the loss of cell cycle control in target neurons, depending on ERK1/2 activation.

Neither the molecular signals that are delivered from the denervated hippocampus and lead to ERK1/2 phosphorylation nor the mechanism determining the duration of its activation are known yet. It has been stated, however, that the ERK1/2 pathway is preferentially activated by mitogenic factors, differentiation

stimuli and cytokines. Along with the numerous changes described in the denervated hippocampus triggered by a lesion on the EC neurons, a rapid glial activation has been a consistent finding. On the one hand, reactive astrocytes may contribute to ERK1/2 activation by secreting growth-promoting molecules, such as S100B, a protein produced largely by activated astrocytes. Interestingly, it has been shown that S100B protein is able to activate ERK and drive cell cycle activation in neuro2 cells in culture (Schmidt et al., 2007). On the other, alternative well-known inducers of ERK activation such a massive release of excitatory transmitters and free radical generation in the denervated hippocampus may also be participating (Hidalgo and Núñez, 2007).

The finding that pharmacological inhibition of MEK prevents the phosphorylation of ERK1/2 and, consequently, reduce the expression of cell cycle markers is suggestive of a mechanistic link, and supports the activation of MEK/ERK signaling in deafferentated hippocampus. The Ras-ERK1/2 signaling pathway associated with neuronal degeneration has been found in various models of neurodegenerative diseases. Particularly in AD brains, some elements of this signaling pathway have been found highly expressed at early stages of the disease (Arendt et al., 1995, Gärtner et al., 1995). ERK is able to phosphorylate tau protein in diverse Ser/Thr residues that has been found associated with the formation of paired helicoidal filaments (Drewes et al., 1992; Goedert et al., 1992). Phospho-ERK1/2 immunoreactivity co-exists with neurofibrillary degeneration in a subpopulation of hippocampal neurons (Ferrer et al., 2001; Ginsberg et al., 1999; Pei et al. 2002; Perry et al., 1999), as do the upstream activators of ERK1/2, Ras and Raf-1 (Gärtner et al., 1999; Gärtner et al., 2003; McShea et al., 1999; Mei et al., 2006). Finally, activated ERK1/2 has been found also associated with amyloid deposits (Medina et al. 2005). In the present model we found that the downstream effectors such as MEK and ERK 1/2 are activated and linked to changes that affect cell cycle control, similarly as reported in neurons in AD (Ferrer et al., 2001).

In summary, cell cycle reactivation is considered an important neuropathological feature of AD. Present results provide a model to analyze the cascade of events that starts with the loss of synaptic input between EC and

hippocampus and culminates with the pathological re-expression of cell cycle markers. These events firstly activate and then drive the cell-cycle to progress into mature neurons, a process which depends, at least in part, on the ERK1/2 as demonstrated.

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FIGURES

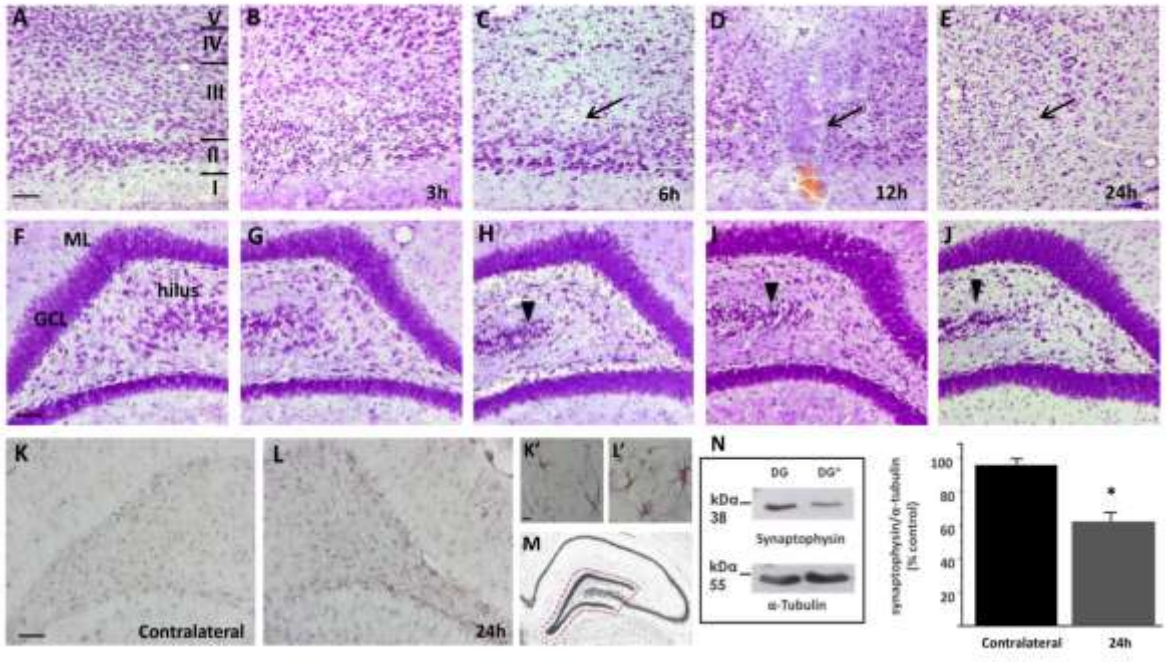

Fig. 1. EC lesion induces changes in cellular morphology in the hippocampus. Time course of neurodegeneration in the EC (A-E) and cellular changes in DG (F-J) assessed by cresyl violet staining. A progressive cellular death is observed in the EC (arrows) and hilus (arrow-heads) at the injected sites as compared with the contralateral sites (A, F, K). In DG: ML, molecular layer; GCL, granule cell layer Scale bar = 100 µm. Inmmunohistochemical staining revealed the increase in GFAP-positive reactive astrocytes in the ipsilateral site of denervated hippocampus (K and L and high magnifications K' and L'). Scale bars = 100 and 20 µm, respectively. Schematic representation of the dissected area in the hippocampal slices to obtain the DG enriched fraction (M). Representative Western blot of synaptophysin (N) content and densitometric analysis showing the decrease of this synaptic marker in DG after 24 h of EC lesion (DG*). Data represent mean ± SEM of 4-6 independent experiments (*p< 0.05).Values were analyzed with an unpaired Student´s *t*- test.

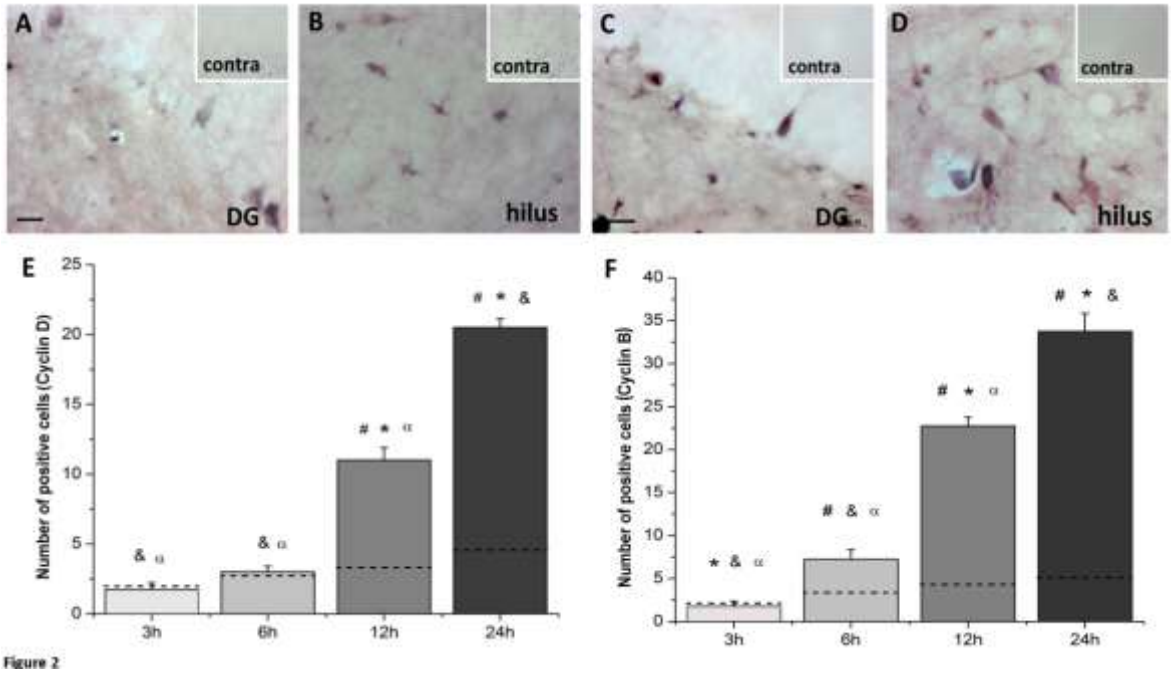

Fig. 2. Number of cyclin D1- and B-positive cells in the hippocampus after EC lesion. Representative micrographs showing the expression of positive cells to cyclin D1 (A and B) and cyclin B (C and D) in the dorsal layer and the hilus of the DG after 24h of KA injection. Inserts show the low content of these cell cycle markers in the contralateral regions. Micrographs are representative of 4-5 independent experiments. Scale bar = 20 μm. A progressive increase of cyclins is observed in the DG (E and F). Bars represent the number of cells positive for cyclin D1 (E) and cyclin B in the DG (F) and the dotted lines indicate the number of positive cells from the contralateral hippocampus. Mean ± SEM of 4 sections from 4–5 independent animals. (# $p < 0.05$ relative to 3h; * $p < 0.05$ relative to 6h, & $p < 0.05$ relative to 12h and α $p < 0.05$ relative to 24h).

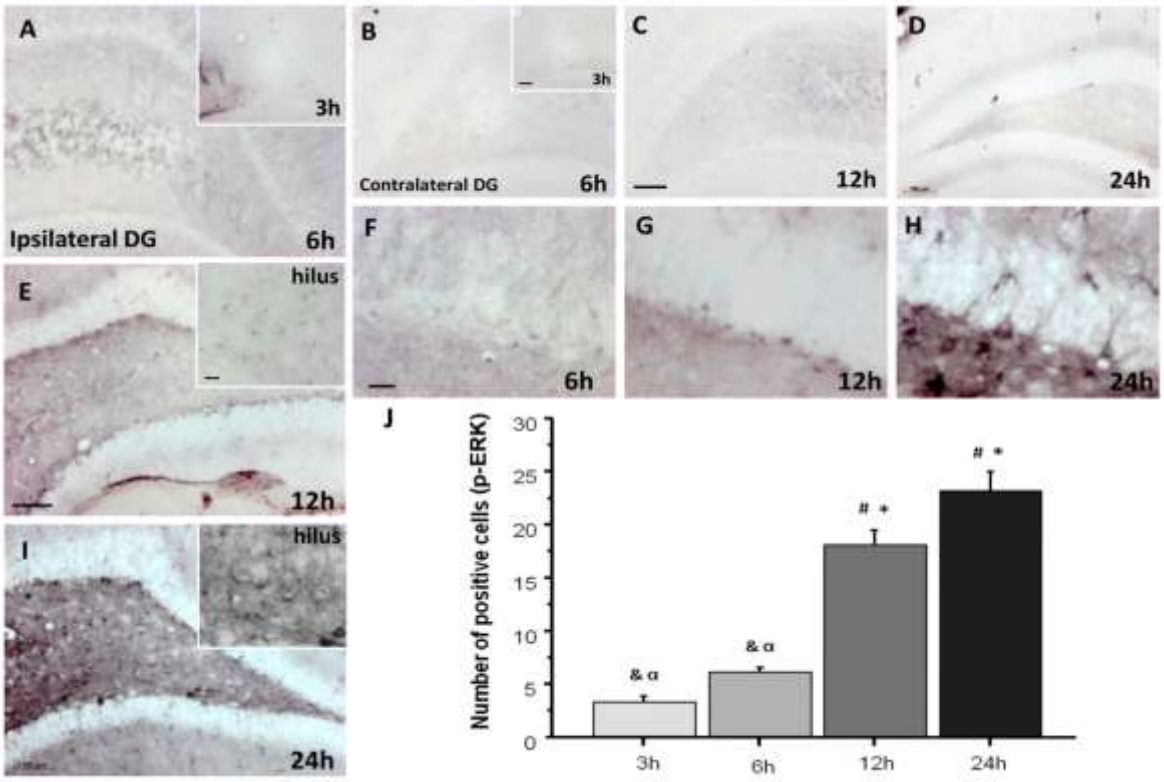

Figure 3

Fig. 3. EC lesion induces the progressive expression of phospho-ERK in the hippocampus. Weak phospho-ERK inmunoreactivity in DG contralateral to the site of KA injection is observed (B-D). In the ipsilateral DG, phospho-ERK 1/2 immunoreactivity was scarcely present 3h after KA (A, insert). However, between 6 to 24 h after KA, activated ERK 1/2 was clearly observed in neuronal somata and neurites in the dorsal layer and in hilar regions of the DG (A, E and I, high magnifications F-H). Micrographs are representative of 5-6 independent experiments. Scale bar = 20 μm and 100 μm (high magnifications). Data are presented as the mean of positive cells for phospho-ERK in the DG (J). Mean ± SEM of 4 sections from 3 independent animals. (# $p < 0.05$ relative to 3h; * $p < 0.05$ relative to 6h, & $p < 0.05$ relative to 12h and α $p < 0.05$ relative to 24h).

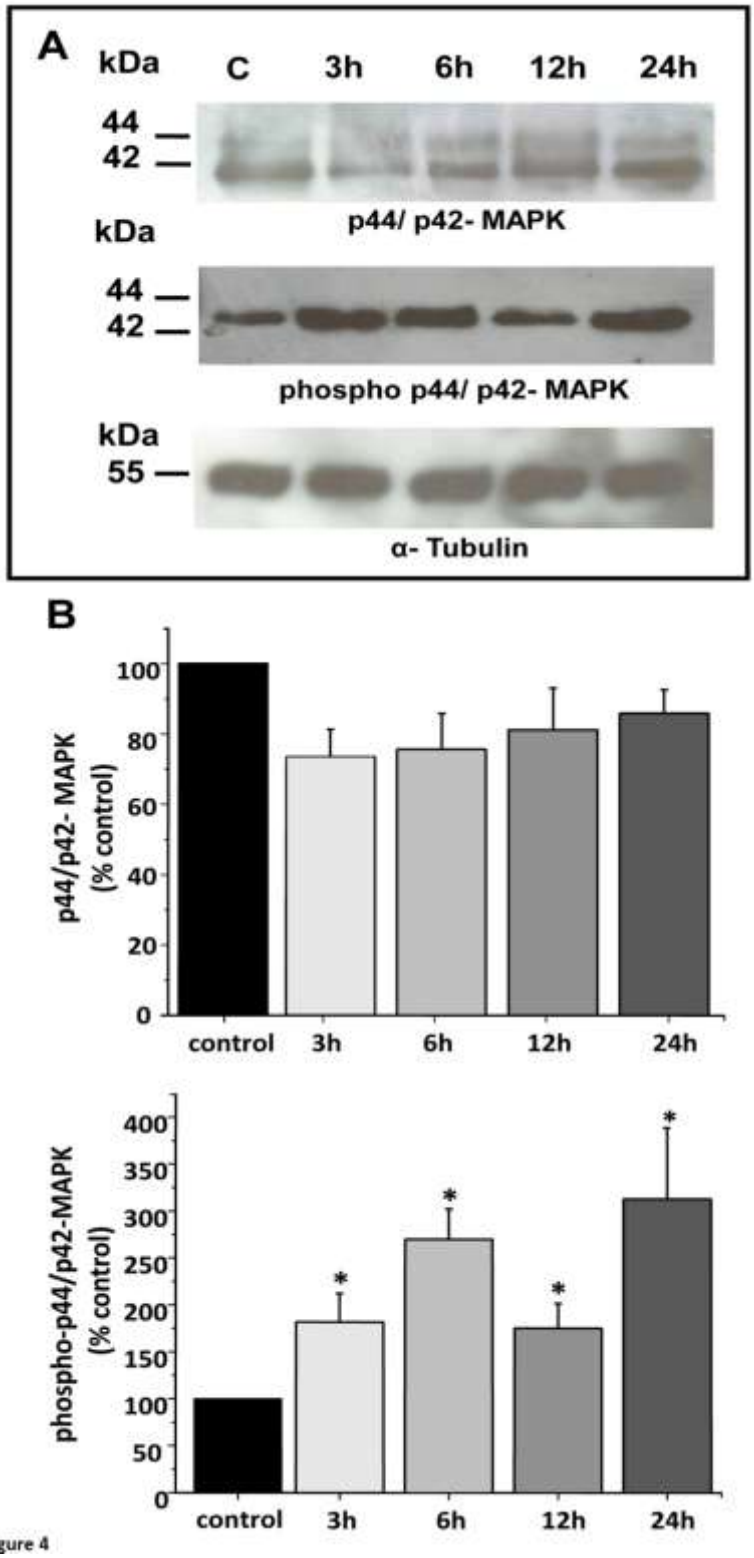

Figure 4

Fig. 4. Progressive hippocampal increase in phospho-ERK 1/2 after EC lesion. Western-blot analysis of basal content of ERK 1/2 (p44/p42-MAPK) (A) and activated phospho ERK2 (phospho-p42-MAPK) in an enriched fraction of DG after 3, 6, 12, and 24 h of KA injection into EC. While total content of ERK 1/2 did not change over time, a significant increase in the activated form of ERK2 is achieved since 3 h after EC lesion. Representative Western blot and densitometric analysis of 6-7 independent experiments. Bars represent means ± SEMs (*p< 0.05 relative to control).

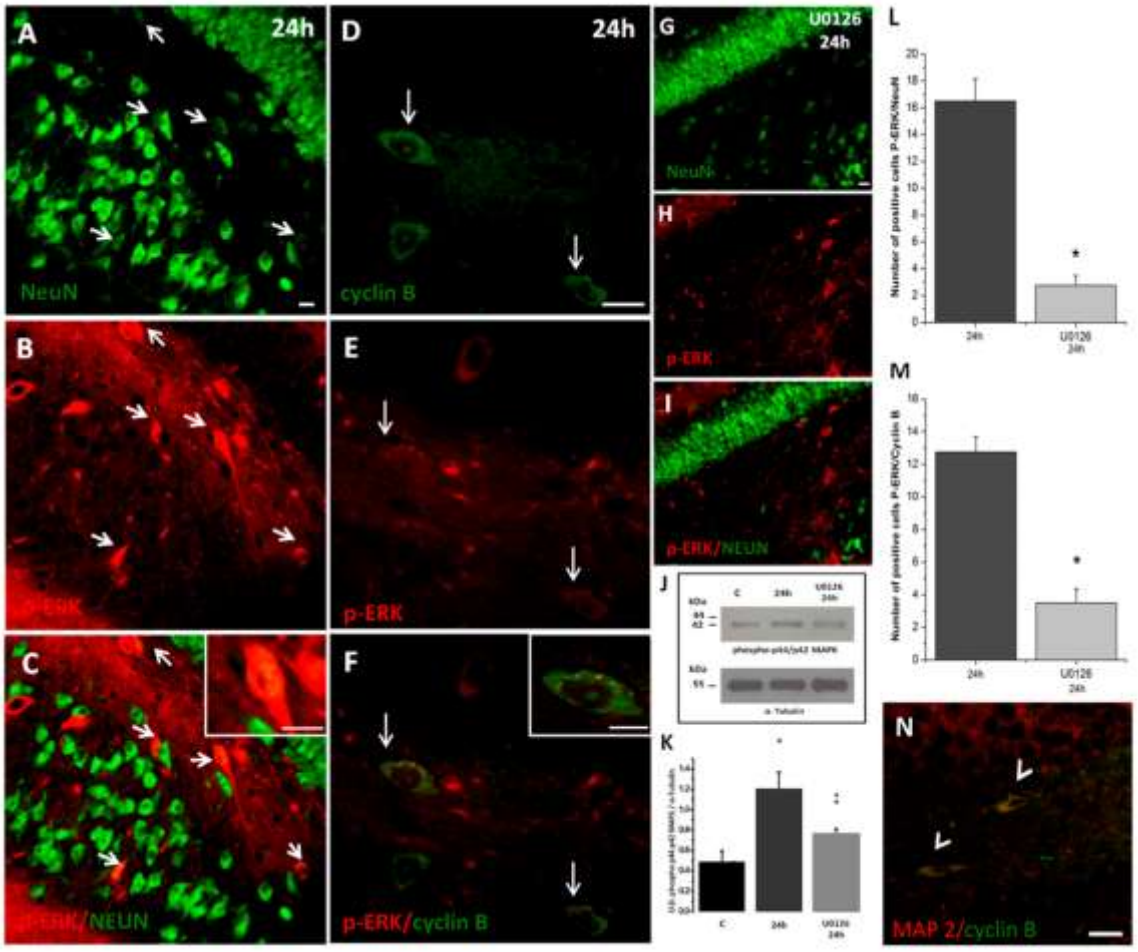

Fig. 5. ERK activation and cyclin B expression in mature neurons are reduced by MEK inhibition. 24 h after EC lesion hippocampal sections were stained with phospho-ERK1/2 antibody (red, B and C) and NeuN antibody (green, A and C). Confocal microscopy images correspond to a single optical section. Arrows denote regions of co-localization of activated ERK and NeuN-positive mature neurons (C) and high magnification (inserts). U0126 administration (H-I) reduced the expression of phospho-ERK in DG NeuN-positive neurons (G and H) (quantification depicted in L). Data represent the mean ± SEM of 4 sections for 4 independent experiments (*p< 0.05, compared with KA after 24). Similarly, the densitometric analysis from Western blot of 5 independent experiments revealed the presence of activated phospho-ERK1/2 after KA and the inhibition of this activation after U0126 administration (J-K). Results are mean ± SEM (*p< 0.05, relative to control and &p< 0.05 relative to the effect of KA after 24h). Cyclin B immunostaining (D and F) colocalizes with activated ERK1/2 (E-F) in some neurons (arrows head in D-F,

magnification see inserts). Micrographs are representative of 4 independent experiments. Scale bar = 20 µm and 10 µm (magnifications). The number of positive cells in the DG that expressed both markers after EC lesion and following U0126 administration is depicted (M) and represent the mean ± SEM of 4 sections for 4 independent experiments (*p< 0.05, compared with 24h after KA).

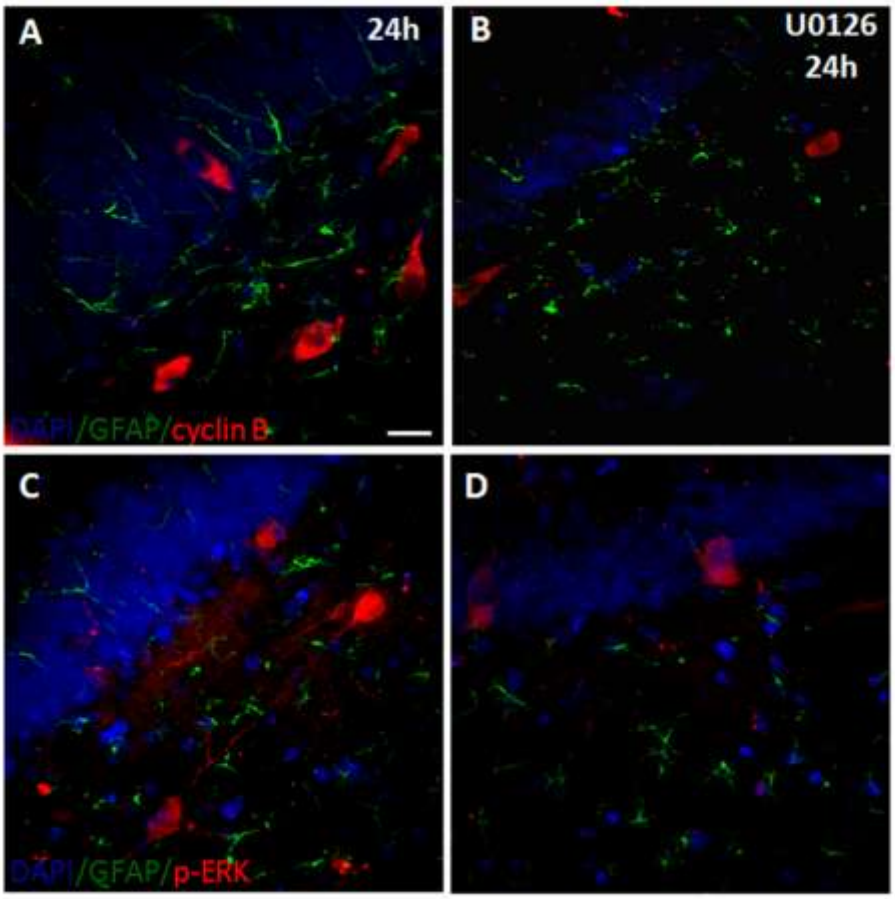

Figure 6

Fig. 6. Double immunofluorescence for GFAP and phospho-ERK1/2 and cyclin B. 24 h after EC lesion hippocampal sections were stained with antibodies against cyclin B (red, A and C) and GFAP (green, B and C) or phospho-ERK1/2 antibody (red, G and I) and GFAP antibody (green, H and I). Note that not colabeling is observed for both markers and GFAP-positive glial cells. The MEK inhibitor, U0126 markedly reduced the number of cyclin B-positive cells (C and I, high magnification in J and L, respectively). Confocal microscopy images correspond to a single optical section. Micrographs are representative of 4 independent experiments. Scale bar = 20 µm.

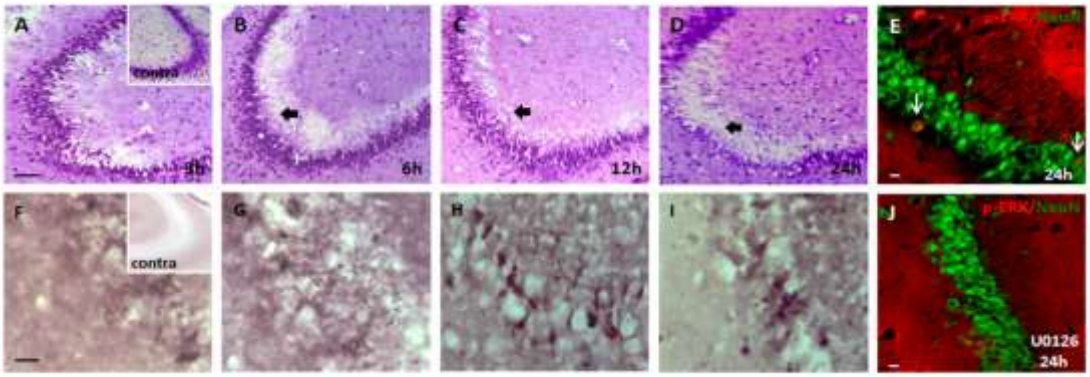

Fig 1 supl

Fig. S. 1. Effects of EC lesion in the morphology of hippocampal CA3 neurons. Time course of neuronal damage in the CA3 region (A-D) after EC lesion. Representative micrographs of cresyl violet staining showing the progressive cellular death in CA3 (dark arrows) ipsilateral to site of KA injection as compared with the contralateral site (A, insert). Scale bar = 100 µm. EC lesion induces the expression of phospho-ERK in hippocampal CA3 region at different times. Scarcely phospho-ERK inmunoreactivity in CA3 contralateral to site of KA injection is detected (F, insert). In the ipsilateral CA3, activated ERK 1/2 was clearly observed in neuronal somata and neurites after 3h (F), 6h (G), 12h (H), and 24h (I) of EC lesion. Administration of the MEK inhibitor, U0126 substantially reduced the expression of phospho-ERK in neurons and fibers of CA3 region (J) compared with the effect observed 24 h after EC lesion without the inhibitor (E). Micrographs are representative of 5-6 independent experiments. Scale bar = 20 µm and 10 µm (E and J).

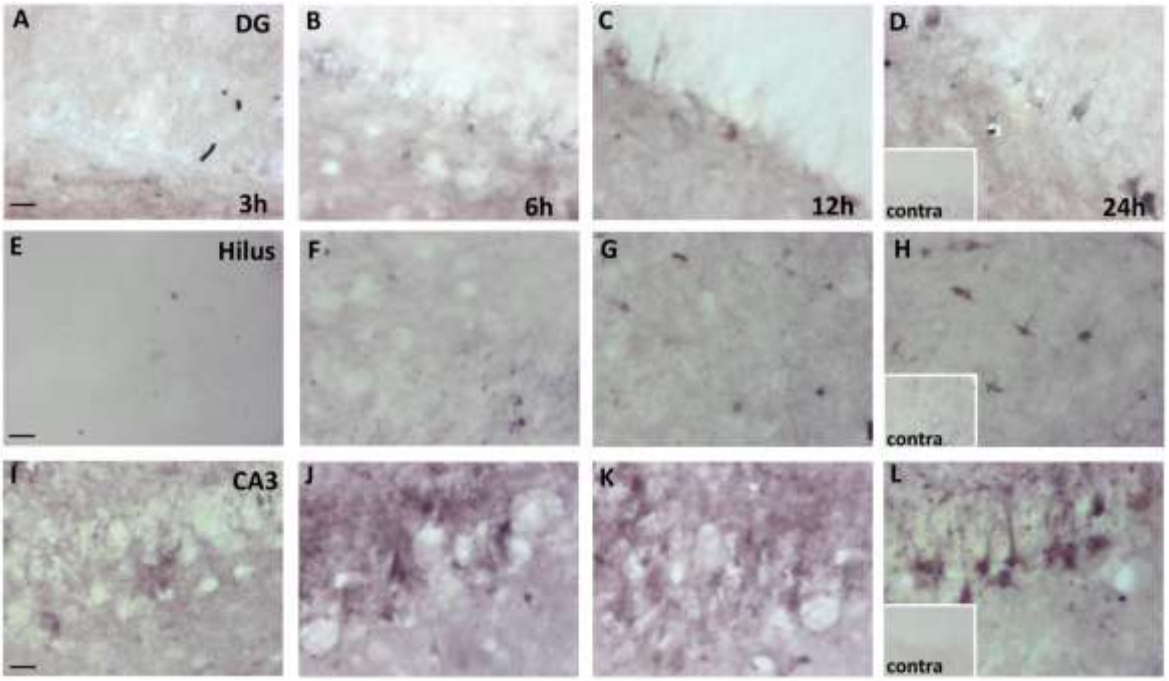

Fig. S. 2. EC lesion induces cyclin D1 expression in the hippocampus. Representative micrographs showing the expression over time of cyclin D1-positive cells in hippocampus after 3, 6, 12 and 24h of KA injection. A progressive increase of cyclin D1 is observed in DG (A-D), hilus (E-H) and CA3 (I-L). Inserts show the absence of this cell cycle marker in the contralateral regions. Micrographs are representative of 4-5 independent experiments. Scale bar = 20 µm.

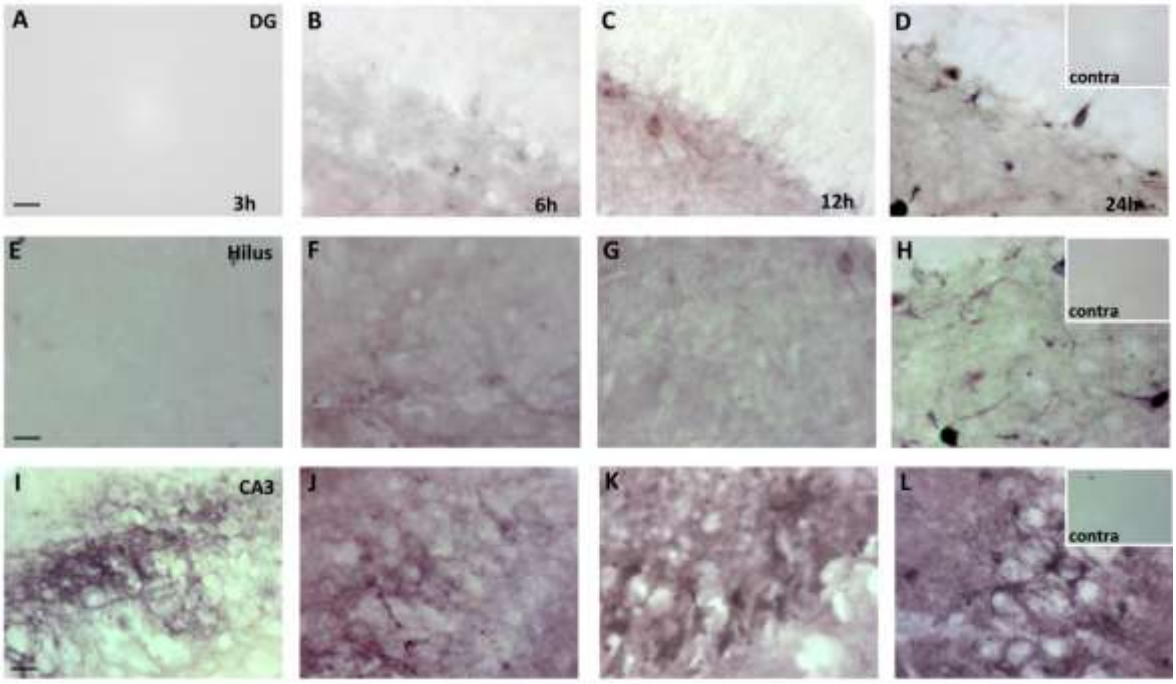

Fig. S. 3. Cyclin B expression is induced in the hippocampus after the EC lesion. Micrographs of representative experiments showing expression over time of cyclin B-positive neurons 3, 6, 12 and 24h after KA injection. A progressive increase of cyclin B immunostaning is observed in DG (A-D), hilus (E-H) and CA3 (I-L). Inserts show the content of this cell cycle marker in the contralateral regions. Micrographs are representative of 4-5 independent experiments. Scale bar = 20 µm.

IX.2. RESULTADOS ADICIONALES

Los resultados de los experimentos referentes al efecto de la administración del inhibidor de MEK 1/2 sobre la expresión de la ciclina D en el GD no están incluidos en el artículo, por lo que se presentan en la siguiente sección.

IX.2.a.EFECTO DE LA ADMINISTRACIÓN DEL INHIBIDOR DE MEK1/2 SOBRE LA EXPRESIÓN DE CICLINA D1 EN EL ÁREA DESAFERENTADA POR LA LESIÓN EN LA CORTEZA ENTORRINAL

Con el objetivo de determinar si existía una relación causal entre la activación de ERK1 /2 y la inducción de los marcadores de Ciclina D1 en

el hipocampo después de la lesión en la CE, se infundió un inhibidor de MEK1/2, el U0126, en el ventrículo lateral. 20min antes de la inyección de AK en la CE, se administraron i.c.v. 2 µg de U0126 (Fig. 5).

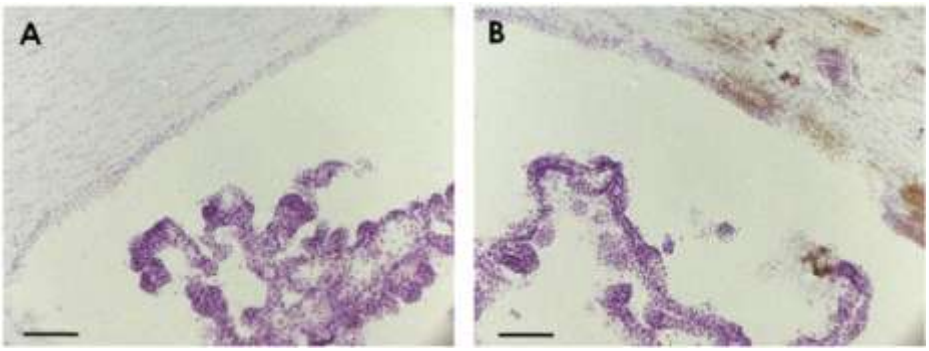

Fig. 5 . Tinción de Nissl en secciones coronales del ventrículo lateral obtenidas 24h posterior a la administración del U0126. Microfotografía del sitio de inyección del inhibidor de MEK 1/2 (B). Ventrículo lateral contralateral a la administración de U0126 y AK (A). Barra de escala: 100µm.

La administración i.c.v de U0126 redujo significativamente la inmunotinción para Ciclina D1 en la región del GD (Fig. 6 B), incluyendo a la capa granular, al hilus, y a CA3 (Fig. 6C) regiones donde se induce mayormente la ciclina D después de la lesión en la CE (Fig. 6A, C). 24h después del daño en la CE, el U0126 disminuyó el número de células positivas a ciclina D en el GD de (21±0.65) a (7.5±0.86). Estos datos, junto con los relativos a la reducción en la inmunoreactividad neuronal para Ciclina B inducida por el U0126 (presentados en el artículo anterior),

respaldan nuestro hipótesis acerca de la participación de fosfo-ERK1/2 en la activación del ciclo celular neuronal inducida por la pérdida de conectividad con la CE.

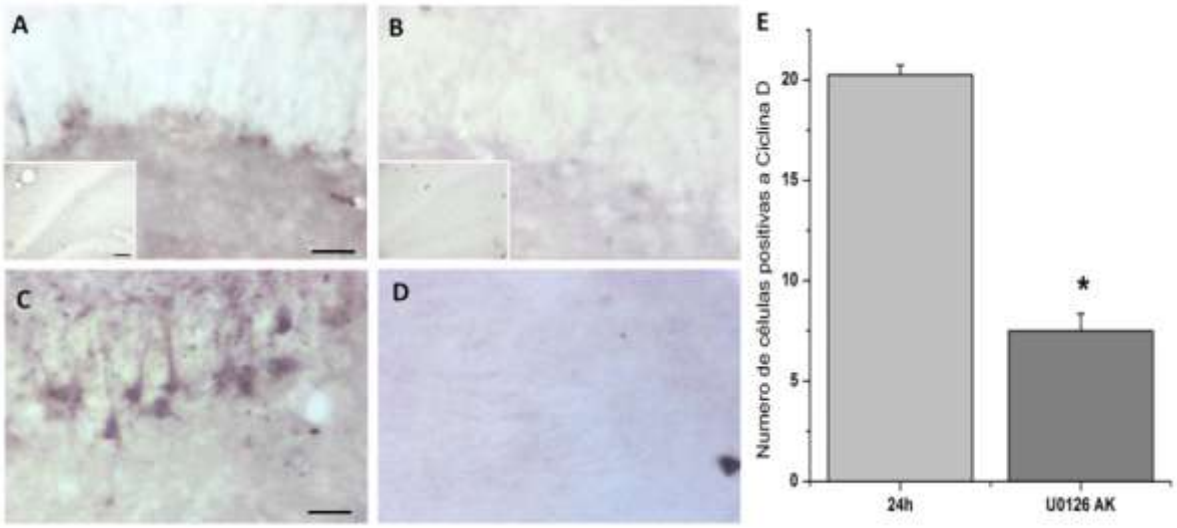

Fig.6. Efecto de la administración i.c.v. del inhibidor de MEK 1/2 en la expresión de Ciclina D1 en el hipocampo 24h después de la inyección de AK. Inmunohistoquímica para Ciclina D1 en secciones de GD (A, B) y CA3 (C, D) correspondientes a ratas tratadas con (B, D) y sin U0126 (A, C). El inhibidor de MEK 1/ 2, U0126, redujo el número de células positivas a Ciclina D en el hipocampo (E). Los insertos en A y D muestran al GD contralateral correspondiente. Las micrografías son representativas de 4 experimentos independientes. Barras de escala: 100µm y 20µm.

X. DISCUSIÓN

En este trabajo utilizamos un modelo de pérdida de aferentes provenientes de la vía perforante hacia el GD en el hipocampo. Describimos la expresión de las ciclina D_1 y ciclina B, proteínas involucradas en la progresión del ciclo celular, asociada a la inducción y activación de ERK1/ 2 en neuronas hipocampales que perdieron proyecciones sinápticas

de la CE. Así mismo, demostramos que la pérdida parcial de aferentes originadas en la CE, es capaz de promover la reactivación del ciclo celular en neuronas maduras del hipocampo.

La lesión en la CE se ha utilizado ampliamente como modelo de desaferentación del hipocampo, el cual permite explorar los mecanismos de neuroplasticidad activados durante la reorganización sináptica secundaria a una lesión cerebral (Bechmann y Nitsch, 2000; Deller y col., 1996; 2007; Fagan y Gage, 1994). La CE y el hipocampo son componentes esenciales del sistema de memoria del lóbulo temporal medial. La CE integra la información proveniente de cortezas sensoriales primarias y cortezas de asociación. Dicha información es transferida hacia el hipocampo, en específico al GD y CA3, a través de la vía perforante (Amaral y col., 1987; Dolorfo y Amaral., 1998; Germroth y col., 1989). Diversos reportes coinciden en que este circuito neuronal es considerablemente vulnerable a lo largo del envejecimiento, tanto exitoso como patológico (Chao y col., 2010; Devanand y col., 2007; Stoub y col., 2011). Además, con relación a la observación de que la pérdida de memoria es un signo primario en la EA, diversos estudios histopatológicos han señalado a este circuito como el sitio inicial en el cual se presentan las alteraciones neurodegenerativas típicas de esta enfermedad (Braak y Braak, 1991; 1995; deToledo-Morrell y col., 2004; Devanand y col., 2007). Incluso, algunos estudios en cerebros de pacientes con EA en etapas tempranas, han reportado la pérdida de contactos sinápticos en el GD (Dekosky y col., 1996; Scheff y Price, 2006), la cual correlaciona con una pérdida neuronas de la capa II de la CE (Gómez-Isla y col., 1996; Price y col., 2001).

En la EA, la reactivación del ciclo celular puede contribuir a la cascada neurodegenerativa, incluyendo la formación de βA y

fosforilación de Tau (Susuki y col., 1997; Vicent y col., 1997). En este sentido, la activación del ciclo celular inducida por la expresión del antígeno T largo del virus 40 de simio indujo una patología tipo Alzheimer en neuronas postmitóticas del hipocampo de ratones (Park y col., 1997). Sin embargo, hasta el momento no se han determinado los factores que pueden conducir a la pérdida del control de la diferenciación neuronal e inducir la activación de la maquinaria del ciclo celular. Los datos acerca de que la expresión de proteínas reguladoras del ciclo celular ocurre en las regiones del hipocampo susceptibles a pérdida sináptica (Dekosky y col., 1996; Scheff y Price, 2006), aunado a la hipótesis de que las neuronas podrían convertir señales derivadas de la reorganización sináptica en señales promotoras de la activación del ciclo celular (Arendt, 2000; 2003; 2012) nos permitieron plantear la hipótesis de trabajo. Esta se refiere a que la pérdida de conectividad sináptica dentro del circuito de la vía perforante podría promover la pérdida del control de la diferenciación neuronal de la región blanco. En un trabajo previo, reportamos la expresión de la proteína precursora del amiloide (PPA) y de Tau hiperfosforilada asociada a la activación del ciclo celular en neuronas de la CE que perdieron la conectividad con el hipocampo (Hernández-Ortega y col., 2007). En el presente trabajo, encontramos que la disminución en las proyecciones aferentes originada por una lesión excitotóxica en la CE y evidenciada por la reducción en el contenido de sinaptofisina en el GD, conduce a la fosforilación de ERK 1/2 y a la expresión de marcadores de ciclo celular en neuronas hipocampales del GD y de CA3. La inducción de Ciclina D1 y Ciclina B alcanzó sus niveles más altos 24h posteriores a la lesión en la CE, de manera sucesiva a la expresión temprana de fosfo-ERK, observada desde las 6 primeras horas posteriores de la lesión. Cabe señalar que las regiones de mayor expresión de estas proteínas, el GD y CA3, corresponden a aquellos sitios inervados

por las neuronas de las capas II y III de la CE (Germoth y col., 1989; Dolorfo y Amaral, 1998), que fueron lesionadas por el AK.

Dado que las aferentes excitadoras originadas en la CE pueden regular la neurogénesis hipocampal (Stone y col., 2011), existe la posibilidad de que aquellas células que expresan los marcadores de ciclo celular y son positivas para fosfo-ERK sean precursores neuronales en proliferación. En este sentido, pese a que la gran mayoría de las células que presentaron inmunoreactividad para fosfo-ERK y para las proteínas de ciclo celular en el hipocampo mostraron fenotipo neuronal, fue necesario determinar su linaje celular y descartar que se tratara de neuronas nuevas localizadas en la zona subgranular del GD.

Existen diversos datos que sugieren que la neurogénesis del hipocampo puede modificarse por lesiones en la CE, sin embargo este efecto se ha observado entre los 3 y 45 días posteriores a la lesión (Gama Sosa y col., 2004). A diferencia de ese estudio, en el presente trabajo nos enfocamos en el análisis de la expresión de reguladores del ciclo celular dentro de las 24h posteriores a la lesión. Por otro lado, los resultados derivados del análisis de las inmunofluorescencias dobles para fosfo-ERK y NeuN, un marcador nuclear de neuronas post-mitóticas, mostraron que una gran cantidad de células positivas a fosfo-ERK son neuronas maduras. De manera similar, encontramos que las células inmunoreactivas para Ciclina B colocalizan con el marcador neuronal MAP2, una proteína asociada a lo microtúbulos presente en neuronas maduras. En concordancia con los datos anteriores, la mayor parte de células que expresan Ciclina B o fosfo-ERK en el hipocampo ipsilateral a la inyección de AK no fueron positivas a GFAP, proteína altamente presente en astrocitos. Queda, sin embargo, por descartar la participación de microglía como un componente celular que al activarse y proliferar podría

representar algún pequeño componente en la expresión de los marcadores estudiados.

De acuerdo a diversas evidencias, dependiendo de la duración e intensidad de la activación de ERK 1/2, esta puede promover muerte neuronal. Por un lado, se ha reportado que la activación sostenida de ERK 1/2 está involucrada en la muerte neuronal inducida por exposición continua a glutamato (Jiang y col., 2000; Hidalgo y Núñez, 2007) y a estrés oxidante (Luo y DeFranco, 2006). Por el contrario, la activación transitoria de ERK 1/2 generada por la presencia de factores tróficos puede tener un papel protector (Almeida y col., 2005; Subramaniam y col., 2005). En este estudio, la desaferentación del hipocampo indujo una activación sostenida de ERK 1/2, sugiriendo su posible participación dentro de las señales que conducen a la disfunción neuronal y por último quizá a la muerte neuronal. Por otro lado, los datos consistentes sobre la participación de ERK 1/2 en el regulación de la proliferación celular (Lavoie y col., 1996; Subramaniam y Unsicker, 2010; Carganello y Roux 2011); aunados al reporte sobre la duración de la activación de ERK 1/2, en el que se determinó que para la progresión a fase S, es necesaria la activación sostenida de ERK hasta la fase G1 tardía (Yamamoto y col., 2006), nos permiten proponer que la pérdida de contactos sinápticos contribuye a la desregulación del ciclo celular dependiente de la activación de ERK en las neuronas maduras. Sin embargo, no excluimos la posibilidad de que otras vías que regulan la proliferación celular y también modulan la plasticidad sináptica como, la señalización mediada por integrinas-FAK-PI3K y Wnt-β-catenina (Patapoutian y Reichardt 2000; Schwartz y Assoian, 2001; para revisión Arendt, 2003) puedan contribuir a la activación del ciclo celular en neuronas afectadas por la pérdida sináptica.

Por el momento, no hemos definido qué señales moleculares se generan en el hipocampo desaferentado y conducen a la fosforilación de ERK 1/2. Así mismo, es necesario explorar los mecanismos que determinan la duración de la activación de ERK 1/2. En este sentido, se sabe que una variedad de factores tróficos y citocinas son capaces de activar a la vía de ERK 1/2. Entre las alteraciones aquí descritas ocasionadas por la lesión en la CE, resalta la rápida activación de células gliales en el hipocampo desaferentado, las cuales pueden producir algunas citocinas. Asimismo, se ha reportado que S100B, una proteína producida abundantemente por astrocitos reactivos, pueden actuar como señal mitogénica, activar a ERK 1/2 y conducir a la activación del ciclo celular en células neuro2 (Schmidt y col., 2007). Por otro lado, es necesario considerar la contribución potencial de otros inductores de la activación de ERK, entre los que se encuentra la liberación masiva de neurotransmisores excitadores y la generación de radicales libres en el hipocampo desaferentado (Hidalgo y Nuñez, 2007).

Los datos de la reducción en la fosforilación de ERK 1/2 y la consecuente disminución en la expresión de los marcadores de ciclo celular derivados de la inhibición farmacológica de MEK 1/2, sugieren una relación causal entre la activación de ERK 1/2 y la reentrada al ciclo celular, a la vez que demuestran la activación de MEK/ ERK en hipocampo posterior a la pérdida de proyecciones aferentes. En este sentido, se ha asociado a la vía Ras-ERK con la neurodegeneración encontrada en varias patologías, en particular en la EA. En cerebros de pacientes con EA en etapas tempranas, se ha descrito la abundante expresión diversos elementos de esta vía de señalización, como Ras, Raf-1, MEK y ERK (Arendt y col., 1995, Gärtner y col., 1995). Además, se sabe que ERK es capaz de fosforilar a la proteína Tau en diversos residuos de Ser/Thr asociados con la formación de los filamentos helicoidales apareados

(Drewes y col., 1992; Goedert y col., 1992). En concordancia con este dato, se ha reportado que la inmunoreactividad para fosfo-ERK 1/2 coexiste con la presencia de neurodegeneración neurofibrilar en neuronas hipocampales (Ferrer y col., 2001; Ginsberg y col., 1999; Pei y col., 2002; Perry y col., 1999). Por último, la expresión de ERK1/2 se ha encontrado asociada con depósitos de βA (Medina et al. 2005). No obstante, cabe señalar que MEK 1/2 puede ser activada directamente por FAK (cinasa de adhesiones focales) y PAK (cinasa activada por p21) componentes de la señalización mediada por integrinas (Schwartz y Assoian, 2001).

En resumen, la reactivación del ciclo celular en neuronas maduras puede ser un evento importante de la neurodegeneración observada en la EA. El modelo utilizado en este trabajo, permite analizar la cascada de eventos generados por la pérdida de conexiones sinápticas entre el hipocampo y la CE, y que conducen a la expresión anormal de proteínas del ciclo celular en neuronas maduras. Dicho proceso, como lo demostramos en este estudio, depende al menos en parte de la activación de la cinasa ERK 1/2, lo que puede plantear la búsqueda de estrategias farmacológicas para limitar el daño causado por la secuencia de eventos patológicos descritos.

XI. CONCLUSIONES

- La inyección de AK indujo una amplia muerte celular en la CE. Esta lesión, tuvo efectos en sus zonas de proyección. En el GD se observó una escasa degeneración celular mientras que en CA3 se presentó una muerte celular significativa.
- La lesión de la CE disminuyó el contenido de sinaptofisina en el GD, sugiriendo una reducción en las proyecciones sinápticas de la CE.

Conjuntamente, se observó una rápida activación glial en el hipocampo ipsilateral a la lesión.

- La desaferentación de hipocampo promovió la fosforilación de ERK 1/2 (Thr 202/185 y Tyr204/187, respectivamente), sugiriendo su presencia en estado activo. Además, se indujo la expresión de ciclina D_1 y ciclina B, indicativo de la reactivación del ciclo celular en neuronas maduras conectadas al sitio de la lesión.
- La administración i.c.v. de U0126, inhibidor de MEK1/2, previa a la inyección de AK, redujo la activación de ERK 1/2 en neuronas hipocampales inducida por la lesión en la CE. Así mismo, el U0126 disminuyó la cantidad de células positivas a Ciclina D_1 y Ciclina B en el hipocampo ipsilateral a la lesión.

De manera general, la pérdida de conectividad sináptica entre la CE y el hipocampo es capaz de activar a ERK 1/2 y promover la expresión de marcadores de ciclo celular en neuronas maduras del hipocampo. Estos resultados apoyan la hipótesis de que ERK 1/2, participa en la reactivación del ciclo celular en neuronas en las que ocurrió pérdida sináptica.

X.II ASPECTOS POSIBLES A DESARROLLAR

A partir del análisis de los resultados obtenidos en el presente trabajo, en la siguiente sección se muestran algunos aspectos relevantes por investigar. Estos permitirían profundizar en el conocimiento de los procesos que acompañan la pérdida de conectividad entre dos regiones cerebrales.

- Considerando la gran activación glial en el hipocampo ipsilateral a la lesión, una posibilidad es que la proteína S100B derivadas de astrocitos activos promueva la expresión de marcadores de ciclo celular. Se sabe que S100B es capaz de interactuar con los receptores de productos avanzados de glicosilación (RAGE), activar ERK y conducir a la expresión de la Ciclina D (Schmidt, 2007). En este sentido, es necesario determinar la cascada de señalización previa a la activación de MEK» ERK en el hipocampo inducida por la lesión en la CE. Sí bien la cinasa Raf es el activador típico de MEK, como ya se mencionó existen otras cinasas capaces de activar a MEK. Otro activador potencial de ERK es la señalización inducida por la interleucina-1β (IL-1β) producidas por la microglía activada (Saud y col, 2005). Un aspecto por evaluar, es la posibilidad de que la pérdida de conectividad con la CE induzca la activación de la microglía en el hipocampo, la cual a su vez, podría promover la reexpresión de proteínas reguladoras del ciclo celular.

- Con el propósito de determinar si las células que expresan a ERK en su forma activa y a los marcadores de ciclo celular son neuronas maduras, es preciso realizar el triple marcado inmunohistoquímico correspondiente. Adicionalmente, el análisis por inmunofluorescencia para los marcadores BrdU/Doblecortina revelaría si la lesión en la CE afecta la tasa de neurogénesis hipocampal y contribuye a la expresión de marcadores de ciclo celular observada después de la lesión en la CE.

- En el planteamiento de nuestro proyecto se mencionó la participación de algunas proteínas reguladoras del ciclo celular en la generación de Tau hiperfosforilada y Aβ, marcadores histopatológicos típicos de la EA. Por lo anterior, es necesario

analizar si en este modelo de pérdida de conectividad estos marcadores se presentan. Así mismo, es preciso determinar si la inhibición de la activación de ERK afecta la expresión de Tau hiperfosforilada y Aβ.

- Un aspecto relevante por determinar es el destino de las neuronas hipocampales que expresan fosfo-ERK y los marcadores de ciclo celular. Es decir, evaluar si ésta reexpresión de marcadores de ciclo celular conduce a las neuronas finalmente a la muerte y a su vez determinar qué tipo de muerte celular ocurre.

- Debido a que la pérdida de memoria es un síntoma inicial y prominente en la EA, diversos estudios histopatológicos han descrito alteraciones tempranas en la formación hipocampal (Hyman, 1984; Braak, 1997; Van Hoesen, 1991). Hasta el momento, en este modelo no se han evaluado las alteraciones funcionales derivadas de la pérdida de conectividad entre la CE y el hipocampo. El análisis de la actividad neuronal mediante parámetros electrofisiológicos y la evaluación de tareas conductuales dependientes del hipocampo, contribuiría a la caracterización integral de nuestro modelo de pérdida de conectividad. Además, proporcionaría un mayor sustento a nuestro modelo como una herramienta útil en el estudio de los procesos implicados en la neurodegeneración que ocurre en la EA.

- El presente modelo de pérdida de comunicación entre dos áreas de la formación hipocampal se basa en una lesión excitotóxica que induce neurodegeneración en la CE y la consecuente reducción en la conectividad con el hipocampo. El planteamiento de un modelo específico de daño sináptico en el hipocampo reduciría los efectos

secundarios generados por la muerte excitotóxica inducida por el AK. Considerando la abundante presencia de la molécula de adhesión NCAM en su forma polisializada (PSA-NCAM) en las sinápsis del hipocampo (Benson y col, 2000), es posible utilizar una microinyección de la enzima endoneuroaminidasa para remover el ácido polisiálico (PSA) de la NCAM (Fedorkova y col, 2002) y así desestabilizar las uniones sinápticas de una región específica del hipocampo.

X.III. REFERENCIAS

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X.III APÉNDICE

X.III.1. Artículo (2): Sequential expression of cell-cycle regulators and Alzheimer's disease–related proteins in entorhinal cortex after hippocampal excitotoxic Damage. Karina Hernández-Ortega, Patricia Ferrera, and Clorinda Arias. Journal of Neuroscience Research 85:1744–1751 (2007).