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Title of Doctoral Thesis:

# Role of brain enriched protein kinases in hippocampal synaptic plasticity and learning and memory

Dissertation for obtaining a doctorate degree at the University of Natural Resources and Applied Life Sciences Vienna

Submitted By

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**Vienna, April 2013**

# I. Acknowledgments

I would like to thank Prof. Gert Lubec, who excellently supervised me during this work. He continuously supported me in all aspects and trusted my ability to work on this project that was performed in a collaborating laboratory.
I also thank Dr. Francisco J. Monje and Dr. Daniella Pollak for the fruitful collaboration and advice and for allowing me to work in their laboratory.
The results of my thesis have been published in a total of 2 papers as a partial requirement for this thesis. These papers are attached as Appendix 1 and 2.
Most of the work for my papers has been performed in the Department of Neurophysiology and Neuropharmacology, Center for Physiology and Pharmacology, Medical University of Vienna.

In paper one, I have performed the morphological work including optimizing the condition of transfection experiments and immunocytochemistry work and I have done behavior experiments and partially participated in electrophysiology work.
Francisco J. Monje performed the electrophysiology experiments. Maureen Cabatic performed the Western blots supervised by Daniella D.Pollak. Arthur Baston partially participated in recording mEPSC. Francisco J. Monje and I analyzed the data and Gert Lubec supervised the whole project and gave advice. Lin Li partially gave us conceptual advice. Francisco J. Monje and Daniella D.Pollak wrote the paper Francisco J. Monje and Gert Lubec designed the experiments.
In paper two, I have performed all the experiments except RNA work and analyzed the data supervised by Francisco J. Monje. Francisco J. Monje and I wrote the paper. Francisco J. Monje interpreted data. Daniella D. Pollak gave advice on experiments of Western blots and RNA work. Harald Höger gave advice on animal work. Gert Lubec supervised the whole project and gave advice.

I appreciate valuable advice and priceless guidance by Dr. Yang Jae Woon especially, when I was in desperate situations. Furthermore, I thank my colleagues in the laboratory, especially, Dr. Sung Ung Kang, Seok Heo, Nark Hyun Bae, Gang Soo Jung, Sunetra Sase, Ajinkya Sase, Maryam Ghafari and Soheil Keihan Falsafi for encouragement and for generating a pleasant work environment. I wish you all have good fortune and happiness.

Lastly, I dedicate this work to my family. I deeply appreciate the support provided by my parents, my brother and sister who were always at my side with encouragement, understanding, support and love. In particular, I sincerely express my gratitude to my precious husband, Ju Hwa Lee.

# Table of Contents



**Appendix 1**

Article: Focal Adhesion Kinase Regulates Neuronal Growth, Synaptic plasticity and Hippocampus-Dependent Spatial Learning and Memory

*Francisco J.Monje*, Eun-Jung Kim*, Daniela D.Pollak, Maureen Cabatic, Lin Li, Arthur Baston, Gert Lubec, Neurosignals, 2012, 20(1):1-14*

**Appendix 2**

Article: Alzheimer's disease risk factor lymphocyte-specific protein tyrosine kinase regulates long-term synaptic strengthening, spatial learning and memory

*Eun-Jung Kim*, Francisco J. Monje*, Lin Li, Harald Höger, Daniela D. Pollak, Gert Lubec, Cellular and Molecular Life Sciences, 2012, Feb;70(4):743-59.*

# I. Introduction

## 1. Protein kinases

Protein kinases are enzymes that modify other proteins by chemically adding phosphate groups to them. This process is called phosphorylation. Protein kinases are involved in most signal transduction processes by modification of substrate activity and control many other cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis and differentiation. [1]

Protein phosphorylation also plays a critical role in intracellular communication during development, in physiological responses and in homeostasis, and in the functioning of the nervous and immune systems.

Protein kinases are among the largest families of genes in eukaryotes [1-4] and have been intensively studied. Specially, in neuroscience, a crucial role for cytoplasmic protein kinases in neuronal plasticity was first recognized in studies of Aplysia, where a form of 5-hydroxytryptamine (serotonin)-induced synaptic plasticity that underlies a simple form of learning was shown to require activation of protein kinase A (PKA) [5].

In a series of studies, Kandel and colleagues have identified critical targets for PKA that include a $K^+$ channel and the transcription factor “cAMP response element binding protein (CREB)”. They have shown how phosphorylation of these targets can mediate both the immediate change in synaptic strength and long lasting changes that involve the formation of new synaptic connections.

In a complementary set of studies in Drosophila, genetic screens have identified the cAMP signaling pathway as a crucial component in behavioral long-term memory. This combination of molecular, genetic, electrophysiological and behavioral studies clearly establish that the cAMP signaling pathway through PKA plays a crucial role in both synaptic and behavioral plasticity, and have encouraged similar approaches in mammals.

Wide studies in mammals have focused on neuronal activity dependent forms of synaptic plasticity, such as long-term potentiation (LTP) and a variety of learning and memory paradigms. Several protein kinases which are involved during LTP are shown in Figure 1.[6]

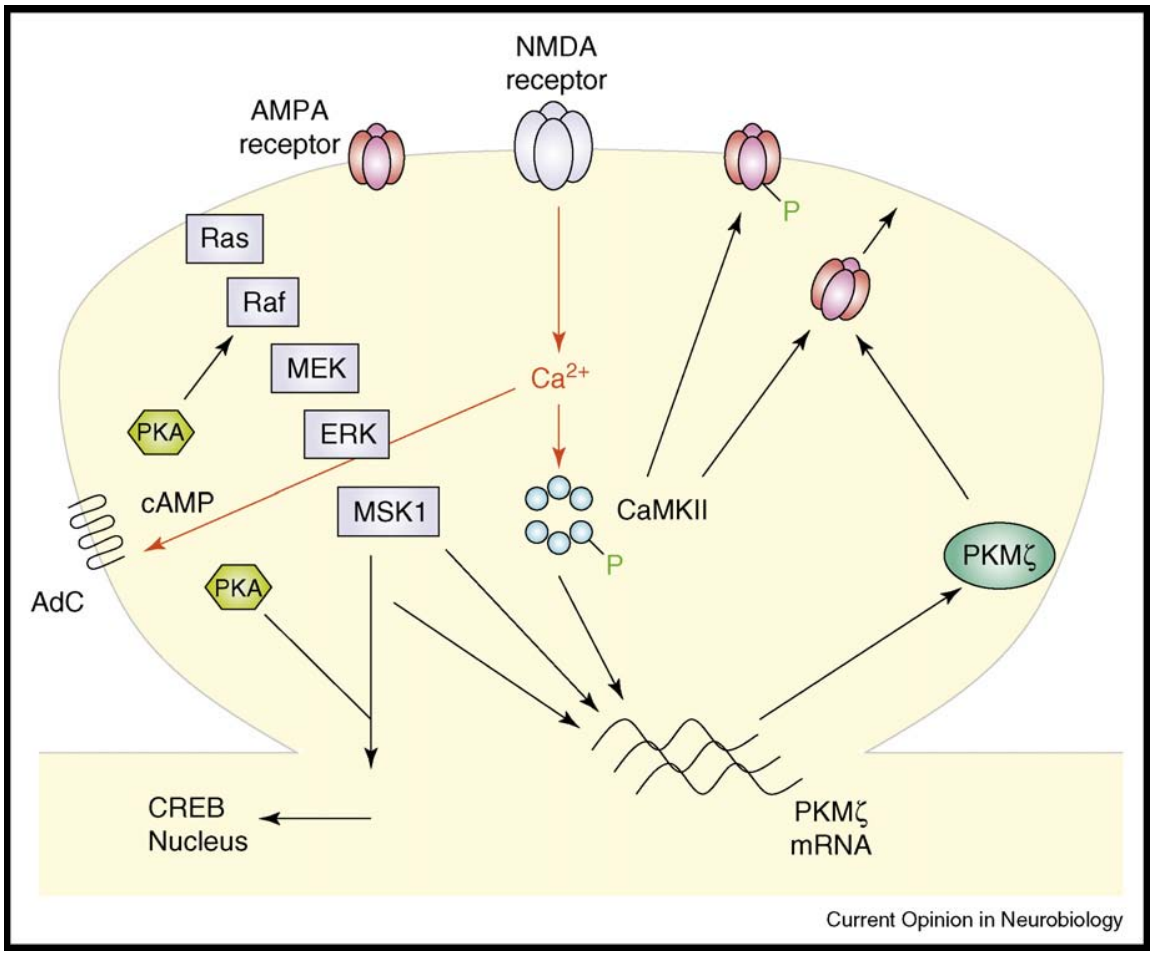


Figure 1 An involvement of Protein kinases during LTP. (Mayford M. *et al*., Curr Opin Neurobiol, 2007)

Following activation in pre-synaptic neurons, $Ca^{2+}$ ions are entering through NMDA-receptors and at least four protein kinase pathways are activated. CaMKII (Calmodulin-Dependent Protein Kinase II) phosphorylates immediately both itself and AMPA-type glutamate receptors which can cause rapid potentiation of synaptic transmission. In addition, CaMKII activation can cause the recruitment of new AMPA receptors to the synapse. The synaptic potentiation is maintained at later time points by the expression of a constitutively active form of PKC, PKM ζ. These kinases also act on the AMPA-type receptors.

The same $Ca^{2+}$ signal also activates the PKA and ERK (MAPK) pathways, by causing $Ca^{2+}$ -dependent adenylyl cyclase (AdC) to produce cAMP. One of the roles in this pathway is to stimulate transcriptional changes which are required for the ultimate stabilization of both LTP and behavioral memories via phosphorylation of CREB.

Most of these kinases involved in this signaling pathway have been tested for a role in synaptic and behavioral plasticity. Genetic or pharmacological disruption of most kinases caused altered synaptic

plasticity, or memory, or both. Although the multifunctional properties of these kinases and the wide-ranging cross-talk in the signaling pathways complicate the interpretation of the data, extensive studies are beginning to define discrete roles and mechanisms for individual kinases in the different temporal phases of both synaptic plasticity and in learning and memory.

## 1.1. Tyrosine kinases

A Tyrosine kinase is an enzyme that can transfer a phosphate group from ATP to the amino acid tyrosine on the protein. Protein tyrosine kinases are divided into two main families, receptor and non-receptor protein tyrosine kinases.

Tyrosine kinases catalyze the phosphorylation of tyrosine residues in proteins. The phosphorylation of tyrosine residues in turn causes a change in the function of the protein that they are contained in.

Phosphorylation controls a wide range of properties in proteins and functions as a switch in many cellular functions such as enzyme activity, subcellular localization, and interaction between molecules.[7]

Furthermore tyrosine kinases are involved in many signal transduction cascades wherein extracellular signals are transmitted through the cell membrane to the cytoplasm and often to the nucleus where gene expression may be modified.[7]

In neuroscience, evidence is accumulating that several protein kinases are critically involved in the production of LTP. Although protein tyrosine phosphorylation is thought to play an important role in the regulation of neuronal function, relatively little is known about the involvement of PTK in LTP induction and maintenance. The idea of the involvement of the PTK related signalling cascade in mechanisms of LTP induction was first presented by O'Dell et al. (1991) [8], who found that pharmacological inhibitors of PTK produce diminished LTP at synapses in the CA1 area of the hippocampus. In addition, gene knockout mice lacking the gene for the nonreceptor tyrosine kinase Fyn but not Src show greatly impaired LTP induction and spatial learning [9, 10].

Several studies have shown that protein tyrosine kinase inhibitors can inhibit the induction of LTP, a candidate synaptic mechanism involved in memory formation[8, 11-13] However, how protein kinases activity might contribute to LTP induction remains elusive.

## 1.2. Focal Adhesion Kinase (FAK)

Focal Adhesion Kinase is encoded by the PTK2 gene in humans. [14] FAK is a non-receptor tyrosine kinase that is widely expressed in different cell types. It is concentrated in the focal adhesions, which are multi-protein structures that link the extracellular matrix to the cytoplasmic cytoskeleton. Focal adhesions additionally include actin, filamin, vinculin, talin, paxillin and tensin.[15]

FAK was originally identified as a substrate for the oncogene protein tyrosine kinase v-src.[16]

FAK is implicated in a wide range of biological processes, including cellular adhesions, cell migration, differentiation, proliferation, tissue formation and tumor progression [17-19]

An engagement of integrins and growth factor receptors on the cell surface induces catalytic activation of FAK, and also promotes its recruitment and activation of Src kinases and other signaling proteins.

FAK is ubiquitously expressed, and is required for diverse developmental processes including neuronal path-finding and epithelial and vascular morphogenesis [20, 21]. FAK deficient mouse embryos are not viable due to a poorly developed vascular system[22, 23].

FAK has a domain structure that includes an N-terminal FERM domain, followed by a linker region, a central kinase domain (~220 residue proline rich low complexity region) and a C-terminal focal adhesion targeting (FAT) domain (Figure 2A). The key tyrosine phosphorylation sites are indicated in Figure 2A.

N-terminal FERM domain and the Kinase domain form an auto-inhibitory interaction. In the auto-inhibited state, the FERM domain (Figure 2B, blue ribbon representation) binds the kinase domain (Figure 2B, red), primarily through an interaction between the FERM F2 lobe and the kinase C-lobe. A section of the linker that contains the auto-phosphorylation site Tyr 397 (Figure 2B, yellow) is located between the FERM F1 lobe and the kinase N-lobe. The FERM domain also blocks access to the active site cleft and to the kinase activation loop (Figure 2B, green). [24] Therefore, this interaction prevents the activation of the Kinase domain, in that way, preventing the signalling functions of FAK.

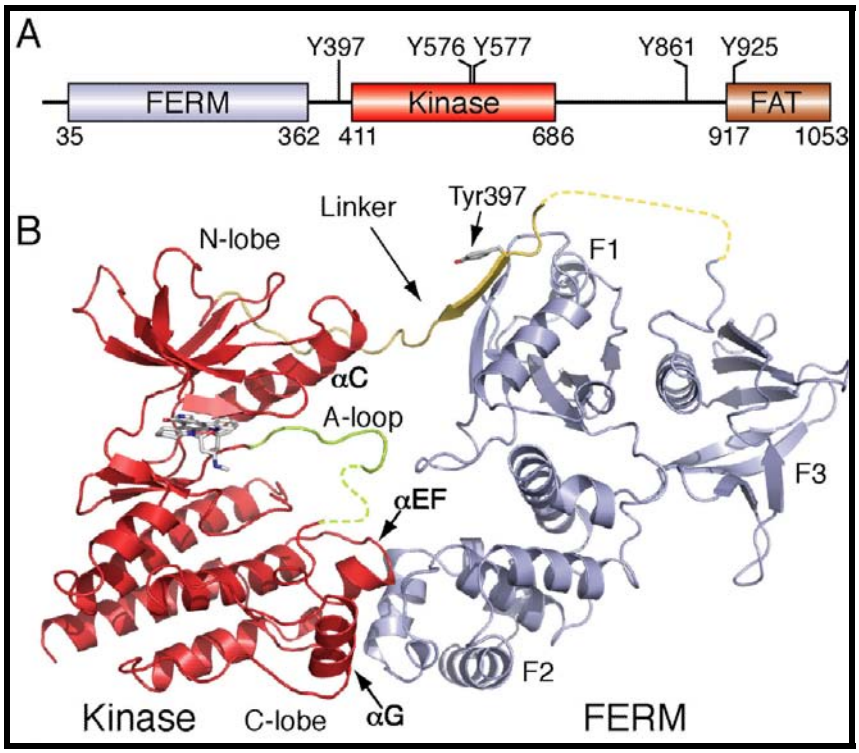


Figure 2 Structure of auto-inhibited FAK. (Lietha D. *et al.*, Cell, 2007)

In the nervous system, the focal adhesion kinase (FAK) is abundantly expressed particularly in the cortex and in the hippocampus [9, 25]. These brain regions are thought to be central importance for learning and memory.

FAK has been shown to regulate neurite outgrowth and branching in developing neurons[26-29] and to modulate synaptic plasticity, processes importantly involved in the neurobiological mechanisms underlying learning and memory[30].

However, the precise role of FAK in neuritic outgrowth, synaptic strengthening and plasticity are still unclear. Actually, conflicting data have been reported regarding its function as a regulator of neuritic outgrowth.

For example, it has been shown that cell-specific inhibition of FAK activity can enhance the number of axonal terminals and synapses and induces a greater numbers of axonal branches, indicating FAK as a negative regulator of axonal branching and synapse formation [29]. In contrast, recent work suggest that FAK promotes neuritic elongation, possibly through inhibition of ATP-gated P2X7 receptors [31] .

## 1.3. Lymphocyte-specific Cell Kinase (LCK)

Lck (or p56Lck) is a non-receptor tyrosine kinase and a member of the Src family of tyrosine kinases (SFKs). It has been shown that five members of the SFKs are expressed in the mammalian central nervous systems (CNS) which are Src, Fyn, Yes, Lyn and Lck. These kinases share a common domain structure that includes the Src homology 4 (SH4) domain, unique domain (U), SH3 domain, SH2 domain, linker region, catalytic domain (SH1 domain, N- and C-lobes) and regulatory domain. The regulation of SFK catalytic activity is mediated by intra-molecular interactions and tyrosine phosphorylation (P) or de-phosphorylation of the kinase itself.

An inactive conformation of the SFK is shown in Figure 3 (left). The SH2 domain interacts with phosphorylated Y527 in the regulatory domain, the SH3 domain interacts with a ligand in the linker region, and Y416 in the activation loop is de-phosphorylated. The inactive conformation is supported by the activity of Csk or Ctk, which phosphorylates Y527.

An active conformation of SFK is shown in Figure 3 (right). A displacement of the intra-molecular interactions by binding of SH2 and/or SH3 domain ligands and by de-phosphorylation of Y527 by protein tyrosine phosphatases (such as PTPα) leads to the active conformation. Auto-phosphorylation of Y416 results in a conformational change of the activation loop, which renders the kinase fully active. Small-molecule inhibitors (such as PP2 (4-amino-5-(4-chlorophenyl)- 7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)) bind to the ATP-binding site in the catalytic domain and block phosphoryl transfer to target proteins.

The SH4 domain is a short sequence (about 15 amino acids) at the amino (N) terminus of the protein that contains signals for lipid modifications, such as myristoylation and palmitoylation that are important for

anchoring the protein to cell membranes. [32]

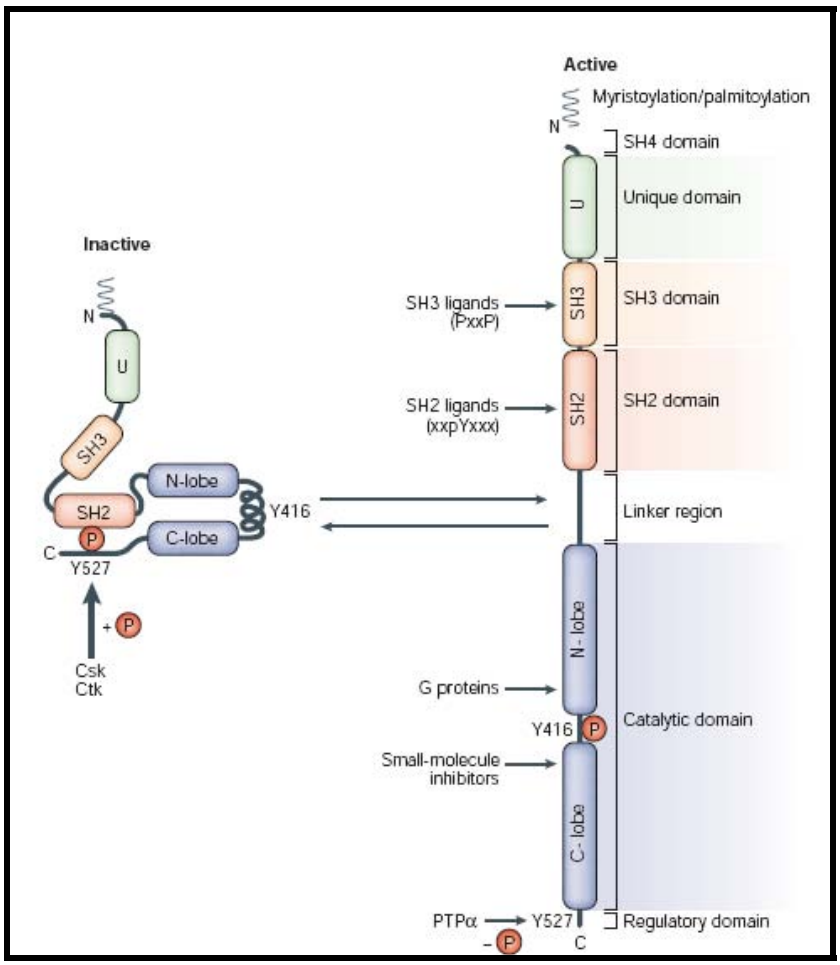


Figure 3 Structure and regulation of Src family kinases (SFKs). (Salter M.W. *et al.*, Nat Rev Neurosci, 2004)

Members of the Src family were originally studied to control cell proliferation and differentiation because Src, the prototypical member of this family, was first identified as a proto-oncogene. However, subsequently it was reported that these kinases are also expressed in differentiated, post-mitotic neurons in CNS. In the developing CNS, they are important for neuronal differentiation and neurite outgrowth [33, 34] and they have been shown to play important roles in regulating ion channel activity and synaptic transmission.[35-38]

Recent clinical reports described significantly down-regulated levels of Lck in the hippocampus of Alzheimer's disease patients [39]. Moreover, it was shown that human Lck gene locates in the Alzheimer's disease-associated genetic linkage region 1p34-36 [40] and further proposed as a novel risk-gene for Alzheimer's disease [41] suggesting a relationship of Lck with memory-related functions.

The role of Lck in memory remains mostly unexplored, although Lck was identified in the brain approximately 15 years ago [42].

## 2. Hippocampus

The hippocampus is named for its shape similar to that of the sea horse. It is a part of the cerebral cortex and contains two main interlocking parts which are Ammon's horn and the dentate gyrus. Together with the adjacent amygdala, hippocampus forms the central axis of the limbic system, which have been shown to play a critical role in all aspects of emotions, fear, learning and memory [43].

The initial insights on the role of the hippocampus came from an attempt to relieve epileptic seizures. There is a famous report by Scoville and Brenda Milner. They attempted to relieve epileptic seizures from Henry Molaison who is known as patient H.M. During surgery his hippocampus was destroyed, resulting in an unexpected postoperative outcome. Patient H.M. showed severe anterograde and partial retrograde amnesia. He was not capable to form new episodic memories after his surgery and could not remember any incident that occurred just before his surgery, but maintained memories which happened years earlier. Afterwards, extensive evidence implicated the hippocampus in the formation of episodic memories in humans [44, 45] and in consolidating information into long-term declarative memory [46].

Moreover, in Alzheimer's disease, the hippocampus is one of the first regions of the brain becoming destroyed which means that memory problems and disorientation occur at the first symptoms.

The hippocampus has different neuronal cell types which are neatly organized into layers and a uni-directional network so it has frequently been used as a good model system for studying neurophysiology. The form of synaptic plasticity known as long term potentiation (LTP) underlying long term memory was first discovered to occur in the hippocampus and has often been examined in this brain region.

A diagram of the hippocampal neural network is shown in Figure 4. Input starts from the axons of layer II neurons in Entorhinal cortex (EC) and it forms connections with dentate gyrus(DG) and CA3 pyramidal neurons via the perforant path including lateral(LPP) and medial perforant path(MPP). CA3 neurons also receive input from DG via the Mossy fibers (MF). Afterwards, this information is sent to CA1 pyramidal neurons through the Schaffer Collateral pathway (SC). CA1 pyramidal neurons send back projections into deep-layer neurons of the EC. [47]

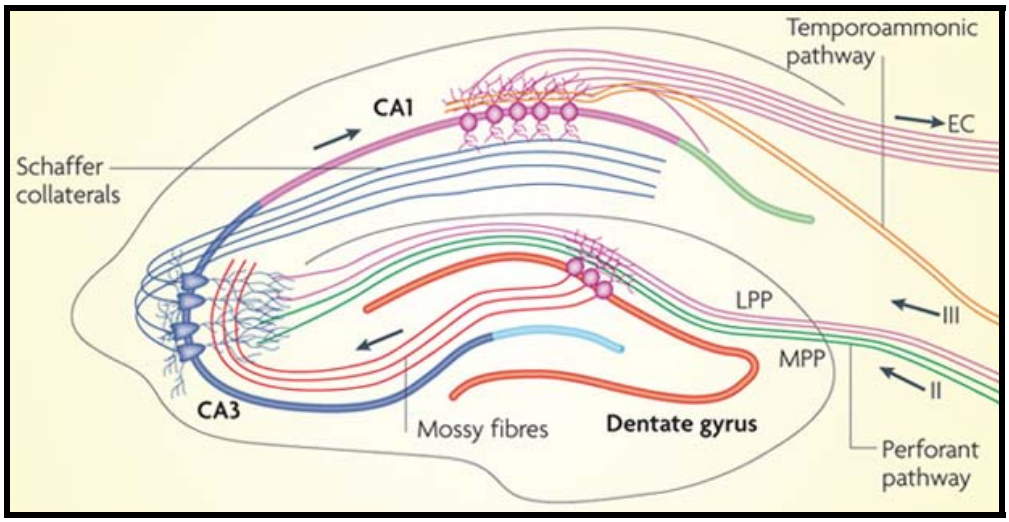


Figure 4 Diagram of the hippocampal neural network. (Deng W. *et al*., Nat Rev Neurosci., 2010)

## 2.1. Hippocampal neuronal culture

Neuronal cultures are potent and highly resourceful tools to examine molecular and cellular mechanisms in neuroscience. They have been established from different species, such as rat, mouse and chicken, and different organs, such as brain, dorsal root ganglia and retina. Furthermore, they have been established by differentiation from precursor cells, such as embryonic or neural crest stem cells [48-51]. Hippocampal neurons have become the most widely used to study and analyze dendritic spine morphology and synaptic plasticity [52-54].

An image of the hippocampal neurons is shown in Figure 5A. This primary culture is from embryo E16.5 mouse. The figure shows typical hippocampal pyramidal neurons that are stained for specific marker proteins. The axon is stained in yellow with anti-tau antibody and several dendrites are stained in red with anti-neuron specific β3 tubulin (Figure 5A). In matured neurons (four week old, 28DIV), mature spines which are synaptic compartment of dendrites can be visualized with FITC-labeled phalloidin (Figure 5B and C)

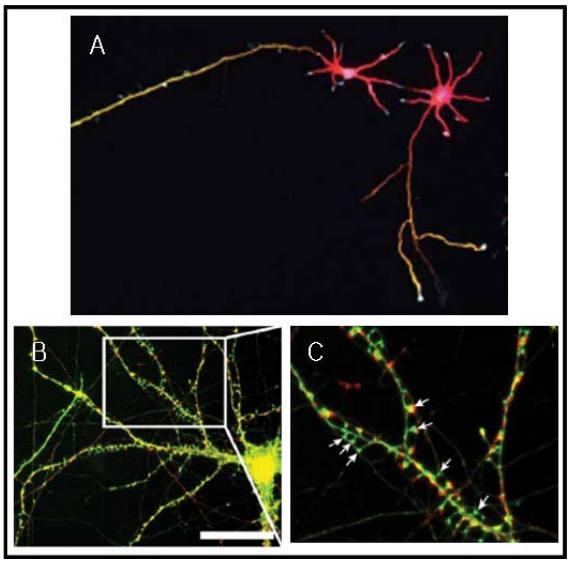


Figure 5 Primary hippocampal neurons in culture. (Fath T. *et al*., Nat Protoc. 2009)

In culture, hippocampal neuronal cells can grow to express neuronal antigens and acquire mature neuronal morphologies, including axons, complex dendritic trees, and synapses that can be visualized at the electron microscopy level.

Also they form functional synaptic connections with each other in culture so one can measure their functional properties electrophysiologically. These culture system provide us to explore the mechanisms of neuronal degeneration as a valuable model as well [55].

Together, this technique of primary hippocampal culture is essential and is used by applying on many neuroscience studies.

## 3. Neurotrophin and growth

The neurotrophin family of target-derived growth factors regulates neuronal survival and physiology in both the CNS (Central Nervous System) and PNS (Peripheral Nervous System). Members of this family include the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5).

Neurotrophin signaling occurs through the Trk receptor tyrosine kinases (TrkA, TrkB, and TrkC) and the low-affinity neurotrophin receptor p75. Although all neurotrophins bind to p75 with similar affinity, Trk receptors display selectivity toward different neurotrophins. TrkA binds to NGF, whereas TrkB binds preferentially to BDNF and NT-4/5. TrkC, on the other hand, binds to NT-3[56]

It has been demonstrated that neurotrophins regulate the dendrite morphology in a variety of preparation. As neurotrophins are released in an activity-dependent manner, they can play a major role in the structural adjustment of developing and mature neuronal circuits[57].

## 4. Synaptic plasticity

In the nervous system, a great deal of effort has focused on understanding the conversion of patterns of synaptic activity into long-lasting changes in synaptic efficacy. Synaptic plasticity is the ability of the synapse to modify the strength of synaptic transmission. The strength of a synapse can change; it is plastic. Synaptic plasticity is thought to be the main mechanism of learning and memory.

Neural activity dependent changes can alter synaptic transmission at pre-existing synapses and can elicit the growth of new synaptic connections or the pruning away of existing ones. Also, it can modulate the excitability properties of individual neurons by changing of the number of receptors located at a synapse.[58]

In an enormous amount of work, many forms of synaptic plasticity as well as their underlying mechanisms, including changes in the quantity of neurotransmitters released into a synapse, and changes in how effectively cells respond to those neurotransmitters, have been described. [59]

Synaptic plasticity has been shown to play a critical role in the outstanding capacity of the brain to translate transient experiences into infinite numbers of memories that can last for decades. Since memories seem to be represented by large interconnected networks of synapses in the brain, synaptic plasticity is one of the important mechanisms underlying learning and memory.

Synaptic transmission can either be enhanced or depressed by activity. These alterations extend from milliseconds to continuing modifications that may last for days or weeks and possibly even longer.

Transient forms of synaptic plasticity have been associated with short-term memory and more enduring changes are regarded to play essential functions in the construction of neural circuits during development and with long-term memory in the mature nervous system.

## 5. Long Term Potentiation (LTP)

Long-term potentiation (LTP), an alteration of synaptic strength for a long period, is one component of synaptic plasticity. Also it is widely believed to be one of the main neural mechanisms by which memory is stored in the brain and to be a correlate for memory consolidation and recall.

In 1966, LTP was originally discovered by Terje Lomo in Oslo, Norway. He performed experiments to examine the function of short term memory using anesthetized rabbits. Actually, he recorded excitatory postsynaptic potentials (EPSPs) in dentate gyrus in response to single pulses of electrical stimulation in the perforant pathway. During these experiments, he unexpectedly observed enhanced excitatory postsynaptic potentials (EPSPs) over a long period of time that were elicited by a high frequency stimulation in the presynaptic fibers. This phenomenon was initially called long lasting potentiation.[60]

Timothy Bliss, who had joined Lomo’s laboratory in 1968, firstly characterized the long lasting potentiation in the rabbit hippocampus and published his findings in a collaboration with Lomo in 1973.

In 1975, Douglas and Goddard named this new phenomenon of long-lasting potentiation as long term potentiation (LTP) [61].

After its initial discovery in the rabbit hippocampus, LTP has been shown in a different brain areas such as cerebral cortex, cerebellum, amygdala and so on. Robert Malenka, a well known LTP researcher, has suggested that LTP could be exhibited at all excitatory synapses in the mammalian brain [62].

Due to its well known organization and due to an easily inducible LTP, the CA1 region of the hippocampus, in particular the Schaffer collateral pathway that is NMDA receptor dependent has become the most popular brain region for studying LTP in mammals.

Memory storage in the mammalian brain consists of at least two distinct stages, which are an early and short term stage of LTP (E-LTP) and a late stage of LTP (L-LTP).

E-LTP which lasts for minutes involves localized, transient changes in synaptic efficacy and is independent of new protein synthesis and RNA synthesis. L-LTP , persistent forms of memory, lasts longer, for up to 8 hr in hippocampal slices [63] and for days in the intact animal [64]. L-LTP requires an elevation of cAMP, recruitment of the cAMP-dependent protein kinase A (PKA), and ultimately the activation of transcription and translation for new RNA and protein synthesis.

L-LTP shares with long term memory the requirement for the synthesis of new RNA and protein [65, 66]. It has been shown that brief inhibition of either protein synthesis or transcription selectively blocks induction of long term memory without affecting short term memory[67, 68].

An enormous number of studies performing electrophysiological recordings in hippocampal slices have been reported that selectively changing the number of conditioning tetanic trains or different conditioning procedures can produce the two mechanistically distinct forms of LTP that are E-LTP and L-LTP [69].

For example, the conditioning protocols that are inducing E-LTP are blocked by inhibitors of calcium/calmodulin kinase II (CaMKII) but are not affected by an inhibitor of cAMP-dependent protein kinase A (PKA), the protein synthesis inhibitor (anisomycin) and cAMP activator forskolin. In contrast, the protocol that can induce L-LTP requires new protein synthesis and is blocked by an inhibitor of cAMP-dependent protein kinase A (PKA) and is occluded by forskolin.

LTP has been studied extensively in hippocampal slices [70]. In Fig 6, the setup I used is shown. In Fig 7, the picture of one slice I used is shown. In our experiments, the field excitatory postsynaptic potentials (fEPSPs) were obtained from the CA1 stratum radiatum area of the hippocampus in a stimulation of Schaffer collateral pathway which is the most popular pathway for a LTP studies as are shown in Figure 7.

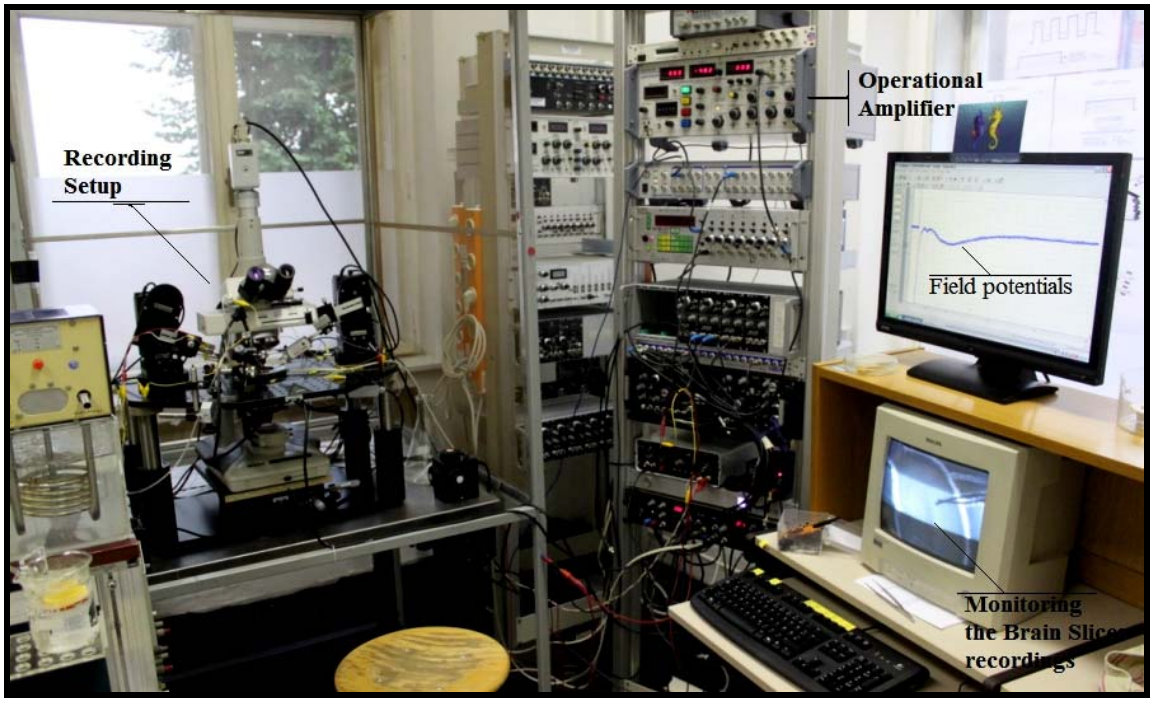


Figure 6 Electrical set-ups for long term extracellular recording used by my experiments.

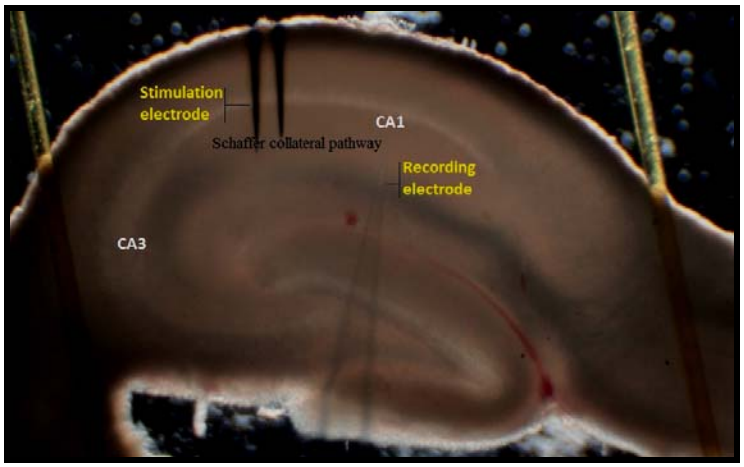


Figure 7 Microscopic view of a hippocampal slice I measured together with electrodes.

LTP has been the object of intense investigation because it is widely believed that LTP provides an critical key to understanding the molecular mechanisms by which memories are generated and, more generally, by which experience modifies behavior.

## 6. Morris water maze

Morris water maze (Morris water escape task) has been widely used in behavioral neuroscience for studying the neuronal mechanisms of spatial learning and memory. It was developed by Richard Morris at

the University of St Andrews in Scotland and was first described in the early 1980s. [71] Morris developed this test to study learning in animals and he showed that the spatial learning can be impaired after disruption of hippocampus by performing this task.

In this task, usually rats or mice are placed in a large round pool containing opaque water and subjected to escape from swimming in the water by finding a hidden platform submerged beneath the water surface. The platform location can be identified by using distal extra-maze cues attached to the room walls and remains constant during the entire experiment. Since there are extra-maze cues indicating where the platform is located, animals have to learn using spatial memory. An overview of Morris water maze is shown in Figure 8, A. [72]

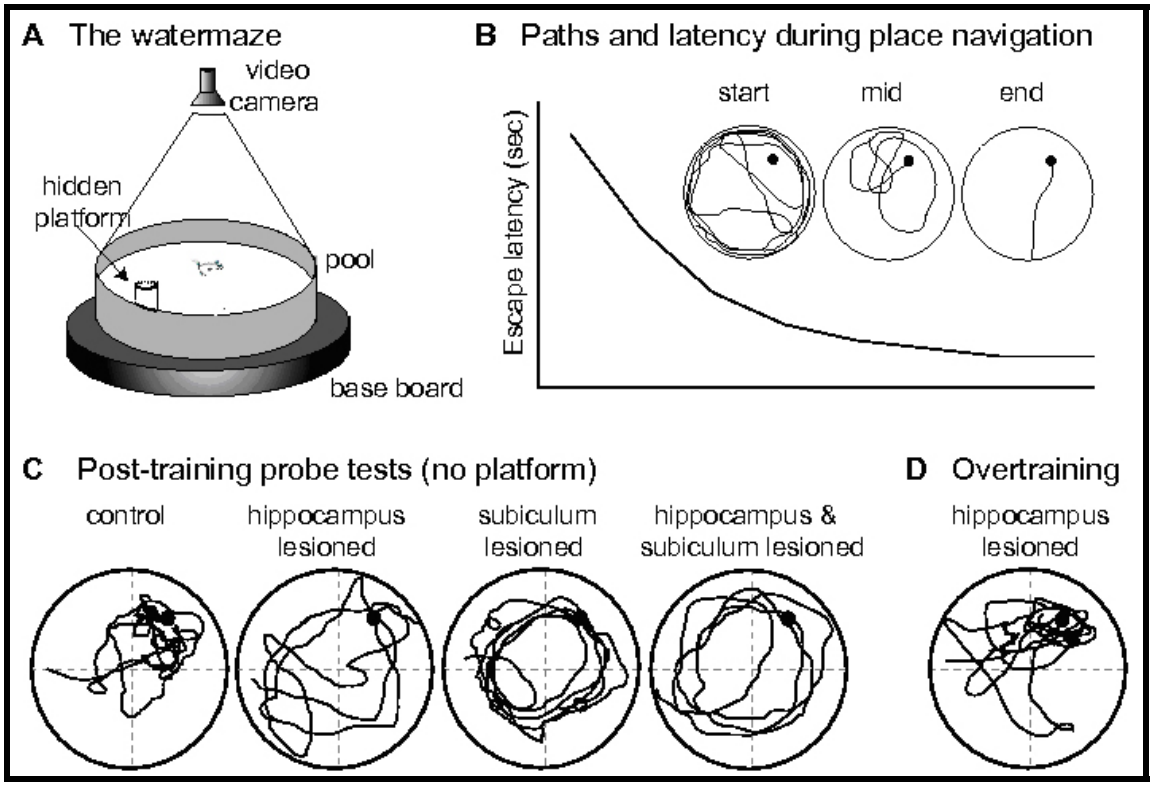


Figure 8 The Morris water maze. A. Drawing of a typical water maze set-up with overhead video camera and hidden platform. B. Representative escape latency graph and swim paths over training session. C. Representative swimming path in post-training probe tests. (Morris R.G. *et al.*, Eur J Neurosci, 1990)

The pool is generally monitored using a digital camera, and the pool image is digitally divided into four quadrants by a computerized tracking/image analyzing system.

The tracking system measures the escape latency in which the animal escape from the water to the platform and parameters such as path-length, swimming speed and so on.

The experiments consist of the pre-acquisition session, acquisition session (training session) and post-

training probe test and depending on the protocol of the laboratory the procedures are slightly different. The day before starting the training session, each mouse receives a pre-acquisition session in which it was placed directly on the platform and required to stay for a minimum of 15 s. The mice are then allowed to have 30 s of a free swimming period before being guided back to the platform. This pre-acquisition consists of 3–4 test trials in which each mouse is required to climb by itself onto the platform.

The acquisition phase consists of three trials per day (with intertrial intervals of 15 min) in which the mice are placed into the pool containing the hidden platform and are allowed to swim freely. The behavioral measurements for each acquisition's trial start when the mouse is released into the water, and the recordings are obtained for at least 90 s. The animals are placed into the water facing the sidewalls of the pool at different start quadrants (except the quadrant in which the platform is located) across trials. They are learning over a number of trials the correct location of the platform and are swimming with more direct swim paths. By measuring the time when mouse escapes to the platform over trials, the examiner can check whether the training session is well performed or not. As shown in Figure 8 B; escape latency is gradually decreasing during trials and the mouse is swimming more directly to the platform.

After the acquisition phase (training session) is finished, the animals are allowed to swim for 60 sec in the pool in which the escape platform is removed and this is called as probe trial. Normally, the training session is well performed, the animal swim to the target quadrant of the pool and repeatedly swim across the position where the escape platform has been located as shown in Figure 8, C.

# II. Aims of the study

It has already been demonstrated well that protein kinases critically regulate synaptic plasticity in the mammalian hippocampus which is a critical brain region for learning and memory. Protein phosphorylation mediated by protein kinases is involved in regulation of important neuronal processes including neuronal development, growth and plasticity such as long term potentiation (LTP).

However, most studies to define discrete roles for individual protein kinases in synaptic plasticity such as LTP and behavioral plasticity have been performed only at early stages. Although there are many different protein kinases in the brain, their function in learning and memory is still unclear.

To this end, the aim of my thesis is to understand the role of brain enriched protein kinases FAK and LCK in memory-related functions (especially synaptic plasticity) of the mouse hippocampus. For that I used primary hippocampal neuronal cultures, electrophysiology tools and behavior experiments to answer the following questions;

First, the relevance of the protein kinase investigated for neuritic outgrowth of hippocampal neurons in culture should be checked using loss-of-function or gain-of function approaches. For loss of function, both pharmacological and genetic strategies should be used.

Second, the involvement of protein kinase should be investigated in the spontaneous and neurotrophin-induced neurotransmitter release at hippocampal synapses.

Third, the role of the protein kinases should be defined in long-term potentiation (LTP) in acute hippocampal slices and as a final point, their contribution to hippocampus-dependent learning and memory should be evaluated at the behavioral level in mice.

Details of my work are given in the two publications;

1) Focal Adhesion Kinase Regulates Neuronal Growth, Synaptic plasticity and Hippocampus-Dependent Spatial Learning and Memory, *Francisco J.Monje*, Eun-Jung Kim*, Daniela D.Pollak, Maureen Cabatic, Lin Li, Arthur Baston, Gert Lubec, Neurosignals,* 2012, 20(1):1-14

2) Alzheimer's disease risk factor lymphocyte-specific protein tyrosine kinase regulates long-term synaptic strengthening, spatial learning and memory, *Eun-Jung Kim*, Francisco J. Monje*, Lin Li, Harald Höger, Daniela D. Pollak, Gert Lubec, Cellular and Molecular Life Sciences,* 2012, Feb;70(4):743-59.

## III. Articles (Appendix)

**Appendix 1**

Article: Focal Adhesion Kinase Regulates Neuronal Growth, Synaptic plasticity and Hippocampus-Dependent Spatial Learning and Memory

*Francisco J.Monje*, Eun-Jung Kim*, Daniela D.Pollak, Maureen Cabatic, Lin Li, Arthur Baston, Gert Lubec, Neurosignals, 2012, 20(1):1-14*

**Appendix 2**

Article: Alzheimer's disease risk factor lymphocyte-specific protein tyrosine kinase regulates long-term synaptic strengthening, spatial learning and memory

*Eun-Jung Kim*, Francisco J. Monje*, Lin Li, Harald Höger, Daniela D. Pollak, Gert Lubec, Cellular and Molecular Life Sciences, 2012, Feb;70(4):743-59.*

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# Focal Adhesion Kinase Regulates Neuronal Growth, Synaptic Plasticity and Hippocampus-Dependent Spatial Learning and Memory

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**Key Words**
Focal adhesion kinase · Neurotrophic tyrosine kinase receptor type 1 TrkA · Hippocampus · Long-term potentiation · Learning and memory

**Abstract**
The focal adhesion kinase (FAK) is a non-receptor tyrosine kinase abundantly expressed in the mammalian brain and highly enriched in neuronal growth cones. Inhibitory and facilitatory activities of FAK on neuronal growth have been reported and its role in neuritic outgrowth remains controversial. Unlike other tyrosine kinases, such as the neurotrophin receptors regulating neuronal growth and plasticity, the relevance of FAK for learning and memory in vivo has not been clearly defined yet. A comprehensive study aimed at determining the role of FAK in neuronal growth, neurotransmitter release and synaptic plasticity in hippocampal neurons and in hippocampus-dependent learning and memory was therefore undertaken using the mouse model. Gain- and loss-of-function experiments indicated that FAK is a critical regulator of hippocampal cell morphology. FAK mediated neurotrophin-induced neuritic outgrowth and FAK inhibition affected both miniature excitatory postsynaptic potentials and activity-dependent hippocampal long-term potentiation prompting us to explore the possible role of FAK in spatial learning and memory in vivo. Our data indicate that FAK has a growth-promoting effect, is importantly involved in the regulation of the synaptic function and mediates in vivo hippocampus-dependent spatial learning and memory.



## Introduction

Activity-related neuritic and synaptic growth is a fundamental mediator of the functional strengthening of synapses mediating the formation and storage of long-term memories [1]. Several different signaling cascades are known to contribute to the regulation of learning-related synaptic plasticity, including receptor tyrosine kinases mediating the actions of neurotrophins [2, 3] as well as non-receptor tyrosine kinases [4–6]. The focal adhesion kinase (FAK), a non-receptor tyrosine kinase, is highly expressed in the nervous system and particularly enriched in the cortex and in the hippocampus [7, 8],

F.J. Monje and E.-J. Kim contributed equally to the work.

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brain regions with pivotal importance for learning and memory. FAK has been proposed to mediate neuronal outgrowth and modulate synaptic plasticity, processes importantly involved in the neurobiological mechanisms underlying learning and memory [9]. However, the specific contribution of FAK to the molecular pathways underlying neuronal growth, synaptic function and plasticity remains poorly understood. In fact, conflicting data regarding its role as regulator of neuritic outgrowth have been reported.

For example, it has been shown that cell-specific ablation of FAK increases the number of axonal terminals and synapses formed by neurons in vivo and induces a greater number of axonal branches in culture, indicating FAK as a negative regulator of axonal branching and synapse formation [10]. In contrast, more recent studies suggest that FAK promotes neuritic extensions, possibly through inhibition of ATP-gated P2X7 receptors [11]. Similarly, it has also been shown that the leucine-rich repeat protein netrin recruits and activates FAK and requires FAK function for mediating its effects on axonal guidance and outgrowth [12]. Costimulation of integrins and growth factor receptors also activates proteins of the FAK family to promote neuritic outgrowth and expression of the C-terminal domain of FAK is sufficient to block neurite outgrowth [13]. Since these observations support a positive neurite-promoting effect of FAK in neurons, it is plausible to speculate that FAK might have a dual role depending on the type of cell, the associated proteins or on the mechanisms of activation. For example, the relevance of FAK as a downstream effector of EphB receptors and the importance of FAK to maintain mature dendritic spines by regulating cofilin activity has been demonstrated [14]. Additionally, treatment of hippocampal slices with ephrin-A3 has been demonstrated to decrease FAK and it has been proposed that FAK might be related to the maintaining of proper spine morphology [15]. On the other hand, Moeller et al. [16] showed hat FAK activation is important for spine shortening and morphogenetic changes also downstream of EphB receptors in cultured hippocampal neurons.

To better understand the role of FAK in learning-related modulation of synaptic growth and function, we have undertaken a systematic study of its role in neuronal growth as well as in the synaptic activity and plasticity of hippocampal neurons and evaluated its importance for learning and memory in vivo. First, we studied the relevance of FAK for the neuritic outgrowth of hippocampal neurons in culture using loss-of-function and gain-of-function (FAK overexpression) approaches. For loss of function we used both a pharmacological and a genetic strategy. For pharmacological inhibition we used the specific FAK inhibitor 1,2,4,5-benzenetetraamine tetrahydrochloride (also known as Y15). For loss-of-function experiments using a genetic strategy we performed transfections of FAK shRNA following an approach that has been successfully used previously [17–21]. The small molecule inhibitor Y15 has been very recently described as a very powerful, specific and selective inhibitor of the FAK which also targets the position Y397 of the protein [22–25]. The Y15 molecule was identified by a combined informatics modeling/screening, pharmacological and molecular-biological approach [22–25]. The remarkably selective inhibitory properties of this molecule, which has already been tested in human cells, makes it a promising tool for therapeutic interventions against several forms of cancers, as Y15 can specifically inhibit the activity of tumor cell FAK resulting in inhibition of tumor cell adhesion and tumor regression in vivo [22–25].

Second, we investigated the involvement of FAK in the spontaneous and neurotrophin-induced neurotransmitter release at hippocampal synapses. Third, we defined the role of FAK in long-term potentiation (LTP) in acute hippocampal slices and as a final point evaluated its contribution to hippocampus-dependent learning and memory at the behavioral level in mice. We found that FAK positively modulates both neuritic outgrowth and synaptic plasticity and is importantly involved in the formation and retention of long-term spatial memory.

## Materials and Methods

*Animals and Housing*

Adult C57Bl/6J male mice (Janvier, France), 10–12 weeks old, were housed in pairs in standard transparent laboratory cages in a temperature-controlled colony room (21 ± 1°C) and were provided with food and water ad libitum. Mice were maintained on a 12-hour light/dark and cages were cleaned once a week. All animal experiments are in line with the UK Animals (Scientific Procedures) Act, 1996 and associated guidelines (86/609/EEC) and were approved by the local animal committee.

*Hippocampal Culture*

Postnatal mouse (days 0–3) hippocampal neurons were dissociated and cultured following standardized procedures [26] and complemented with glial support cultures as previously described [27].

*Morphological Evaluation*

On the day of plating (day 0), cells were untreated (control), incubated with 1 μM Y15 (Sigma-Aldrich, Austria), incubated with neuronal growth factor (NGF) (25 ng/ml) or incubated with NGF (25 ng/ml) in the presence of 1 μM Y15. Morphological anal-

ysis was carried out 24 h after addition of the respective cell treatment. Images were acquired using Axiovert 200M (Zeiss) and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). Neuritic outgrowth was evaluated according to parameters described elsewhere [28, 29]. For transfection of hippocampal neurons, 1 μg of each DNA (GFP plasmid and FAK-GFP plasmid) was incubated with 15 μg of Nupherin-neuron (Nupherin™ Transfection Reagent; ENZO Life Sciences) in 150 μl of neurobasal medium without antibiotics for 15 min. In parallel, 1 μl of Lipofectamine™ LTX Reagent (Invitrogen) was separately mixed in 150 μl of neurobasal medium. After 15 min, the two solutions were combined (mix) and incubated for 45 min. Cell culture media was removed (and stored at 37°C) and neurons (cultured in 24-well plates) were incubated in the 300 μl of mix for 5 min, centrifuged (in a swinging bucket centrifuge) at 233 *g* for 5 min and additionally incubated for 2.5 h. The mixture was then removed and replaced with the original cell culture media and neurons continued in culture until experiments were performed. This transfection procedure was also used to transfect specific FAK shRNAs (Santa Cruz) in order to inhibit FAK expression as previously described [17–21]. For this, coexpression experiments were carried out combining GFP and FAK shRNAs (0.5 μg of GFP DNA plasmid with 0.5 μg of FAK shRNA plasmid or 0.5 μg of Control-A shRNA (Santa Cruz) plasmid with 0.5 μg of GFP DNA plasmid), a genetic strategy successfully used previously for the inhibition of FAK and other proteins [17–21]. Standardized confocal microscopy was performed 24 or 48 h after transfection for either FAK overexpression or inhibition.

*Cell Viability Assay*

Cell viability was monitored using the colorimetric MTT assay, as previously described [30]. Briefly, cells cultured in 96-well plates were treated with increasing concentrations of Y15 (0.5, 1, 2, 6, 10, 40 μM) or media alone as control and incubated with 0.5 mg/ml MTT-Formazan (Sigma), at 37°C under 5% $CO_2$, for 1 h and then washed in PBS. The blue Formazan reduction product, produced by the action of succinate dehydrogenase in living cells on the dye, was dissolved in 200 μl DMSO, incubated for 10 min and the optical density was read at 550 nm using a BioTek Ultra Microplate Reader BL808 (BioTek Inc., Winooski, Vt., USA). Data are expressed as the percentage of viable cells compared with control cells (100% viability) determined by MTT reduction.

*Western Blotting*

Animals were sacrificed by neck dislocation 4 h after termination of behavioral experiments. The hippocampus was rapidly dissected, snap-frozen in liquid nitrogen and the tissue was stored at –80°C until use. Hippocampal tissue (n = 6–8 per group) was ground under liquid nitrogen and homogenized in a protein lysis buffer containing of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% SDS, 0.5% Triton X-100, 1 mM PMSF and protease inhibitor cocktail (1×) (Roche Diagnostics, Mannheim, Germany). Western blotting was carried out essentially as previously described [31]. Membranes were blocked by incubating with 5% non-fat dry milk in 100 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween 20 (TTBS). Membranes were then incubated with primary antibodies (Phospho-FAK (Tyr397) [1:500], Cell Signaling Technology; total FAK [1:1,000], Cell Signaling Technology; phospho-PYK2 [1:500], Cell Signaling Technology; total PYK2 [1:500], Cell Signaling Technology; drebrin [1:1,000], MBL International, Woburn, Mass., USA; PSD95 [1:1,000], Millipore; synapsin I [1:1,000], Abcam plc, Cambridge, UK; Anti-Erk1/2 (p44/p42) [1:500], Millipore; Anti-phospho-MAP Kinase1/2 (Erk1/2) [1:500], Millipore) overnight at 4°C, rinsed three times with TTBS and incubated 1 h at 21 ± 1°C with horseradish peroxidase-conjugated secondary antibody (goat-anti rabbit IgG [1:3,000], Cell Signaling Technology; horse-anti mouse IgG [1:3,000], Cell Signaling Technology). Immunoreactivity was visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, N.J., USA). Quantification was performed by chemiluminescent imaging with a FluorChem HD2 (Alpha Innotech, San Leandro, Calif., USA) using the respective software.

*Single-Cell Recordings and Data Analysis*

For recording of miniature excitatory postsynaptic currents (mEPSCs), mouse primary hippocampal neurons 14 days after plating were used. Cells were untreated (control), treated with 2 μM Y15 30 min prior to recording, incubated with NGF (25 ng/ml) 24 h prior to recordings or treated with 2 μM of Y15 for 30 min and after washout incubated with NGF (25 ng/ml) for 24 h before recordings. mEPSCs were acquired in the whole-cell configuration of the patch-clamp technique at room temperature (21 ± 1°C) on neurons at 14 days in vitro. The neurons were clamped at –70 mV. The extracellular solution contained 140 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 2 mM MgCl, 29 mM glucose and 10 mM Hepes, and was adjusted to pH 7.4 with NaOH. 0.5 μM tetrodotoxin and was added to inhibit sodium-channel conductance and prevent the occurrence of action potential propagations. 30 μM bicuculline-methiodide was used to suppress miniature inhibitory postsynaptic currents. Recording electrodes from borosilicate glass capillaries were pulled using a horizontal puller (Sutter Instruments) to yield tip resistances of 2.5–5 MΩ and filled with a solution containing 140 mM KCl, 1.6 mM $CaCl_2$, 10 mM EGTA, 10 mM Hepes and 2 mM Mg-ATP, adjusted to pH 7.3 with KOH. Events <5 pA and events with a slow rise time (>5 ms) were excluded from the analysis. Currents were measured using the FitMaster software package (Heka Electronics). All data are given as means ± SE.

*Brain Slice Preparation*

Brain slice preparations and electrophysiological experimental protocols were performed as previously described [32, 33] with minor modifications. In brief, male (7–11 weeks old) C57BL/6J mice (Janvier, France) were sacrificed by cervical dislocation. Brains were rapidly removed and immersed in cold (~4°C) artificial cerebrospinal fluid (ACSF). Hippocampi were isolated while submerged in ACSF and transverse slices (400 μm) were prepared using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd, Guilford, UK). Slices were transferred to a home-made nylon-mesh plastic holder floating in a home-made interface chamber containing ACSF maintained at 21 ± 1°C. Slices were allowed to recover for 1 h at 21 ± 1°C plus 30 extra minutes at 28°C before recordings were performed. During the whole procedure, slices in ACSF were continuously supplied with a saturating Carbogen mixture (95% $O_2$/5% $CO_2$) leading to a pH of 7.4. Slices were then transferred to the recording chamber perfused with carbogenated ACSF at a rate of 2 ml/min and measurements were performed at a temperature of 28°C. ACSF contained (in mM): NaCl 125, KCl 2.5, $NaHCO_3$ 25, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 25, and $NaH_2PO_4$ 1.25 (all from Sigma-Aldrich, Austria).

*Slice Electrophysiology and Data Analysis*

Slice electrophysiology was performed essentially as previously described [32, 33] with minor modifications. Extracellular measurements of field excitatory postsynaptic potentials (fEPSPs) were performed by placing the recording electrode in the CA1 stratum radiatum of the hippocampus using borosilicate glass micropipettes (2–5 MΩ) filled with ACSF. Data acquisition and storage were performed using an AxoClamp-2B amplifier in the Bridge mode, a Digidata 1440 interface and the pClamp-10 Program software (all from Axon Instruments, Foster City, Calif., USA/Molecular Devices, USA). fEPSPs were evoked by electrical stimulation of the Schaffer collateral/associational commissural pathway with a home-customized bipolar tungsten electrode insulated to the tip (50-μm tip diameter). Stimuli were delivered every 15 s. The initial slope of the rising phase (20–80% of peak amplitude) of the fEPSPs was used as a measure of the strength of synaptic transmission [32, 33]. Baseline recordings were taken using stimulation intensities eliciting ~40% of the maximal obtainable response. Baselines comprised a minimum of 15 min of stable baseline recordings (5 min before theta-burst stimulations are shown). After theta-burst stimuli were applied to the Schaffer collateral/associational commissural pathway, elicited field recordings were obtained for at least 30 min. Slope values for each obtained fEPSPs were then normalized with respect to the initial steady baseline responses recorded during the 5 min preceding the theta-burst stimulation. Test stimuli (50-μs pulse width) were delivered every 15 s. LTP was induced using a theta-burst stimulation protocol previously described [34].

*Behavioral Analysis*

Y15 (2 mg/kg) or saline were injected intraperitoneally in an injection volume of 10 ml/kg and behavioral analysis was carried out 30 min after injection.

Basic Neurological Examination

The procedure followed the set-up by Irwin [35]. A battery of tests was applied to reveal defects in gait or posture, changes in muscle tone, grip strength, visual acuity and temperature. To complete the assessment, vitally important reflexes were scored. Throughout the manipulations, incidences of abnormal behavior, fear, irritability, aggression, excitability, salivation, lacrimation, urination and defecation were recorded.

Open Field

The open field (OF) was a rectangular polyvinyl chloride arena (30 × 40 × 60 cm). Locomotor activity was recorded during 15 min using a CCD camera and analyzed using the Limelight video tracking system (Limelight Actimetrics, Elmwood, Ill., USA). Total distance traveled (cm) as parameter for locomotor activity was automatically generated by the software. The equipment was cleaned using 70% ethanol between animals.

Morris Water Maze

The Morris water maze (MWM) consisted of a circular pool (122 cm diameter, walls 76 cm depth) in which mice were trained to escape from water by swimming to a hidden platform (1.5 cm beneath the water surface) whose location could be only identified using distal extra-maze cues attached to the room walls. Visual cues had different colors and dimensions and were kept constant during the whole experiment. Water temperature was maintained at 21 ± 1°C. The pool was divided into four quadrants by a computerized tracking/image analyzing system (Limelight Actimetrics). The platform was placed in the middle of the SW quadrant and remained at the same position during the acquisition phase (training). The acquisition phase consisted of 12 training trials: 3 training trials per day and 5 training days with an inter-trial interval of 60 min. Mice were injected with Y15 or saline 30 min before the first training trial on each day. Mice were released randomly with their heads facing the pool wall from the four compass locations (NE, NW, SW, and SE), and allowed to swim and search for the platform for 120 s. If mice did not locate the platform after 120 s, they were manually placed on the platform and allowed to remain on it for 15 s. On the first training day, mice were given an acclimatization training session in which they were placed on the hidden platform, allowed to swim for 30 s, and then subsequently guided back to the platform. The day after the acquisition phase, mice underwent a probe trial in which the platform was removed. Animals were released from the longest distance to the platform and were allowed to swim freely for 60 s. The path the mouse swam was tracked and analyzed for the proportion of swimming time and/or path length spent in each quadrant of the pool was recorded.

*Statistical Analysis*

For comparisons between two groups, two-tailed Student's t tests and Fisher's exact test were used. Two-way ANOVAs were employed to assess the effects of NGF and Y15 in a 2 × 2 design. LTP data and latencies in the MWM were analyzed by repeated measures ANOVAs, with time points or training days respectively, as the repeated measure (within-subject factor) and pharmacological treatment as between-subject factor. Significant main effects or interactions were followed by Scheffé's post hoc test. An $\alpha$ level of 0.05 was adopted in all instances. All analyses were carried out using BioStat 2009 professional software (AnalystSoft Inc., Alexandria, Va., USA).

## Results

*FAK Is Required for Neuritic Outgrowth in Mouse Hippocampal Neurons*

We started evaluating the involvement of FAK in neuritic outgrowth by examining the effects of pharmacological inhibition FAK on NGF-induced enhancement of the number and length of extensions of neuritic processes of mouse hippocampal neurons in culture. To this aim, Y15 was used at non-cytotoxic concentrations (1 μM, cell viability 88.89% of control cells as revealed by the MTT assay) to block FAK activity. FAK inhibition by application of 1 μM Y15 significantly impaired the growth enhancement induced by NGF evaluated by the average length of neuritic extensions, as revealed by two-way ANOVA (effect of Y15: $F_{(1,55)} = 16.29$: $p < 0.001$) and the number of neuritic processes longer than 22.5 μm (effect of Y15: $F_{(1,55)} = 15.93$; $p < 0.001$). FAK blockade induced by Y15 also significantly reduced the total number of neuritic extensions ($F_{(1,55)} = 10.09$; $p < 0.01$) (fig. 1A–D). Y15 at a lower dosage (0.5 μM)

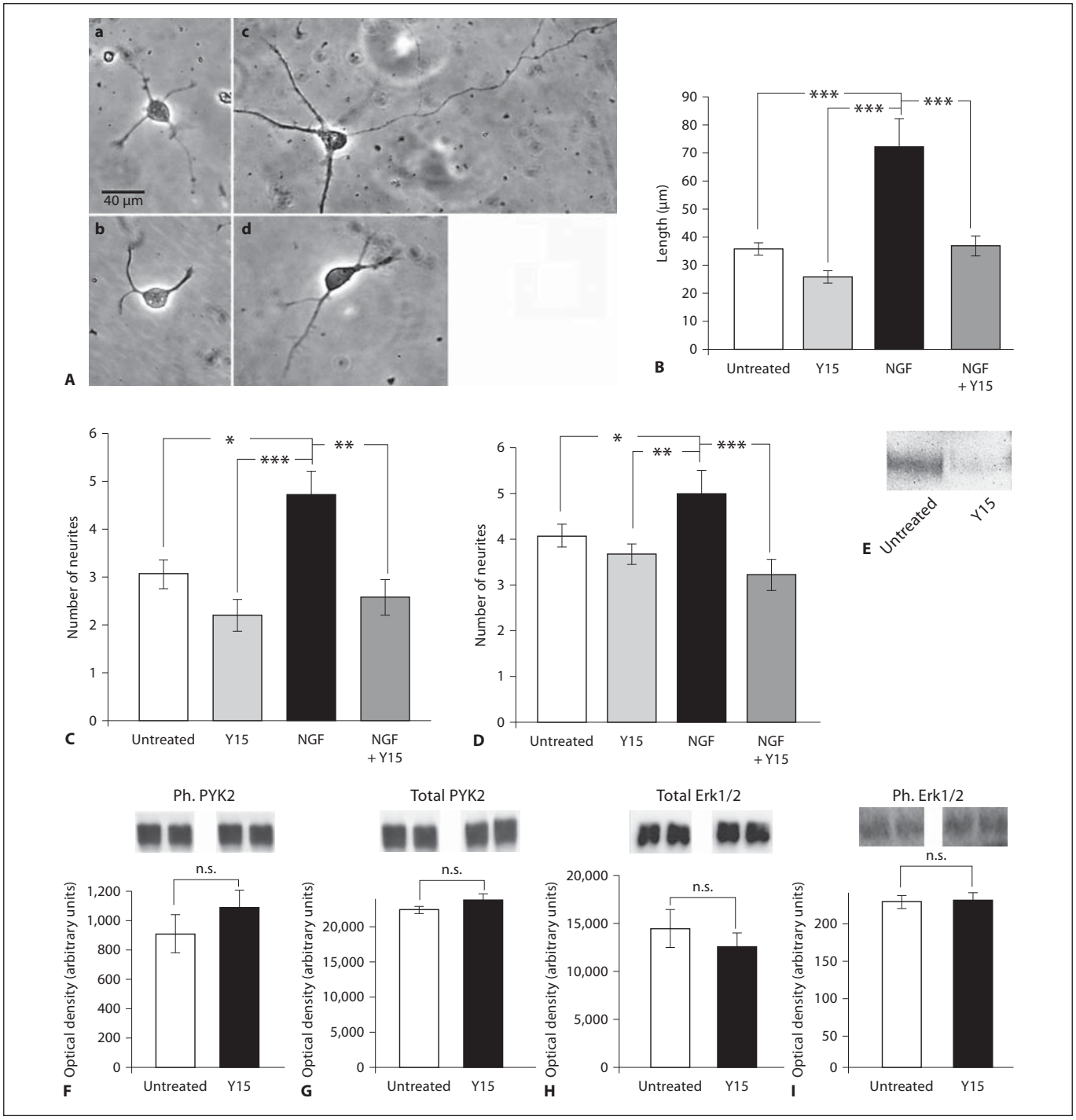


**Fig. 1.** FAK is required for neuritic outgrowth induced by NGF. **A** Y15 treatment results in a reduction of promotion of growth of neuritic processes induced by NGF in mouse hippocampal neurons: mouse hippocampal neurons without stimulation (**a**), after treatment with Y15 (**b**), after treatment with NGF (**c**), and after treatment with NGF in the presence of Y15 (**d**). The average length of neuritic extensions (**B**), the number of neuritic processes longer than 22.5 μm (**C**), and the total number of neuritic extensions (**D**) are enhanced by treatment with NGF and reduced in the presence of Y15. **E** Treatment with Y15 reduces levels of Y397 phosphorylated FAK in mouse hippocampal neurons. Hippocampal protein levels of phosphorylated (Ph.) PYK2 (**F**), total PYK2 (**G**), total ERK1/2 (**H**), and phosphorylated (Ph.) ERK1/2 (**I**) are not affected by in vivo Y15 treatment. Protein levels are displayed as optical densities in arbitrary units. All data are displayed as mean ± SEM. Statistical evaluation resulting from post hoc analysis is depicted. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = Not significant.

did not affect neuritic outgrowth, while, in agreement with the MTT assay, higher dosages (6 and 10 μM) were not compatible with survival of hippocampal neurons (see online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000330193).

The inhibitory effect of Y15 on FAK activity in our experimental conditions was revealed by reduced phosphorylation levels of Y397 FAK in hippocampal cells as evaluated by Western blot (a representative image of three independent experiments is depicted in fig. 1E). The specificity of Y15 as selective inhibitor of FAK over other protein kinases was tested in mouse hippocampal tissue after in vivo treatment with Y15 (2 mg/kg). No significant differences in the protein levels of phosphorylated and total PYK2 and phosphorylated and total Erk1/2 were detected (fig. 1F–I).

To further test the role of FAK in the morphological properties of hippocampal neurons using a genetic approach, we examined the effects of inhibition of FAK on neuritic outgrowth using specific FAK shRNA following a silencing genetic strategy used previously with success during the inhibition of FAK and other proteins [17–21]. Cotransfection of FAK shRNA plasmid and GFP plasmid resulted in a significant reduction in the total length and number of neurites per cell when compared to data obtained from cells cotransfected with scrambled control shRNA and GFP plasmids ($p < 0.05$; fig. 2A–C).

A gain-of-function experiment was then carried out to evaluate the effects of FAK overexpression on hippocampal neurons' neuritic outgrowth. Overexpression of FAK-GFP resulted in a significant enhancement in the total length and number of neuritic extensions per cell when compared to neurons expressing GFP alone ($p < 0.001$; fig. 2D–F). Although specific FAK shRNAs have been proven to constitute an effective method to hinder FAK expression [17–21], we nevertheless examined this effectiveness in our experimental conditions. To this aim, we examined by immunocytochemistry the changes in signal detection in hippocampal neurons transfected with either FAK shRNA or scrambled control shRNA. Lower detection signals in cells transfected with FAK shRNA were observed (fig. 2G).

*FAK Regulates Spontaneous Neurotransmitter Release of Hippocampal Neurons*

To determine the possible role of FAK in regulating – besides growth – also the electrophysiological properties of hippocampal neurons, we first tested the effect of Y15 on spontaneous neurotransmitter release and on its modulation induced by NGF. To this aim, we measured by single-cell voltage clamp the frequency and amplitude of mEPSCs in cultured hippocampal neurons treated with either NGF or Y15, or a combination of both. While NGF increased the frequency but not the amplitude of mEPSCs with respect to control cultures, treatment with Y15 significantly decreased the frequency of mEPSCs (effect of Y15: $F_{(1,43)} = 48.36$; $p < 0.0001$) (fig. 3A, B) and of their amplitude ($F_{(1,43)} = 51.36$: $p < 0.0001$) (fig. 3A, C). Taken together, these findings show that FAK activity regulates spontaneous neurotransmitter release and that this kinase is a key mediator of the effect of NGF on the frequency of mEPSCs.

*Inhibition of FAK Impairs Activity-Dependent LTP in Hippocampal Slices*

Given the evidence that FAK modulates spontaneous neurotransmitter release of hippocampal neurons, we investigated its possible role as a mediator of hippocampal synaptic plasticity at the network level. To this aim we measured fEPSPs in the CA1 dendritic layer of hippocampal slices induced by stimulation of the Schaffer collateral pathway, before and after high-frequency theta-burst stimulation. Y15 treatment did not prevent the theta-burst-induced post-tetanic potentiation but resulted in a significant impairment of the LTP ($F_{(1,12)} = 4.98$, $p < 0.05$) (fig. 4A) as also reflected in a reduction in fold change from baseline at 15 and 30 min after LTP induction (fig. 4B, C). These results indicate that FAK has a fundamental role in the long-term maintenance of hippocampal activity-dependent synaptic plasticity.

*Inhibition of FAK Impairs Hippocampus-Dependent Spatial Learning and Memory*

Hippocampal LTP is one of the neural substrates of long-term memory formation [36–39]. Our observations of the involvement of FAK in the Schaffer collateral-CA1 hippocampal LTP, together with previous observations [40], prompted us to further explore in vivo the potential role of FAK in hippocampus-dependent learning and memory. To this aim, we used the MWM to study the effect of Y15-induced FAK inhibition on hippocampus-dependent spatial learning and memory. We found that, at a dosage of 2 mg/kg, Y15 did not significantly alter basic neurological parameters of mice as evaluated by the Irwin observational battery [35] (table 1), while still leading to a significant reduction in Y397 phosphorylation of FAK in the hippocampus (fig. 5A) without affecting protein levels of total FAK (fig. 5B). Thirty minutes after injection with either Y15 or saline, control mice (n = 10 per group) underwent evaluation of locomotor and explor-

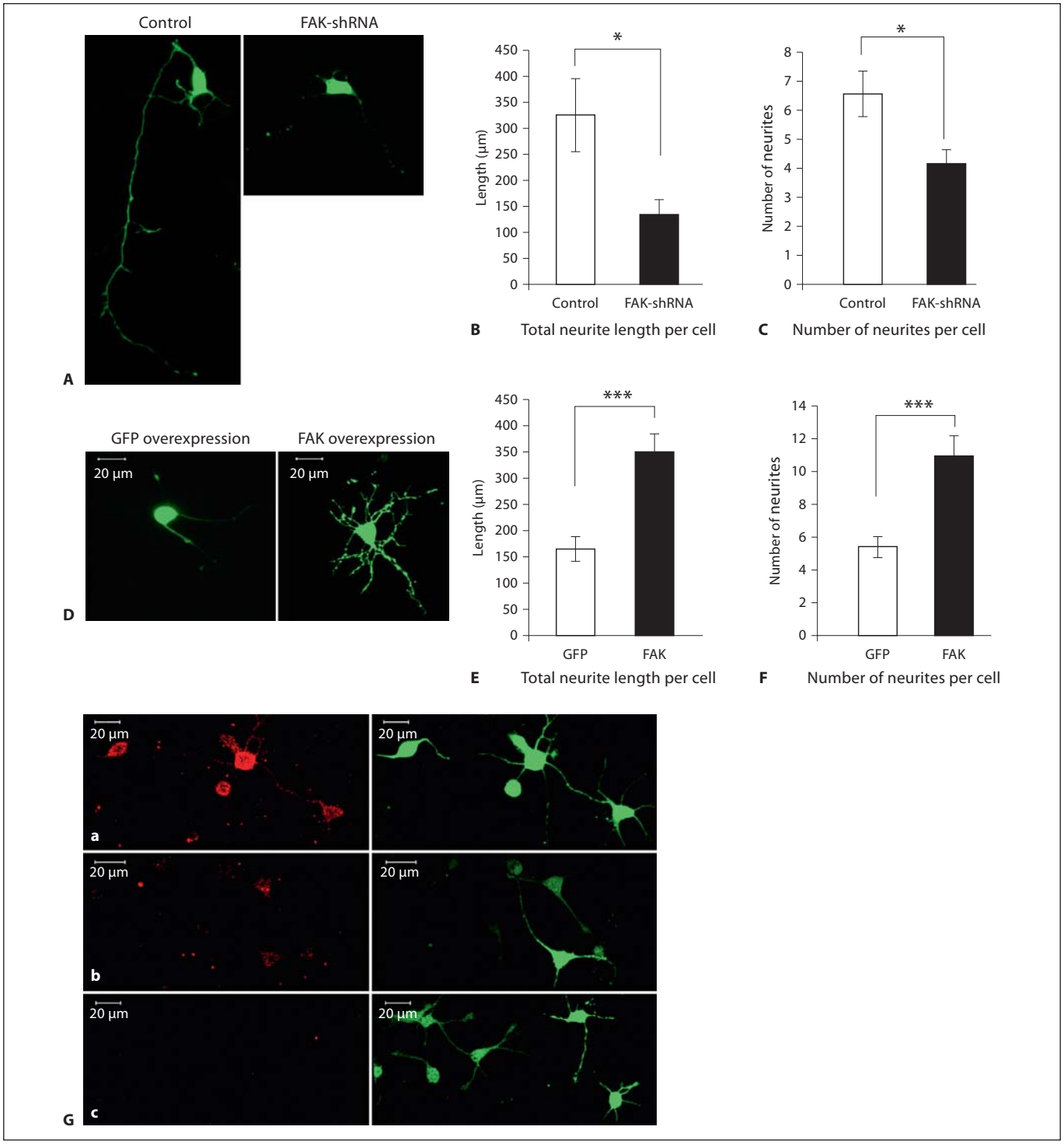


**Fig. 2.** FAK regulates neuritic outgrowth and interacts with neurotrophic tyrosine kinase receptor type 1 (TrkA). **A** Transfection of shRNA plasmids encoding plus hairpin sequences corresponding to FAK siRNA Gene Silencer sequences reduces neuritic outgrowth in mouse hippocampal neurons as evaluated by the length of all neurites (**B**) and the number of neurites per cell (**C**). **D** Expression of a FAK-GFP containing plasmid enhances neuritic outgrowth in mouse hippocampal neurons as evaluated by the length of all neurites (**E**) and the number of neurites per cell (**F**). **G** FAK shRNA is effective to obstruct FAK. Specific phospho-FAK antibody was used to detect signal levels in hippocampal neurons transfected with either scrambled control shRNA (**a**) or FAK shRNA (**b**); **c** negative control using secondary antibody alone. * $p < 0.05$, *** $p < 0.001$.

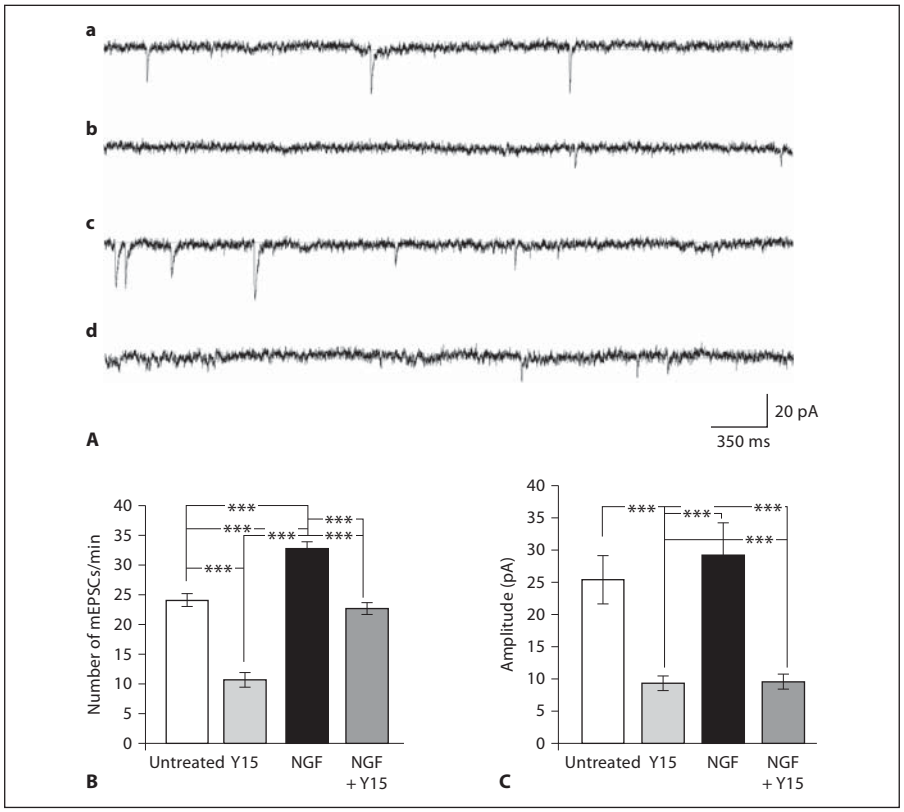


**Fig. 3.** FAK modulates miniature excitatory postsynaptic currents in mouse hippocampal neurons (mEPSCs). **A** Traces of recordings from mouse hippocampal neurons displaying mEPSCs in control cells (**a**), cells treated with Y15 (**b**), cells treated with NGF (**c**), and cells treated with NGF and Y15 (**d**). mEPSCs frequencies (**B**) and amplitudes (**C**) in control cells, cells treated with Y15, cells treated with NGF, and cells treated with NGF and Y15. All data are displayed as mean ± SEM. Statistical evaluation resulting from post hoc analysis is depicted. *** $p < 0.001$.

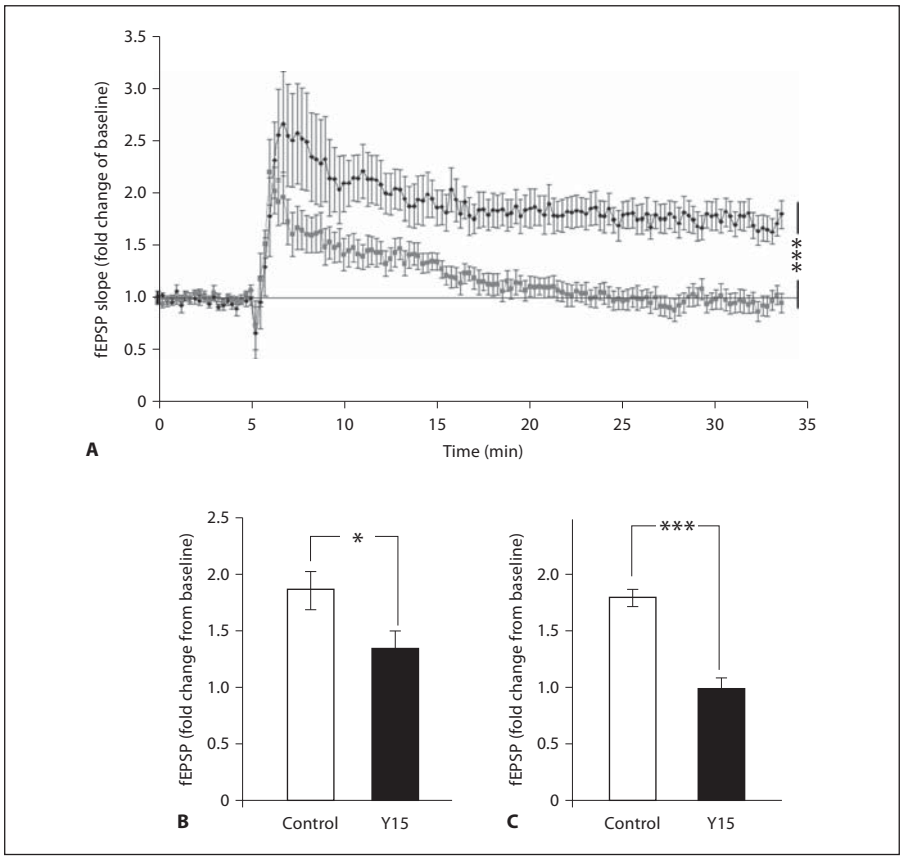


**Fig. 4.** Inhibition of FAK impairs LTP in mouse hippocampal slices. **A** Field potential (fEPSP) slopes in mouse hippocampal slices treated with Y15 (grey squares) and untreated controls (black diamonds). Fold changes in response to theta-burst stimulation are depicted. Statistically significant differences in changes of fEPSP slope are observed 15 min (**B**) and 30 min (**C**) after theta-burst stimulation. All data are displayed as mean ± SEM. Statistical evaluation resulting from repeated measure analysis (**A**) and post hoc analysis (**B**, **C**) is depicted. * $p < 0.05$, *** $p < 0.001$.

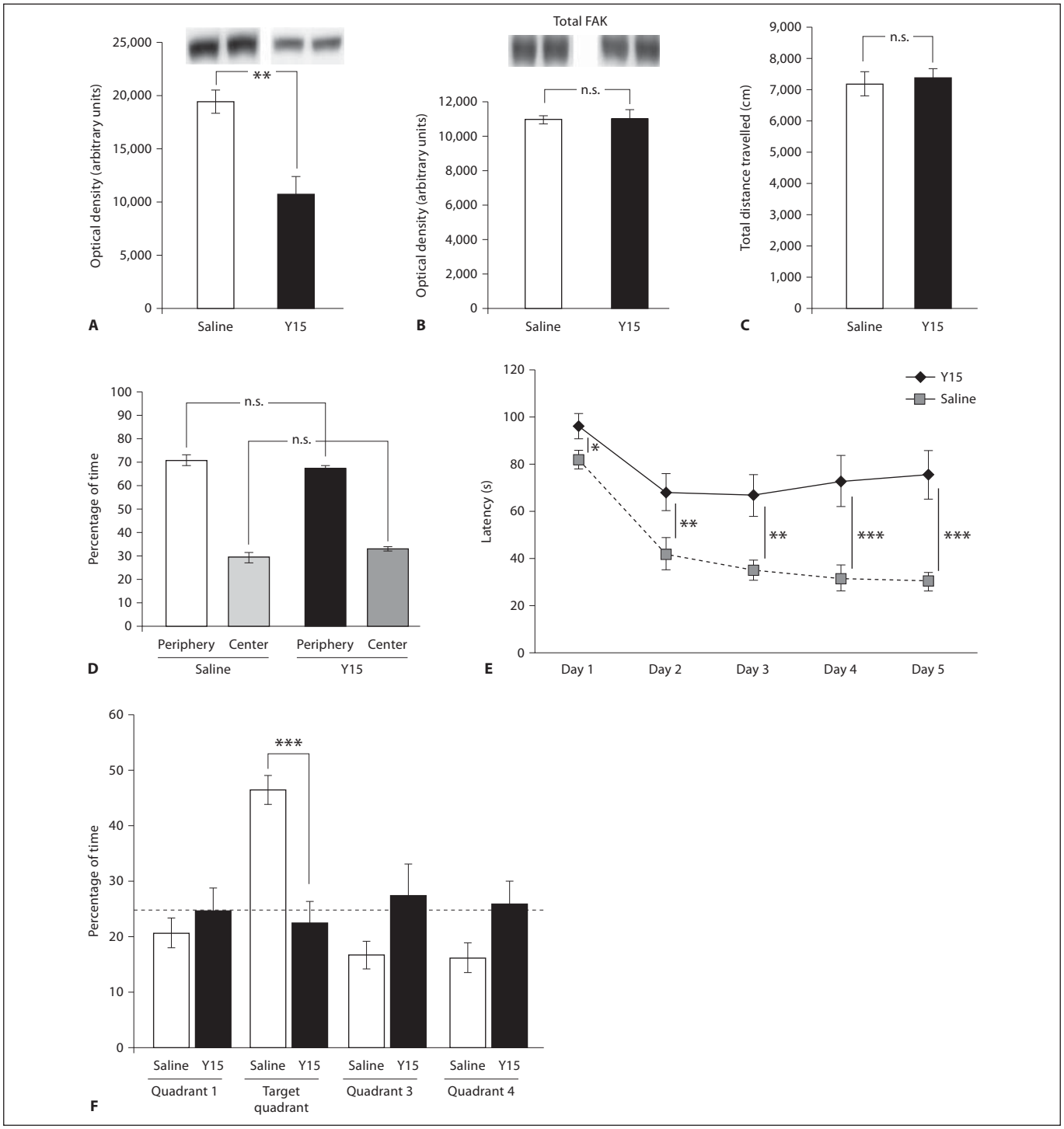


**Fig. 5.** Inhibition of FAK impairs hippocampus-dependent spatial learning and memory. Protein levels of Y397 phosphorylated FAK (**A**) and total FAK (**B**) in hippocampal tissue of Y15-treated (2 mg/kg) and saline control mice. Protein levels are displayed as optical densities in arbitrary units. Total distance traveled (**C**) and time spent (**D**) in the center and in the periphery in the OF in Y15-treated and saline control mice. **E** Latency to find the hidden platform in the MWM during 5 days of training (average of three trials per day) in Y15-treated and saline control mice. **F** Percentage of time spent in the target quadrant (original location of the platform during training) versus the other three quadrants during the probe trial in Y15-treated and saline control mice. Data are displayed as mean ± SEM. Statistical evaluation resulting from pairwise comparison and post hoc analysis is depicted. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = Not significant.

**Table 1.** Results of the primary behavioral observation screen in Y15-treated and saline control mice

| | Saline | Y15 |
|---|---|---|
| Muscle/lower motor neuron and spinocerebellar function | | |
| Body position | 5.9 ± 0.3 | 5.8 ± 0.3 |
| Atactic gait | 0 | 0 |
| Hypotonic gait | 0 | 0 |
| Impaired gait | 0 | 0 |
| Limb rotation | 0 | 0 |
| Spatial locomotion | 2.1 ± 0.6 | 1.8 ± 0.3 |
| Locomotor activity | 4.3 ± 0.4 | 4.1 ± 0.5 |
| Wire maneuver | 1.0 ± 0.4 | 0.7 ± 0.4 |
| Pelvic elevation | 2.0 ± 0.3 | 2.1 ± 0.1 |
| Tail elevation | 3.4 ± 0.5 | 4.4 ± 0.7 |
| Visual placing | 4.3 ± 0.6 | 4.4 ± 0.5 |
| Abdominal tone | 3.8 ± 0.4 | 3.5 ± 0.5 |
| Limb tone | 3.8 ± 0.4 | 3.1 ± 0.5 |
| Grip strength | 4.4 ± 0.9 | 4.5 ± 0.8 |
| Vestibular drop | 1 | 1 |
| Proprioception | 1 | 1 |
| Sensory function | | |
| Transfer arousal | 4.2 ± 0.6 | 4.3 ± 0.7 |
| Pinna reflex | 3.8 ± 0.4 | 3.5 ± 0.7 |
| Corneal reflex | 3.7 ± 1.4 | 3.3 ± 0.9 |
| Tail pinch | 2.8 ± 1.0 | 2.6 ± 0.5 |
| Toe pinch | 1.8 ± 0.3 | 1.3 ± 0.4 |
| Finger approach | 1.9 ± 0.5 | 1.7 ± 0.6 |
| Finger withdrawal | 3.9 ± 0.6 | 3.6 ± 0.5 |
| Neuropsychiatric function | | |
| Bizarre behavior | 0 | 0 |
| Tremor/twitches | 0 | 0 |
| Provoked biting | 0 | 0 |
| Provoked freezing | 0 | 0 |
| Biting tendency | 2.5 ± 0.8 | 2.3 ± 0.9 |
| Autonomous function | | |
| Palpebral closure | 0 | 0 |
| Skin color | 4.1 ± 0.4 | 4.3 ± 0.8 |
| Respiratory rate | 4.5 ± 0.4 | 4.8 ± 0.5 |
| Hypothermia | 0 | 0 |
| Urination/defecation | 1.2 ± 1.2 | 1.2 ± 0.5 |
| Salivation | 0 | 0 |
| Piloerection | 1.9 ± 0.5 | 1.8 ± 0.4 |

Scores (mean ± SD) as evaluated by Irwin [35]: higher score presents more (better, higher) activity (performance, response) or parameters are scored as present (1) or absent (0).

atory activity in the OF. No significant differences in total distance travelled ($p > 0.05$) (fig. 5C) and time spent in the center ($p > 0.05$) (fig. 5D) were observed among groups. Forty-eight hours after the OF test, mice of both groups were subjected to training in the MWM. Latency to reach the hidden platform averaged over three daily trials was used as parameter for spatial learning and memory. Significant differences between Y15-injected mice and saline controls were observed on all 5 days of training (effect of day of training $F_{(4,23)} = 15.07$: $p < 0.0001$, effect of Y15 $F_{(1,26)} = 28.68$: $p < 0.0001$) (fig. 5E). Twenty-four hours after the last day of training, a probe trial in which the platform had been removed was carried out and the percentage of time spent in the quadrant which had originally contained the platform was used as indicator of spatial memory. While control mice showed a significant preference for the target quadrant, Y15-injected mice did not spend significantly more time in the target than in adjacent quadrants (fig. 5F). Taken together, these findings indicate that FAK activity is required for hippocampus-dependent spatial learning and for its long-term retention.

*FAK Inhibition Alters the Levels of Hippocampal Synaptic Proteins*

To evaluate the effects of FAK inhibition on synapse composition, levels of pre- and post-synaptic marker proteins were determined in hippocampal tissue of Y15-treated and saline-treated control mice. A significant reduction of synapsin I ($p < 0.05$), PSD-95 ($p < 0.01$) and drebrin ($p < 0.01$) protein levels induced by Y15 treatment was revealed by Western blot (fig. 6A–C).

## Discussion

The results of our experiments indicate that the non-receptor tyrosine kinase FAK is a fundamental player in the regulation of neuronal growth, synaptic function and plasticity of hippocampal neurons, as well as a necessary element for hippocampus-dependent spatial learning and memory.

*FAK and Hippocampal Neuronal Outgrowth*

Using both loss- and gain-of-function approaches, we here provide evidence that FAK plays a major role as a positive regulator of the morphological properties of hippocampal neurons. Our data suggest that FAK might act as mediator conveying the effects of neurotrophins, such as NGF, on cytoskeletal rearrangement underlying neuritic outgrowth, similarly as proposed for growth factors including platelet-derived and epidermal growth factors (PDGF and EGF, respectively) [41]. It has been shown that neuronal stimulation with NGF results in rapid and specific activation of both the FAK and the MAPK signaling pathways and that inhibition of FAK activity in this con-

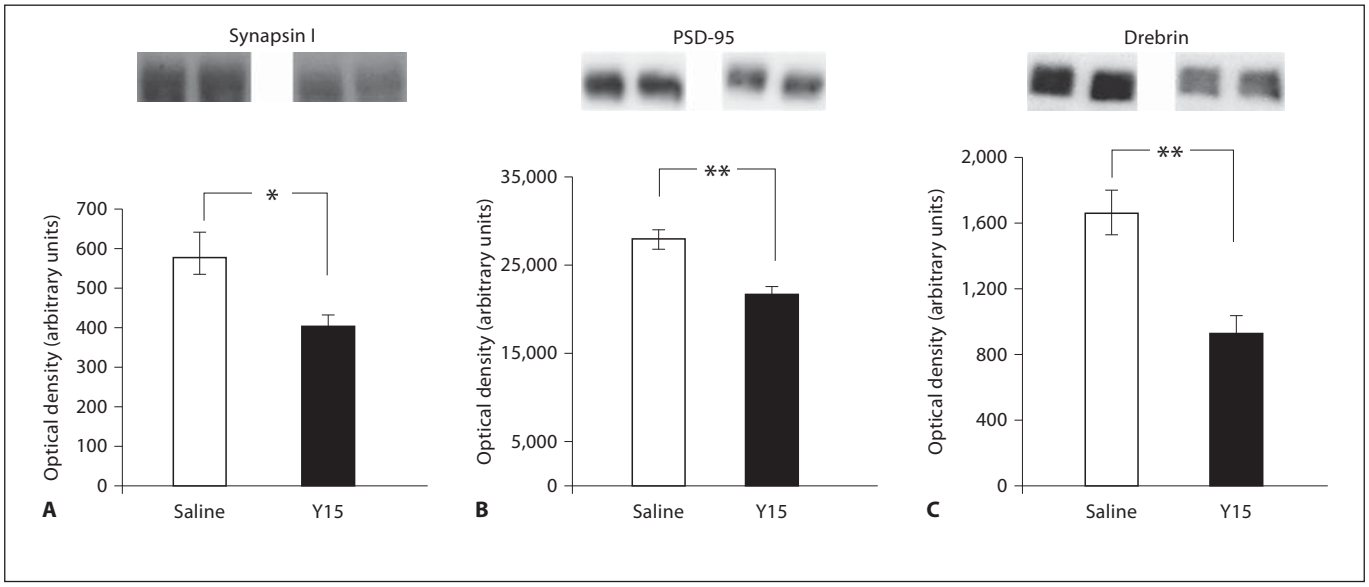


**Fig. 6.** Inhibition of FAK modulates levels of synaptic proteins in the mouse hippocampus. Synapsin I (**A**), PSD-95 (**B**) and drebrin (**C**) protein levels determined by Western blot experiments in hippocampal tissue of saline control and Y15-treated (2 mg/kg) mice. Protein levels are displayed as optical densities in arbitrary values. Data are displayed as mean ± SEM. Statistical evaluation resulting from pairwise comparison is depicted. * $p < 0.05$, ** $p < 0.01$.

text attenuates both NGF-induced PI3-K/Akt and MEK/MAPK signaling pathways, as well as neurite growth [42]. Previous reports indicate that FAK is an important modulator of the NGF signaling pathway, suggesting the possibility that FAK might interfere with the regulation of TrkA receptor levels induced by NGF [43]. However, the specific mechanism linking the mediation of FAK in neurotrophin signaling still remains unclear. Our data indicate that FAK activity related to NGF signaling is importantly related to the modulation of structural properties of maturing neurons, morphological features which are prerequisites for memory-related synaptic activity in hippocampus [42].

*FAK as a Regulator of Hippocampal Synaptic Function and Plasticity*

We found that FAK is an important regulator of spontaneous and evoked synaptic activity and plasticity in hippocampal neurons. Electrophysiological recordings in cultured hippocampal neurons revealed an important role for FAK as a mediator of both frequency and amplitude of spontaneous mEPSCs and as mediator of the NGF-dependent modulation of spontaneous release. The observation that FAK inhibition impacts on mEPSCs amplitude and the frequency suggests a role for FAK signaling in pre- and postsynaptic plasticity-related mechanisms. Postsynaptic modulation of growth factor and neurotrophin receptor function may account for the effect of FAK on mEPSCs amplitude. Moreover, FAK is also necessary for the EphB receptor tyrosine kinase-induced formation of dendritic spines [16] and may thus impact postsynaptic architecture. Modifications of mEPSCs frequency point towards an involvement of FAK in the regulation of presynaptic neurotransmitter release probability. FAK is importantly involved in signaling pathways originating from integrin-mediated cell adhesions sites and cell adhesion molecules have been described to be required for excitatory synapse formation [44, 45] and to critically affect mEPSCs frequency [45]. Indeed, integrin signaling has been found to be important for coordinating exocytotic events [46] and disruption of FAK activity might partially impair the neuronal exocytotic machinery, thus hampering neurotransmitter release. Important roles for mEPSCs in the regulation of neuronal firing [47], synaptic plasticity [48], dendritic spine density [49] and local protein synthesis have been proposed [50]. Thus, FAK may contribute to these processes also through a regulation of spontaneous neurotransmitter release at hippocampal synapses.

Given the evidence provided by our morphological and electrophysiological studies on the involvement of

FAK in hippocampal synaptic activity, we set out to investigate the role of this kinase in the plasticity of hippocampal synaptic circuits. A pioneering study has shown that FAK is required for the induction phase of LTP in the dentate gyrus of rats [40]. However, other aspects of FAK involvement in long-term plasticity in the hippocampal circuitry remain unexplored. We therefore studied here the role of FAK in the memory-related Schaffer collateral-CA1 pathway. The results of our experiments show that inhibition of FAK does not affect basal synaptic transmission or high-frequency-induced post-tetanic potentiation but significantly impairs the protein synthesis-dependent maintenance phase of hippocampal LTP. Interestingly, the pioneering study on the involvement of FAK in hippocampal synaptic plasticity at the network level in the dentate gyrus also showed that inhibition of FAK activity resulted in a deficit of the protein synthesis-dependent induction of LTP [40]. Thus, our studies on the Schaffer collateral-CA1 neuronal pathway functionally complement the findings of the Yang et al. [40] study of the dentate gyrus. Together with our here first described in vivo studies, these findings point towards a general relevance of the role of FAK within the hippocampal formation and consequently in learning and memory formation and retention.

There are several possible mechanisms by which FAK inhibition may lead to impairment in the maintenance of Schaffer collateral-CA1 LTP. First, FAK is a scaffolding molecule which is known to form complexes with integrin receptors which convey signals from the extracellular matrix to the cytoskeleton [for a review, see 51]. In fact, the effects of integrin antagonists, preventing the establishment of a stable phase of synaptic potentiation after LTP induction [52], are similar to those observed herein after FAK inhibition. This observation suggests that deficient integrin signaling might contribute to the LTP impairment induced by inhibition of FAK. Moreover, FAK directly activates target proteins, such as neural Wiskott-Aldrich syndrome protein (N-WASP), that are crucial regulators of the actin complex during the cytoskeletal reorganization required for the formation of dendritic spines [53]. Additionally, FAK is also required for the EphB receptor-modulated regulation of dendritic spine morphology through RhoA activation [16]. Considering the pivotal role of dendritic spines as postsynaptic terminals of excitatory synapses for synaptic plasticity, it can be hypothesized that deregulation of the molecular processes mediating spine formation, such as insufficient activation of N-WASP or RhoA, resulting from inhibition of FAK, may also contribute to altered LTP [16]. Third, FAK is known as cellular partner for the Src family of protein kinases and FAK and Src are known to activate each other [for a review, see 54]. Inhibition of Src kinases has been shown to prevent potentiation of glutamatergic N-methyl-D-aspartate (NMDA) receptor-mediated transmission, critical for the stabilization of LTP at hippocampal synapses [55]. FAK inhibition might thus also disrupt NMDA receptor plasticity, ultimately affecting hippocampal LTP [4–6]. Finally, the effects of FAK on LTP may also be related to the interaction of FAK with the NGF/Trk pathway which we are indicating herein. In fact, it has been demonstrated that increase in the levels of NGF facilitate the induction of hippocampal LTP, and that blocking endogenous NGF results in a significant reduction of hippocampal LTP [56–58]. TrkA activation by NGF is also inhibited by treatment with an inhibitor of Src family kinases [55]. Since FAK and Src are capable of mutual activation [54] and given that activated Src can also phosphorylate TrkA directly in vitro [55], alterations of FAK activity might also impact LTP by regulating the heteromeric signaling complexes associated to the NGF/TrkA pathway.

*FAK Is Involved in Hippocampus-Dependent Spatial Learning and Memory*

Since the role of FAK for learning and memory in vivo has not yet been determined, we here examined hippocampus-dependent spatial learning and memory in mice while pharmacologically inhibiting FAK activity as a final step in characterizing the role of FAK in synaptic plasticity. Our findings that FAK inhibition resulted in deficient learning in the MWM task, as well as in impaired retention and retrieval of the spatial memory, are well in line with our studies of spontaneous synaptic activity carried out on individual neurons and also in agreement with our results obtained from the LTP analysis of hippocampal slices, and therefore are most likely to be mediated by the same molecular mechanisms. Our data indicate that at dosages effectively inhibiting FAK activity, Y15 can be used as a valuable tool in the study of the physiological role of FAK in the nervous system and its involvement in memory-related synaptic plasticity.

By demonstrating reduced hippocampal levels of synaptic proteins, including synapsin I, a nerve terminal-specific synaptic vesicle-associated marker protein, the postsynaptic marker protein PSD-95 and the actin-binding protein drebrin, a marker for dendritic spines, we provide evidence that the involvement of FAK in hippocampal-mediated spatial memory formation and mainte-

nance could be in part attributable to a participation of FAK in the regulation of synapse formation and composition. These data are also in agreement with results suggesting a functional link between the FAK and the NGF pathway in the regulation of both structural/morphological and electrical synapse-related properties of neurons. This first demonstration of a role for FAK in cognitive processes in the intact animal complements our results from morphological and functional analyses at the single cell and network level and allows a comprehensive evaluation about the role of FAK as critical positive regulator of learning-related synaptic plasticity in the mouse hippocampus.


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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

# Alzheimer's disease risk factor lymphocyte-specific protein tyrosine kinase regulates long-term synaptic strengthening, spatial learning and memory

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**Abstract** The lymphocyte-specific protein tyrosine kinase (Lck), which belongs to the Src kinase-family, is expressed in neurons of the hippocampus, a structure critical for learning and memory. Recent evidence demonstrated a significant downregulation of Lck in Alzheimer's disease. Lck has additionally been proposed to be a risk factor for Alzheimer's disease, thus suggesting the involvement of Lck in memory function. The neuronal role of Lck, however, and its involvement in learning and memory remain largely unexplored. Here, in vitro electrophysiology, confocal microscopy, and molecular, pharmacological, genetic and biochemical techniques were combined with in vivo behavioral approaches to examine the role of Lck in the mouse hippocampus. Specific pharmacological inhibition and genetic silencing indicated the involvement of Lck in the regulation of neuritic outgrowth. In the functional pre-established synaptic networks that were examined electrophysiologically, specific Lck-inhibition also selectively impaired the long-term hippocampal synaptic plasticity without affecting spontaneous excitatory synaptic transmission or short-term synaptic potentiation. The selective inhibition of Lck also significantly altered hippocampus-dependent spatial learning and memory in vivo. These data provide the basis for the functional characterization of brain Lck, describing the importance of Lck as a critical regulator of both neuronal morphology and in vivo long-term memory.

**Keywords** Lck kinase · Hippocampal synaptic strengthening · Learning and memory · Alzheimer's disease

## Introduction

The non-receptor Src protein kinase was discovered more than 30 years ago by identifying the transforming single viral gene of the avian Rous sarcoma virus [1] and determining that this viral gene encoded a protein kinase associated with a phosphoprotein [2]. Several years later, the identification of Src homologues led to the proposal of a new family of non-receptor protein kinases: the Src kinase family. This family is characterized by the presence of a distinctive molecular structure consisting of several SH (Src homology) functional domains including a highly conserved catalytic (SH1) region flanked by a C-terminal auto-inhibitory phosphorylation motif [3–5]. The entire Src family is divided into two major subfamilies: subfamily-A (including Src, Fyn, Fgr and Yes) and subfamily-B (including Hck, Lyn, Blk and the protein Lck, which is also known as p56Lck [6]). An additional type of Src proteins, the Frk proteins, has also been described [3, 4]. Members from both the A and B subfamilies have been shown to play important roles in neuritic outgrowth and synaptic activity [7–14], but only Fyn, Src, Yes, Lyn and Lck have

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been identified in the central nervous system [3, 5, 9, 15–18].

Recent clinical reports described significantly down-regulated levels of Lck in the hippocampus of Alzheimer's disease patients [19], suggesting an association of Lck with this memory-related disorder. Indeed, the human Lck gene has been located in the Alzheimer's disease-associated genetic linkage region 1p34-36 [20]. Lck has been further proposed to constitute a novel risk-gene for Alzheimer's disease [21]. These observations have increased the interest in Lck in clinical and basic neuroscience research [19–21] because, although Lck was identified in the brain approximately 15 years ago [22], it had previously been classically known for its role in T cell activation [23–25]. The role of Lck in memory remains mostly unexplored, and it is still unclear whether the down-regulation of Lck plays a pathogenic role in Alzheimer's disease.

Here, the role of Lck in the memory-related functions of the mouse hippocampus was studied, and Lck is proposed as a key regulator of both the morphological and electrical properties of neurons. It is reported that Lck critically modulates neuritic outgrowth and long-term memory. The inhibition of Lck selectively impairs the protein synthesis-dependent long-term forms of memory-related synaptic plasticity without affecting the protein synthesis-independent short-term forms of synaptic strengthening. Additionally, the selective in vivo inhibition of Lck significantly impairs hippocampus-dependent spatial learning and memory.

To the best of our knowledge, these data constitute the first comprehensive in vitro and in vivo functional characterization of Lck in the mammalian brain, suggesting that Lck is a critical mediator of the acquisition and long-term maintenance of memories, which are the most salient processes impaired in Alzheimer's disease. Because down-regulated levels of Lck have been reported in the hippocampus of patients with Alzheimer's disease [19], current animal studies may provide the first functional evidence linking the effects of the in vivo down-regulation of Lck with some of the memory-related deleterious structural and functional features that are also observed in the pathophysiology of Alzheimer' disease in humans.

## Materials and methods

### Animals and housing

Adult C57BL/6J male mice (Janvier, France) that were 10–12 weeks old were housed in pairs in standard transparent laboratory cages in a temperature-controlled colony room (21 ± 1 °C) and were provided with food and water ad libitum. The mice were maintained on a 12-h light/dark rhythm, and the cages were cleaned once a week. The experiments were performed under license of the federal ministry of education, science and culture, which includes an ethical evaluation of the project (Project: BMBWK-66.009/0036/BrGT-2006). The housing conditions and the maintenance of the animals were in compliance with European and national regulations. All efforts were exerted to minimize the degree of animal suffering and the number of animals used.

### Hippocampal culture

Postnatal mouse (day 0–2) hippocampal neurons were dissociated and cultured according to standardized procedures [26] and complemented with glial support cultures as previously described [27].

### Cell viability assay

To evaluate the effects on cell survival of the different compounds used in this work (damnacanthal, Herbimycin-A, and 4A6H), hippocampal neurons (DIV1) were plated on 96-well plates at an average density of $5 \times 10^4$ cells/well, and MTT assays were performed in quintuplicate for each experimental compound. The colorimetric MTT assays were conducted as previously described [28]. The cells were treated with increasing concentrations of the respective compound or DMSO alone as a control and incubated with 0.5 mg/mL MTT-formazan (Sigma-Aldrich, Poole, Dorset, UK) at 37 °C under 5 % $CO_2$ for 1–4 h and then washed in PBS. The blue formazan reduction product, which is produced by the action of succinate dehydrogenase in living cells, was dissolved in 100 µL of DMSO and incubated for 10 min. The optical density was then measured at 550 nm using a BioTek Ultra Microplate reader BL808 (BioTek, Winooski, VT, USA). The data are expressed as the percentage of viable cells compared with the number of control cells (100 % viability), as determined by MTT reduction.

### Morphological evaluation

DIV0 cells were treated with different concentrations of Damnacanthal (Enzo Life Sciences) for 24 h as described in the text. A morphological analysis was performed using conventional confocal microscopy (Axiovert 200M; Zeiss, Vienna, Austria). The images were analyzed using the public domain open source software ImageJ (http://rsbweb.nih.gov/ij/). The degree of neuritic outgrowth was evaluated using previously described parameters [29, 30]. Briefly, low-density hippocampal neuronal cultures were established and used for the morphological analysis. Only those neurons that were growing in isolation, that is,

neurons whose branches did not make visible contacts with any other cell as determined using a ×40 objective and bright field microscopy, were used for the analyses. The field areas were visualized under the microscope, and representative pictures from the neurons obtained from the different experimental conditions were obtained. The ImageJ software was used to manually trace-track and measure the length of individual neurites for each of the different cells measured, and only primary (derived from the soma) neuritic branches were analyzed. Because no significant differences in the number of neuritic extensions derived from the soma were observed, we arbitrarily selected for analysis only those cells with neuritic branches more than 10 μm in length. The cells were imaged by a researcher blinded to the experimental conditions. Comparisons between the experiments (triplicate per group) were performed using different animals and cell batches. A solution of 1 % B27 supplement media was used during drug studies. On average, 25–35 isolated cells per group were analyzed.

## Transfection

The DIV0 cells were transfected with Lipofectamine® LTX (Invitrogen, Carlsbad, CA, USA) and Nupherin™-neuron (Biomol, Plymouth Meeting, PA, USA) according to the manufacturer's protocols with minor modifications that have been previously described [31]. A genetically based approach that was previously used to successfully induce silencing of the Lck protein [32] was followed in this work using the murine Lck shRNAs and the scrambled nonspecific shRNAs controls in the pGeneClip hMGFP vector (Qiagen, Hilden, Germany). Briefly, 0.5 μg of the murine Lck shRNAs or the scrambled nonspecific shRNA control were incubated with 15 μg of Nupherin™-neuron in 150 μL of Neurobasal-A medium (Invitrogen) while 0.5 μL of Lipofectamine® LTX (Invitrogen) was mixed in 150 μL of Neurobasal-A medium. After 15 min, the two solutions were mixed and incubated for 45 min at 21 ± 1 °C. The neuronal cultures were incubated in 24-well plates in the resulting 300 μL mixture for 5 min, centrifuged in a swinging bucket centrifuge at 233*g* for 5 min and incubated for 2.5 h. The mixture was then substituted with 1 mL of culture medium, and the cells were transferred to a culture incubator in 5 % $CO_2$ at 37 °C. After 24 h, the cells were fixed in 4 % paraformaldehyde at 21 ± 1 °C for 30 min, and the paraformaldehyde was subsequently removed by washing with PBS. The confocal imaging was performed as previously described [33]. The neuritic outgrowth was also evaluated using ImageJ software (http://rsbweb.nih.gov/ij/). PC12 cells were cultured at a density of $5 \times 10^5$ cells on 10-cm Petri dishes coated with collagen (Sigma-Aldrich) and maintained in DMEM supplemented with 5 % fetal bovine serum, 10 % horse serum, and 1 % penicillin/streptomycin. The PC12 cells were transfected 12 h after plating using Lipofectamine® LTX (Invitrogen) according to the manufacturer's protocols. Briefly, 10 μg of the murine Lck shRNAs or the scrambled nonspecific shRNA control were incubated with 10 μL of PLUS™ reagent (Invitrogen) in 1 mL of Opti-MEM® reduced-serum media while 20 μL of Lipofectamine® LTX was diluted in 1 mL of Opti-MEM® reduced-serum media. After 10 min, the two solutions were combined and incubated for 25 min at 21 ± 1 °C, and the PC12 cells were then incubated in the resulting 2 mL mixture in 5 % $CO_2$ at 37 °C. The efficiently transfected cultures (60–70 %) were used for the subsequent RNA extraction and qRT-PCR analysis.

## RNA isolation, cDNA synthesis and qRT-PCR

The RNA from the PC12 cells was isolated using the RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's instructions. A 1-μg aliquot of the total RNA was used for cDNA synthesis using SuperScript III (Invitrogen) according to the protocol supplied.

The qRT-PCR experiment was performed using specific primers that have been previously described for Lck [34] and *β*-actin [35]: Lck forward (CCAGCTCACAATGCCAGCAG) and reverse (GCTCGGGGAGGGTTCATTC); *β*-actin forward (ATGGTGGGAATGGGTCAGAAG) and reverse (TCTCCATGTCGTCCCAGTTG). The qRT-PCR was performed on diluted cDNA samples using the StepOne Real-Time PCR system (Applied Biosystems) and the Power SYBR Green PCR Mastermix (Applied Biosystems) under universal cycling conditions (10 min denaturing step; 10 min at 95 °C; 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and 15 s at 72 °C and a melting point analysis in 0.1 °C steps). The data were analyzed using the cycle threshold method and normalized relative to the *β*-actin expression control [35]. The experiments were performed in triplicate.

## Single-cell electrophysiology

Primary hippocampal neurons (10–14 DIV) were used for electrophysiological measurements of the mEPSCs. The cells were untreated (control) or treated with either 200 nM or 10 μM damnacanthal (Biomol) 2 h prior to the recordings. The mEPSCs were acquired using the whole-cell configuration of the patch-clamp technique at 21 ± 1 °C and clamped at −70 mV. The bath solution contained 125 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 15 mM glucose, and 10 mM Hepes, and the pH was adjusted to 7.4 with NaOH. Solutions of 0.5 μM tetrodotoxin (TTX) and 30 μM

bicuculline-methiodide were used to inhibit sodium-channel conductance and to suppress miniature inhibitory postsynaptic currents, respectively. The borosilicate-glass recording electrodes were pulled using a horizontal puller (Sutter Instruments) to obtain resistances of 5–7 MΩ when filled with a solution containing 140 mM KCl, 1.6 mM $CaCl_2$, 10 mM EGTA, 10 mM Hepes, and 2 mM Mg-ATP, adjusted to pH 7.3 with KOH. Events <5 pA and events with a slow rise time (>5 ms) were excluded from the analysis. The currents were obtained and analyzed using a NPI SEC-10L amplifier (NPI Electronic, Tamm, Germany) and Axon and Heka amplifiers, the FitMaster software package (HEKA Elektronik, Lambrecht, Germany) and a pClamp-10 (Axon Instruments, Foster City, CA, USA). All data are given as the mean ± SEM.

## Brain slice preparation

The hippocampal slices were prepared and the electrophysiological measurements were recorded according to previously described procedures [36, 37] with minor modifications. In brief, male (7–11 weeks old) C57BL/6J mice (Janvier, France) were narcotized with $CO_2$, swiftly sacrificed by cervical dislocation and decapitated. The brains were quickly extracted and immersed in ice-cold (~4 °C) "dissection" artificial cerebrospinal fluid solution (D-ACSF) containing 125 mM choline-chloride, 2.5 mM KCl, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 6 mM $MgCl_2$, 25 mM D-glucose, and 1.25 mM $NaH_2PO_4$ (all from Sigma-Aldrich, Vienna, Austria).

The hippocampi were isolated while the tissue was submerged in D-ACSF. Transverse slices (400 µm) were obtained using a McIlwain Tissue Chopper (Mickle Laboratory Engineering, UK). The slices were quickly transferred to a nylon-mesh floating in a home-customized interface chamber containing "recording" artificial cerebrospinal fluid solution (Rec-ACSF) maintained at 21 ± 1 °C. The slices were allowed to recover for 1 h at 21 ± 1 °C and 30 min at 28 °C before the recordings were performed. During the dissection, recovery and recording, the slices were continuously bubbled with a saturating carbogen mixture (95 % $O_2$/5 % $CO_2$) adjusted to pH 7.4. The field potential recordings of the slices were performed at a temperature of 28 °C. The Rec-ACSF solution contained 125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM D-glucose, and 1.25 mM $NaH_2PO_4$ (all from Sigma-Aldrich).

## Slice electrophysiology and data analysis

It has been reported that, whereas a brief period of hippocampal theta-burst stimulation induces a protein synthesis-independent short-term synaptic potentiation, prolonged stimulation gives rise to a sustained or long-term protein synthesis-dependent synaptic potentiation [38–40]. Here, an electrical stimulation was applied to the hippocampal Schaffer collateral/associational commissural pathway using a home-customized bipolar tungsten electrode insulated to the tip (50 µm tip diameter). The stimuli were delivered using an ISO-STIM 01D isolator stimulator (NPI Electronics, Tamm, Germany). The field excitatory postsynaptic potentials (fEPSPs) were obtained from the CA1 stratum radiatum area of the hippocampus using borosilicate glass micropipettes that yield resistances of 2–5 MΩ when filled with Rec-ACSF. The stimuli (80 µs) were delivered at intervals of 15 s, and the slope of the decaying phase (20–80 % of the peak amplitude) of the fEPSP was used to estimate the strength of the synaptic transmission. Stable baseline measurements were obtained for at least 10 min using stimulation intensities eliciting ~40 % of the maximal inducible response (5 min before the theta-burst stimulation). The early or short synaptic potentiation (E-LTP) was induced by applying a single high-frequency train of pulses (pulses delivered at 100 Hz for 1 s, 80 µs/pulse) as previously described [38–40]. The late or long-term (L-LTP) form of synaptic potentiation was induced by four trains of theta-burst stimulation (15 bursts of four pulses at 100 Hz (80 µs/pulse) with inter-burst intervals of 200 ms and inter-train intervals of 5 min) [38–40]. The recordings ($n$ = 10–20) were obtained for at least 30 min after each standard LTP-inducing stimulation protocol. The slope values from the fEPSPs obtained after the theta-burst stimulation were normalized to the average of their respective baseline slopes. The data were obtained using an AxoClamp-2B amplifier (Bridge mode) and a Digidata-1440 interface (Axon Instruments), and the pClamp-10 Program software (CA/Molecular Devices, USA) was used for the data analysis.

## Behavioral analysis

Two groups of animals (10 per experimental group; one group of untreated controls, which were injected with a saline/DMSO solution, and a second group injected with damnacanthal/DMSO) were used for the behavioral analysis (an extra set of different animals was used for the western blot analysis; see below). For the behavioral studies, damnacanthal (5 mg/kg) or saline with DMSO (final concentration of 8 %) was injected i.p. at an injection volume of 10 mL/kg. All the experimental trials in which the behavior was examined throughout the entire period of study were preceded by the corresponding i.p. injection. The behavioral analyses were performed 45 min after the injection.

*Basic neurological examination*

To examine the effects of the Lck inhibitor damnacanthal on the major neurological behavioral features and reflexes of the experimental subjects, a series of tests were implemented 45 min after injection as previously described [33, 41].

*Open field (OF)*

The open field consisted of a rectangular box of Plexiglas (40 × 40 × 60 cm) in which locomotor activity was monitored for 1 h using a CCD camera (Panasonic, Secaucus, NJ, USA). The locomotor activity, which was evaluated 45 min after the i.p. injection as the total distance traveled (cm), was automatically measured using a Limelight video tracking system (Limelight Actimetrics, Elmwood, IL, USA). A solution of 70 % ethanol was used to clean the equipment between each examination.

*Rota rod*

The motor coordination and balance were tested using an automated 5-station mouse system (Model ENV-575MA; Med-Associates, Georgia, Vt, USA). The animals were tested 45 min after the i.p. injections using a previously described protocol [42] with minor modifications. The mice were placed on the rod when the rod started moving, with the snout of the animal facing the same direction as the moving belt, and the speed of rotation was set to systematically increase from 4 to 40 rpm over a 5-min period. The time until the mouse fell off the drum (latency) was recorded and used for the analysis.

*Morris water maze*

The Morris water maze (MWM) consisted of a standard circular pool (122 cm diameter with walls 76 cm deep). The mice were trained to escape by swimming to a hidden platform located 1.5 cm beneath the water surface. The platform location was identified using distal extra-maze cues attached to the room walls and remained constant during the entire experiment. The pool (with a water temperature of 21 ± 1 °C) was monitored using a digital camera, and the pool image was digitally divided into four quadrants by a computerized tracking/image analyzing system (Limelight Actimetrics). From a total of 20 mice, 10 mice were used per group (control vs. treated). Each group was subdivided into two sub-groups of 5 mice each. The hidden platform was changed from south-east to north-east between sub-groups. The platform was placed in the middle of the respective quadrant and remained at the same position during the acquisition phase (see below). Each day, and 45 min before the first training trial, the mice were injected either with damnacanthal or with a diluting saline control solution. The day before starting the measurements, each mouse received a pre-acquisition session in which it was placed directly on the platform and required to stay for a minimum of 15 s. The mice were then allowed to have 30 s of a free swimming period before being guided back to the platform. This pre-acquisition consisted of 3–4 test trials in which each mouse was required to climb by itself onto the platform. On the first day of the measurements, the mice received single and final reinforcement trials in which the mice were placed directly onto the hidden platform and required to remain for at least 15 s. The acquisition phase then began and consisted of three trials per day (with inter-trial intervals of 15 min) in which the mice were placed into the pool containing the hidden platform and allowed to swim freely. The behavioral measurements for each acquisition trial started when the mouse was released into the water, and the recordings were obtained for at least 90 s. The mice were released while facing the outer edge of the pool at one of the quadrants (except the quadrant in which the platform was located). A trial was terminated when the animal located the platform and remained on it for at least 15 s. During the acquisition, the mice lined up and were tested successively in trains of five. Mice that failed to locate the platform within the first 90 s of the acquisition phase were manually placed onto the platform and required to remain for 15 s. The acquisition phase was extended for a period of 3 days. After the acquisition phase, the mice underwent a 1-day probe trial in which the platform was removed. In this trial, the mice were released from the longest distance to the platform and were allowed to swim freely for 60 s. Measurements of the swimming path taken by the mouse were recorded, and the proportion of swimming time and/or the path length spent in each quadrant of the pool was analyzed.

Western blotting

The dose–response was examined to evaluate the inhibitory effects and specificity of damnacanthal in vivo. Thus, two individual mice per group, which were not involved in the behavioral analysis, were subjected to intra-peritoneal injections with 2.5, 5, or 10 mg/kg of damnacanthal according to the same administration procedures used for the animals subject to the behavioral experiments. The animals were narcotized with $CO_2$ 45 min after injections, rapidly underwent cervical dislocation and were immediately decapitated. The hippocampi were then quickly dissected and snap-frozen in liquid nitrogen, and the tissue was stored at −80 °C until used. The hippocampal tissue was ground under liquid nitrogen and homogenized in a protein lysis buffer containing 10 mM Tris–HCl (pH 7.5),

**Fig. 1** Selective inhibition of Lck impairs neuritic outgrowth. (**A**) *a* An MTT assay was used to evaluate the cytotoxicity of different concentrations of damnacanthal. None of the displayed concentrations induced high levels of cell mortality. *b*, *c* Treatment with the Lck-specific inhibitor damnacanthal (200 nM) did not induce changes in the length of neurites (two-tailed $P > 0.05$) compared with those of untreated control hippocampal cells. (**B**) *a* Representative cells obtained from a group of untreated primary cultured control hippocampal neurons presented a marked development of neuritic processes (*a left*, *b*), whereas the specific inhibition of Lck with 10 μM damnacanthal resulted in a marked reduction (two-tailed $P < 0.0001$) in the development of neuritic processes (*a right*, *b*). In (*b*, *c*), the representative images of the results of western blot assays indicate that 10 μM damnacanthal is more efficient than 200 nM damnacanthal in hampering the levels of 394-phospho-Lck in primary cultured mouse hippocampal neurons. (**C**) Effect of shRNA-based inhibition of Lck on neuritic outgrowth. *a* Representative neuron transfected with the green fluorescent protein (GFP) alone. *b* Representative neuron transfected with a scrambled-control version of the Lck shRNA gene-silencing sequence (scrambled-control shRNA). *c* Representative neuron transfected with a specific and previously characterized Lck shRNA gene-silencing sequence (LCK#1). *d* Histograms representing the changes in the average neuritic length in response to neuronal transfection with four specific and previously characterized Lck shRNA gene-silencing sequences (LCK#1–4), all of which resulted in a pronounced reduction of neuritic outgrowth compared with the neurons transfected with the green fluorescent protein (GFP) alone (*a*) or with cells transfected with the scrambled-control version of the Lck shRNA gene-silencing sequence (*b*). *d* Average of neurite length/cell: ANOVA *P* value is <0.0001; Tukey–Kramer post hoc tests; GFP versus scrambled-control shRNA ns $P > 0.05$; GFP versus LCK#1 *** $P < 0.001$; GFP versus LCK#2 *** $P < 0.001$; GFP versus LCK#3 *** $P < 0.001$; GFP versus LCK#4 *** $P < 0.001$; scrambled-control shRNA versus LCK#1 *** $P < 0.001$; scrambled-control shRNA versus LCK#2 *** $P < 0.001$; scrambled-control shRNA versus LCK#3 ** $P < 0.01$; scrambled-control shRNA versus LCK#4 *** $P < 0.001$; LCK#1 versus LCK#2 ns $P > 0.05$; LCK#1 versus LCK#3 ns $P > 0.05$; LCK#1 versus LCK#4 ns $P > 0.05$; LCK#2 versus LCK#3 ns $P > 0.05$; LCK#2 versus LCK#4 ns $P > 0.05$; LCK#3 versus LCK#4 ns $P > 0.05$. (**D**) A western blot analysis (triplicate) demonstrated the presence of Lck in the PC12 cells (*a*). In *b*, the previously characterized Lck-shRNA [32] was used to inhibit Lck activity in hippocampal neurons and has the ability to hamper the levels of Lck mRNA, as demonstrated by proof-of-principle experiments in which PC12 cells were transfected with Lck-specific shRNA or scrambled-control shRNA. The mean of three experiments is indicated as a percentage of mRNA expression of the negative control (scrambled-control shRNA), as determined using qRT-PCR. (**E**) Effect of the non-receptor tyrosine kinase inhibitor Herbimycin-A on hippocampal cultured neurons neuritic outgrowth. *a* Effect of different concentrations of Herbimycin-A on the survival of neurons, as measured using the MTT assay. *b* Effect of different concentrations of Herbimycin-A on neuritic outgrowth. A one-way ANOVA and the Scheffe post-test indicated highly significant differences for 100 and 500 nM does with respect to the untreated control cells ($P < 0.001$). *c* The representative cultured hippocampal neurons treated with either 100 or 500 nM Herbimycin-A exhibited a pronounced reduction in neuritic length compared with the untreated control cells. (**F**) Effect of the selective Lck inhibitor 4A6H on the neuritic outgrowth of hippocampal cultured neurons. *a* Effect of different concentrations of 4A6H on the survival of neurons, as measured using the MTT assay. *b* Effect of different concentrations of 4A6H on neuritic outgrowth. A one-way ANOVA and the Bonferroni post-test indicated highly significant differences for 500 nM and 1 μM does with respect to the untreated control cells ($P < 0.001$). *c* The representative cultured hippocampal neurons treated with 50 nM, 500 nM or 1 μM 4A6H exhibited a pronounced reduction in neuritic length compared with the untreated control cells. To evaluate the effects on cell survival of damnacanthal, Herbimycin-A and 4A6H, hippocampal neurons (DIV1) were plated on 96-well plates at an average density of $5 \times 10^4$ cells/well, and MTT assays were performed in quintuplicate ("Materials and methods")

150 mM NaCl, 0.05 % SDS, 0.5 % Triton X100, 1 mM PMSF and a protease inhibitor cocktail (1×; Roche Diagnostics, Mannheim, Germany). Western blotting was performed essentially as previously described [43]. The membranes were blocked by incubating them with 5 % non-fat dry milk in 100 mM Tris (pH 7.5), 150 mM NaCl and 0.1 % Tween 20 (TTBS). The membranes were then incubated overnight at 4 °C with primary antibodies [Phospho Lck (Tyr 394) (1:100), Phospho Lck (Y505) (1:100); Santa Cruz Biotechnology, Santa Cruz, CA, USA; Total Lck (1:500), Phospho Fyn (1:500), Total Fyn (1:500), Phospho Fyn (1:500), Total Src (1:1,100); Abcam, Cambridge, UK]. The membranes were rinsed three times with TTBS and incubated for 1 h at 21 ± 1 °C with horseradish peroxidase-conjugated goat-anti-rabbit IgG (1:3,000) and horse-anti-mouse IgG (1:3,000) (Cell Signaling Technology, Danvers, MA, USA). The immunoreactivity was visualized using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ, USA). Quantification was performed by chemiluminescent imaging using a FluorChem HD2 (Alpha Innotech, San Leandro, CA, USA) and the corresponding software.

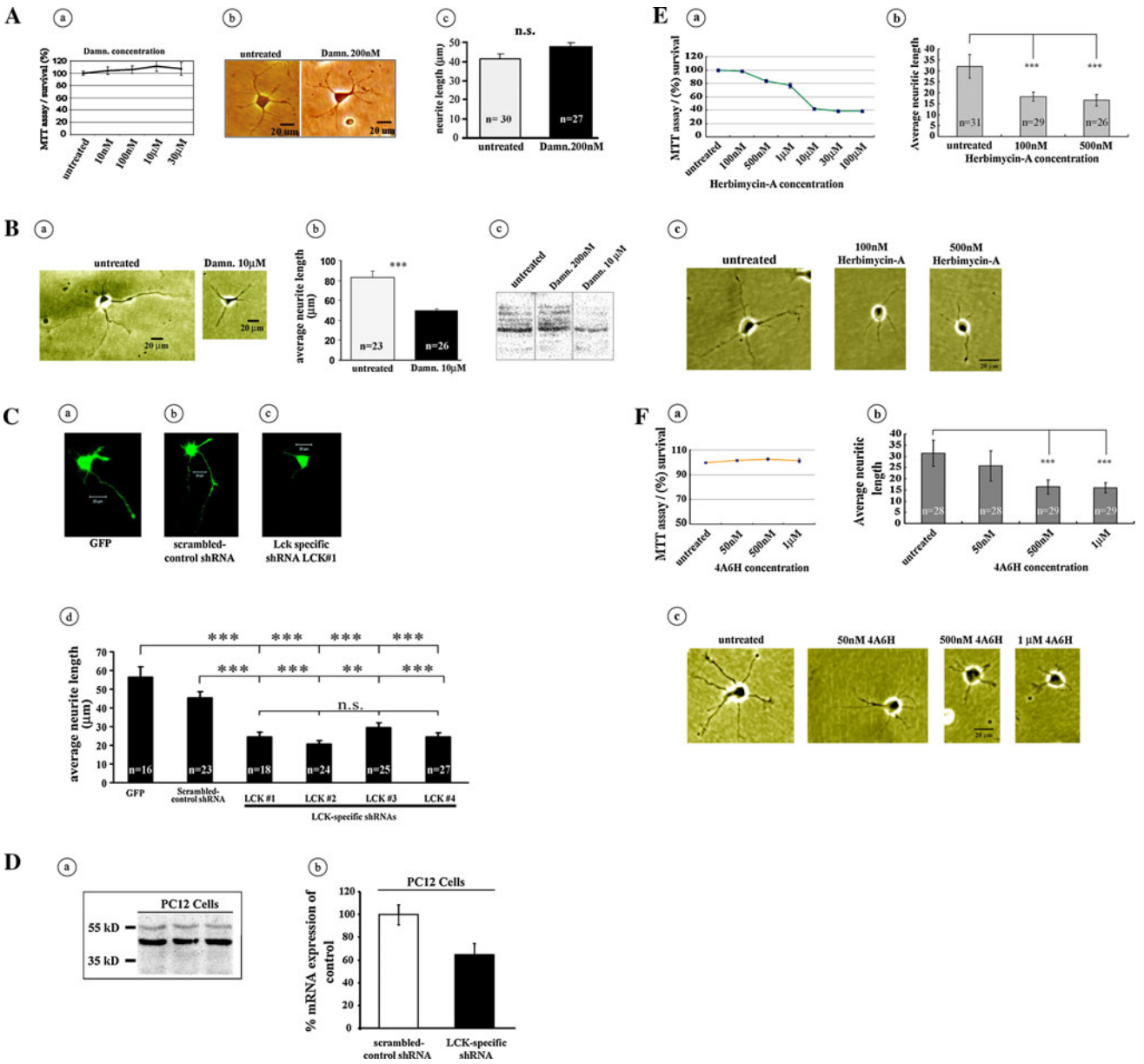
### Statistical analysis

To compare the two groups, two-tailed Student's *t* tests and Fisher's exact test were performed, and to compare more than two groups, a one-way ANOVA was used when appropriate. The LTP data and the latencies in the MWM were analyzed by repeated measures ANOVAs, with time points or training days, respectively, as the repeated measure (within-subject factor) and the pharmacological treatment as the between-subject factor. Significant main effects or interactions were evaluated using Scheffe's post hoc test. An α-level of 0.05 was adopted in all instances. All analyses were performed using BioStat 2009 professional software (AnalystSoft, Alexandria, VA, USA).

## Results

### Lck is required for neuritic outgrowth

Some Src family members have been implicated in the regulation of neuronal outgrowth [44], and the protein Lck




modulates differentiation in non-neuronal cells [45]. However, the involvement of Lck in the regulation of neuritic outgrowth in hippocampal neurons has remained unexplored. Using a combination of pharmacological and genetic down-regulation approaches, we examined the involvement of Lck in the modulation of neuritic outgrowth, a process central for synaptic strengthening [46, 47]. To inhibit Lck pharmacologically, we used damnacanthal, a specific inhibitor of Lck [48, 49] that has previously been used for selective Lck-inhibition in a wide range of non-neuronal cell types [50–53]. In previous reports, the inhibition of Lck with damnacanthal has been achieved using concentration ranges of 100–1,000 nM [52], 0.5–30 μM [54], 60 μM [51], or even 34 mM [53]. However, to the best of our knowledge, there are no reports addressing the specific concentrations of damnacanthal required to induce the inhibition of Lck in hippocampal neurons. Therefore, we first determined the potential neuronal toxicity of damnacanthal on cultured hippocampal neurons using a standard colorimetric MTT assay and observed that concentrations between 10 nM and 30 μM were nearly innocuous in terms of hippocampal neuron survival (Fig. 1A, a).

We next treated primary cultured hippocampal neurons with 200 nM damnacanthal and examined the effects on neuritic outgrowth. No differences in neuritic length were apparent between neurons treated with 200 nM damnacanthal and the corresponding internal untreated control

cells (Fig. 1A, b–c). We then tested the effects of 10 μM damnacanthal on neuritic outgrowth. In agreement with previous reports describing the involvement of other Src family members in the regulation of neuronal growth [44], 10 μM damnacanthal significantly reduced the length of neuritic processes (Fig. 1B, a–b). No significant differences in the number of neurites per cell during the different damnacanthal treatments (data not shown) were observed.

Western blot analysis using equal amounts of protein extracts and specific phospho-Lck antibodies was also used to examine the effects of 200 nM and 10 μM damnacanthal on 394-phospho-Lck-inhibition. A reduction in the levels of 394-phospho-Lck was noticeable only in the cells treated with 10 μM damnacanthal (Fig. 1B, c). We also verified the requirement of Lck for the proper development of neuritic extensions of hippocampal neurons by transfecting the neurons with Lck-shRNA-specific vectors that had previously been used for the selective silencing of Lck [32]. In agreement with the pharmacological experiments, genetic hampering of the cellular function of Lck using several different Lck-shRNA-specific silencing vectors resulted in a significant reduction in the length of neuritic extensions (Fig. C, a–d) compared with those of cells transfected with scrambled nonspecific control shRNA. To further evaluate the general biological capability of the specific Lck shRNAs as a selective inhibitor of Lck, we performed proof-of-principle Lck-shRNA transfection experiments using PC12 cells, a widely known mammalian cell line with neuronal-like morphological and functional properties. We first examined the presence of Lck in the PC12 cells using a western blot assay with a specific Lck antibody and detected a band of the size expected for the Lck protein (Fig. D, a). We then determined the levels of Lck in PC12 cells that had been transfected with the Lck-shRNA-specific silencing vectors and compared these levels with those from the PC12 cells that were transfected with scrambled-control shRNA using qRT-PCR. The PC12 cells that were transfected with the Lck-shRNA-specific silencing vectors exhibited reduced levels of Lck (Fig. 1D, b).

These two independent pharmacological and genetic experiments indicate that, although Lck does not appear to be involved in the genesis of neuritic processes, it might be implicated in the subsequent developmental stages associated with the regulation of neuritic elongation, a protracted process of neuronal maturation that eventually results in the development of functional structures required for the establishment of synaptic interactions.

The agreement between the effects of the Lck-shRNA-specific silencing vectors and those of damnacanthal, together with the data from previous reports [48–54], further endorsed the use of damnacanthal as a viable experimental strategy for the selective inhibition of Lck activity. Other experiments described below provide additional support for the use of damnacanthal as a selective inhibitor of Lck.

To complement our experiments using damnacanthal, we further examined the effects on neurite outgrowth of 4A6H, which is another previously described Lck inhibitor [55]. We also examined the effects of the general non-receptor tyrosine kinase inhibitor Herbimycin-A, a Src-family selective tyrosine kinase inhibitor [6], as a positive-control. We first performed positive-control experiments using Herbimycin-A to test the involvement of Src-family kinases in neuritic outgrowth using primary dissociated hippocampal neurons under our experimental conditions. Because elevated concentrations of Herbimycin-A have been previously described to cause cell death [56], we first performed an Herbimycin-A dose–response assay and examined the survival of the primary dissociated hippocampal neurons (Fig. 1E, a) using the MTT assay ("Materials and methods"). For this assay, cells were treated 12 h after plating with Herbimycin-A (100 nM, 500 nM, 1 μM, 10 μM, 30 μM, and 100 μM) or 1 % DMSO as an untreated control group ("Materials and methods"). After 24 h, the cells were incubated with 0.1 mg/mL MTT-Formazan at 37 °C under 5 % $CO_2$ for 4 h ("Materials and methods"). We observed that concentrations of Herbimycin higher than 10 μM resulted in the death of more than 58 % of the cells, whereas Herbimycin-A concentrations of 100 and 500 nM resulted in a cell survival of more than 98 and 80 %, respectively (Fig. 1E, a). We next examined the effect of Herbimycin-A on neuritic outgrowth in hippocampal neurons using Herbimycin-A concentrations of 100 and 500 nM. Consistent with previous observations [57, 58], the inhibition of Src proteins resulted in marked alterations in the neuritic outgrowth (Fig. 1E, b–c).

We then evaluated the effects of 4A6H on cell survival. For this assay, the cells were treated 12 h after plating with 4A6H (50 nM, 500 nM, and 1 μM) or 1 % DMSO as untreated controls ("Materials and methods"). Neither of the tested concentrations of 4A6H exhibited significant cytotoxicity (Fig. 1F, a), and only 4A6H concentrations of 500 nM and 1 μM induced a reduction in the neuritic length by approximately 50 % (Fig. 1F, b–c), thus confirming the involvement of Lck in the regulation of the morphological properties of hippocampal neurons.

## Lck does not mediate spontaneous excitatory synaptic transmission

We next examined the effects of the selective Lck-inhibitor damnacanthal on the electrophysiological properties of primary-cultured hippocampal neurons by measuring the miniature excitatory postsynaptic currents (mEPSCs). For

these measurements, we used matured neurons that had reached a sufficient age for the establishment of detectable, robust functional connections (10–14 DIV). Spontaneous excitatory mEPSCs were then recorded from damnacanthal-treated and untreated-control neurons. Treatment with either 200 nM or 10 μM damnacanthal did not affect the frequency or the amplitude of the mEPSCs (Fig. 2A, B; two-tailed $P$ values $> 0.05$), suggesting that Lck does not play a major role in the regulation of presynaptic excitatory neurotransmitter release and basal synaptic transmission.

## Inhibition of Lck impairs memory-related long-term hippocampal potentiation

We next explored the functional involvement of Lck in the regulation of NMDAR-dependent long-term synaptic plasticity. Using protocols that have been previously described [37], the fEPSP were obtained in the CA1 dendritic layer of mouse hippocampal slices before and after the application of stimulation protocols that induce either short- or long-term forms of synaptic potentiation ("Materials and methods"). The stimulations were delivered at the Schaffer collateral CA3-CA1 pathway, and recordings were obtained from untreated control slices and from slices exposed to the selective Lck inhibitor damnacanthal ("Materials and methods"). We first measured the so-called paired-pulse facilitation [59–63], a short-term form of synaptic strengthening manifested as an increase in the amplitude of an elicited excitatory post-synaptic potential evoked by a succeeding presynaptic stimulation delivered shortly after an initial conditioning presynaptic stimulation. This form of neuronal facilitation is thought to depend exclusively on the neuronal presynaptic function [59–63]. We did not observe any alterations in the synaptic facilitation induced by a conventional paired-pulse electrical stimulation protocol in any of the different groups of slices treated with damnacanthal (Fig. 3A). This result suggests that Lck might not be primarily involved in the modulation of the presynaptic events associated with the rapid changes underlying hippocampal excitatory synaptic transmission [59–63].

Standardized stimulation protocols during in vitro electrophysiological studies on mouse hippocampal slices can be implemented to functionally distinguish and separately examine both short- and long-term forms of synaptic strengthening [38–40]. Therefore, we next studied the possible involvement of Lck in both of these electrophysiologically distinguishable forms (short-term and long-term) of memory-related synaptic potentiation ("Materials and methods") using the selective Lck inhibitor damnacanthal.

We observed that the treatment of the hippocampal slices with damnacanthal did not impair either the protein

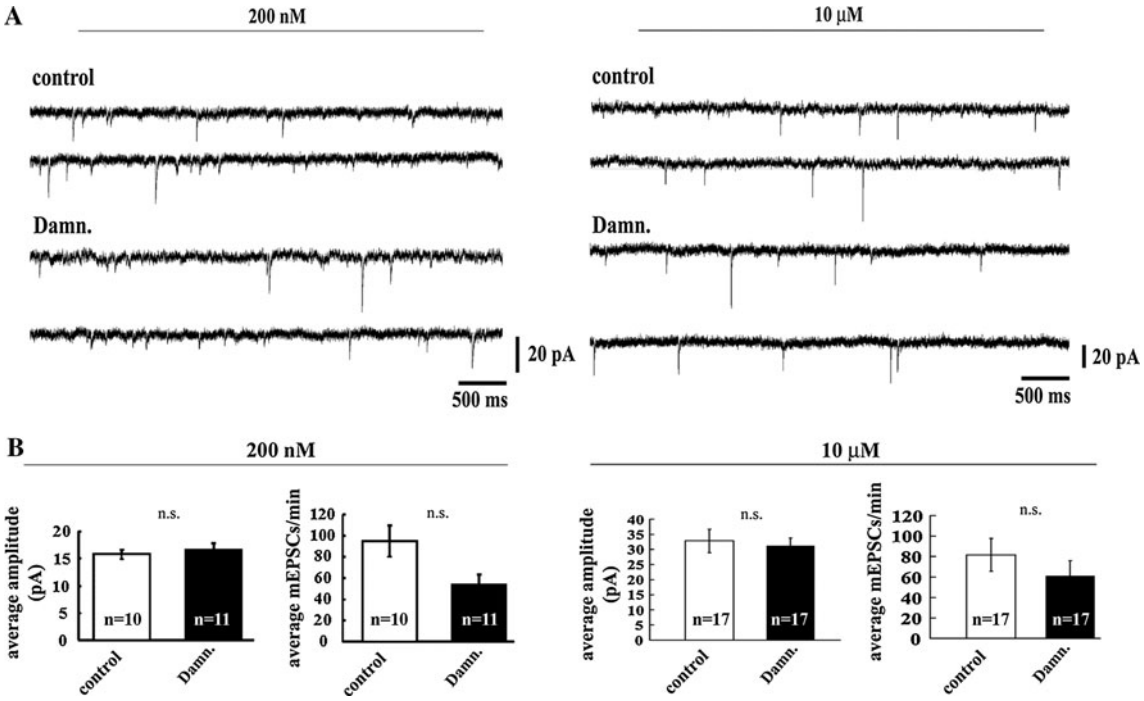



**Fig. 2** Inhibition of Lck with the specific pharmacological inhibitor damnacanthal does not affect AMPA-mediated synaptic properties. **A** Representative mEPSCs traces obtained from untreated primary-cultured hippocampal neurons (control) or from neurons exposed to two different concentrations (200 nM and 10 μM) of the selective Lck inhibitor damnacanthal (*Damn.*). **B** Compared with the untreated control neurons, the inhibition of Lck did not induce statistically significant changes in either the amplitude or the frequency of the mEPSCs obtained from neurons treated with either 200 nM or 10 μM damnacanthal (*Damn.*)

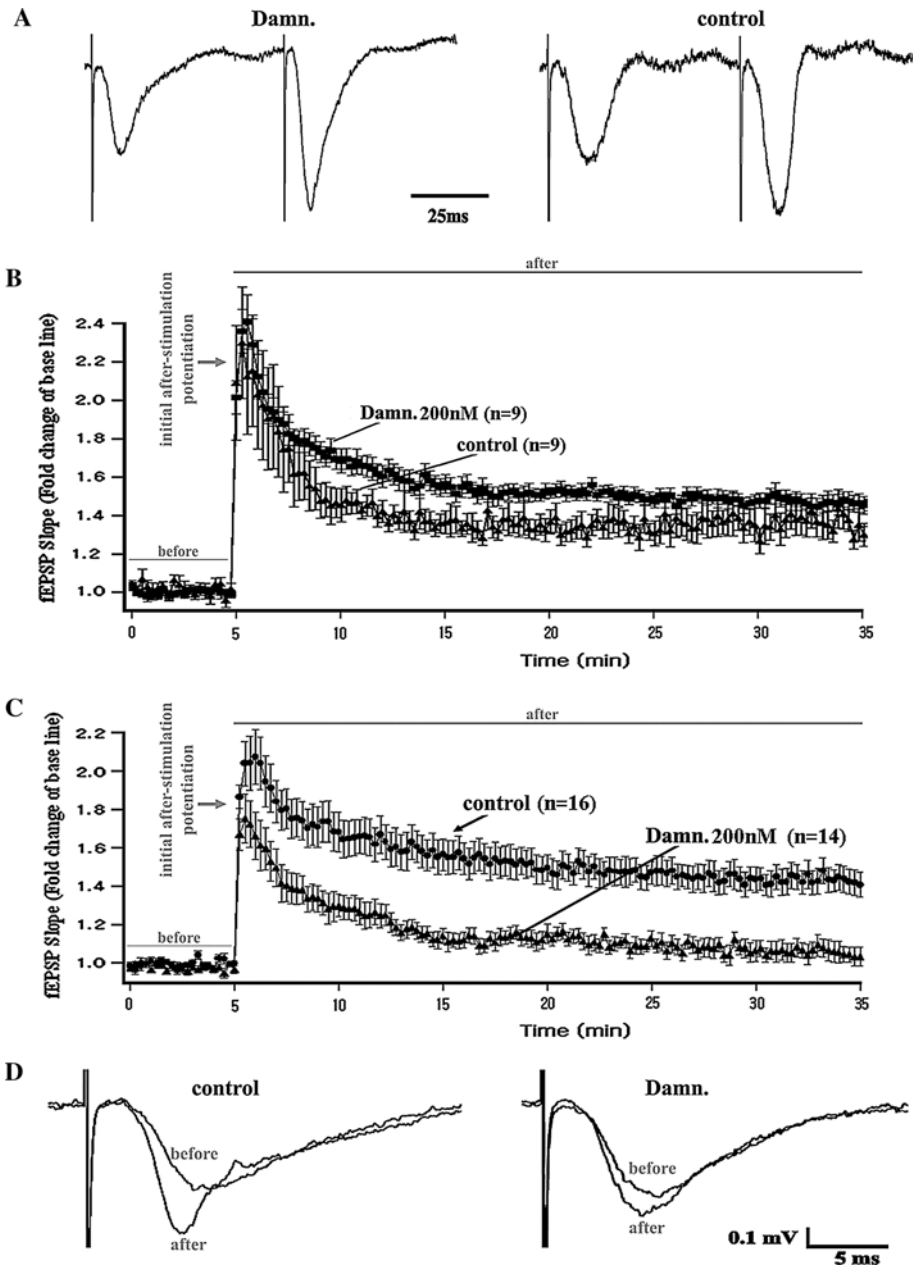



**Fig. 3** Lck-inhibition and synaptic function in the CA3-CA1-Schaffer collateral pathway of hippocampal slices. **A** Representative field-potential traces (normalized to their maximal decay peak values) obtained from the control and Lck-inhibition groups of slices that underwent a paired-pulse-induced synaptic facilitation protocol (two consecutive stimulations are presented in less than 100 ms, see *Time scale bar*). The recordings indicated that the inhibition of Lck does not prevent paired-pulse-induced synaptic facilitation (*left*) but results in field potentials reflecting a synaptic activity comparable to that observed in the untreated control slices (*right*). **B** Temporal course of the fEPSP slopes obtained before (*before*) and after (*after*) the application of a brief theta-burst stimulation protocol (see "Materials and methods") that is known to only induce a protein synthesis-independent short-term form of synaptic potentiation [38–40]. The data indicate that the selective inhibition of Lck by damnacanthal (*Damn.*) did not affect this form of short-term potentiation (also known as Early-LTP). **C** Temporal course of the fEPSP slopes obtained before (*before*) and after (*after*) the application of a prolonged theta-burst stimulation protocol (see "Materials and methods") that is known to induce a long-term form of synaptic potentiation [38–40] and that requires gene transcription and the formation of newly synthesized proteins. The data indicate that the selective inhibition of Lck by damnacanthal (*Damn.*) selectively impairs this long-lasting form of synaptic potentiation (also known as Late-LTP). **D** Representative traces of the field potential recordings obtained during baseline measurements (*before*) and 30 min after the application of the L-LTP-inducing theta-burst stimulation protocol (*after*). The traces were obtained either from a group of untreated control slices (control, *left*) or from slices that underwent selective pharmacological inhibition of Lck using damnacanthal (*Damn.*, *right*; see "Materials and methods")

synthesis-independent short-term form of synaptic strengthening or its concomitant theta-burst-induced post-tetanic potentiation [Fig. 3B; repeated measures ANOVA: main effect of treatment $P > 0.05$ (n.s.); repeat (time course of LTP): $P < 0.0001$; interaction: $P > 0.05$ (n.s.)].

Conversely, in response to a completely different electrophysiological stimulation protocol pattern [38–40], Lck-inhibition resulted in a significant reduction in gene transcription and protein synthesis-dependent long-term synaptic potentiation. This effect was evaluated as the percentage of fold-change in the slope of the field potentials after application of the theta burst-induced L-LTP ("Materials and methods") compared with the low-frequency-induced baseline field responses [Fig. 3C, D; repeated measures ANOVA: main effect of treatment $P < 0.001$; repeat (time course of LTP): $P < 0.0001$; interaction: $P < 0.05$)].

As observed previously, Lck-inhibition did not prevent the generation of the initial after-stimulation potentiation during the induction of the L-LTP (Fig. 3C, D). These data suggested that Lck might play a critical role in the in vivo

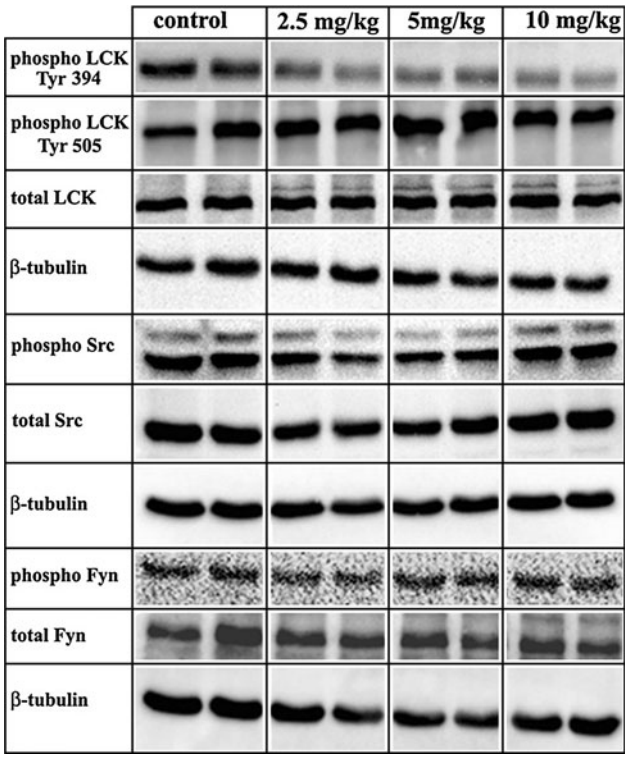



**Fig. 4** Damnacanthal acts primarily as a selective inhibitor of Lck in vivo. Increasing concentrations of damnacanthal selectively inhibited the levels of the catalytically active form of the Lck protein (phospho Lck Tyr 394) in the mouse hippocampus, as determined using western blot experiments with samples of hippocampal tissue obtained from either saline control mice or damnacanthal-treated mice. Samples from one animal (two hippocampi) were used in each lane

**Table 1** Primary behavioral observation screen in damnacanthal-treated and saline control mice

| Type | Saline | Damnacanthal |
|---|---|---|
| Muscle/lower motor neuron and spinocerebellar function | | |
| Body position | 5.6 ± 0.5 | 5.8 ± 0.4 |
| Atactic gait | 0 | 0 |
| Hypotonic gait | 0 | 0 |
| Impaired gait | 0 | 0 |
| Limb rotation | 0 | 0 |
| Spatial locomotion | 1.9 ± 0.3 | 1.9 ± 0.3 |
| Locomotor activity | 4.1 ± 0.3 | 4.0 ± 0.0 |
| Wire maneuver | 0.5 ± 0.5 | 0.7 ± 0.5 |
| Pelvic elevation | 3.2 ± 0.9 | 2.1 ± 0.3 |
| Tail elevation | 2.7 ± 0.7 | 2.1 ± 0.3 |
| Visual placing | 3.7 ± 0.7 | 4.0 ± 0.0 |
| Abdominal tone | 4.0 ± 0.0 | 4.0 ± 0.0 |
| Limb tone | 4.2 ± 0.6 | 4.0 ± 0.5 |
| Grip strength | 4.6 ± 0.7 | 3.9 ± 0.6 |
| Vestibular drop | 1.0 ± 0.0 | 1.0 ± 0.0 |
| Proprioception | 1.0 ± 0.0 | 1.0 ± 0.0 |
| Sensory function | | |
| Transfer arousal | 4.4 ± 0.7 | 3.8 ± 0.6 |
| Pinna reflex | 1.9 ± 0.3 | 2.0 ± 0.0 |
| Corneal reflex | 2.5 ± 0.5 | 2.2 ± 0.4 |
| Tail pinch | 1.7 ± 0.9 | 2.0 ± 0.0 |
| Toe pinch | 1.6 ± 0.5 | 1.7 ± 0.8 |
| Finger approach | 2.3 ± 0.7 | 2.5 ± 0.7 |
| Finger withdrawal | 3.0 ± 0.7 | 2.6 ± 0.8 |
| Neuropsychiatric function | | |
| Bizarre behaviour | 0 | 0 |
| Tremor/twitches | 0 | 0 |
| Provoked biting | 2.1 ± 0.3 | 2.3 ± 0.7 |
| Provoked freezing | 0 | 0 |
| Biting tendency | 1.0 ± 0.0 | 1.4 ± 0.5 |
| Autonomous function | | |
| Palpebral closure | 0 | 0 |
| Skin color | 4.3 ± 0.5 | 4.0 ± 0.0 |
| Respiratory rate | 4.0 ± 0.0 | 4.1 ± 0.3 |
| Hypothermia | 0 | 0 |
| Urination/defecation | 0.25 ± 0.4 | 0.2 ± 0.3 |
| Salivation | 0 | 0 |
| Piloerection | 1.0 ± 0.0 | 1.0 ± 0.0 |

Results of the battery of tests applied [82] to evaluate possible defects in gait or posture, changes in muscle tone, grip strength, visual acuity, temperature and other vitally important reflexes scored in control and Lck-inhibited mice groups. Throughout the manipulations, occurrence of unusual behavior, fear, irritability, aggression, excitability, salivation, lacrimation, urination and defecation were also examined in both experimental conditions. Scoring (mean ± standard deviation) was conducted following previously described parameters [82]. Higher score presents more (better, higher) activity (performance, response) or parameters are scored as present (1) or absent (0)

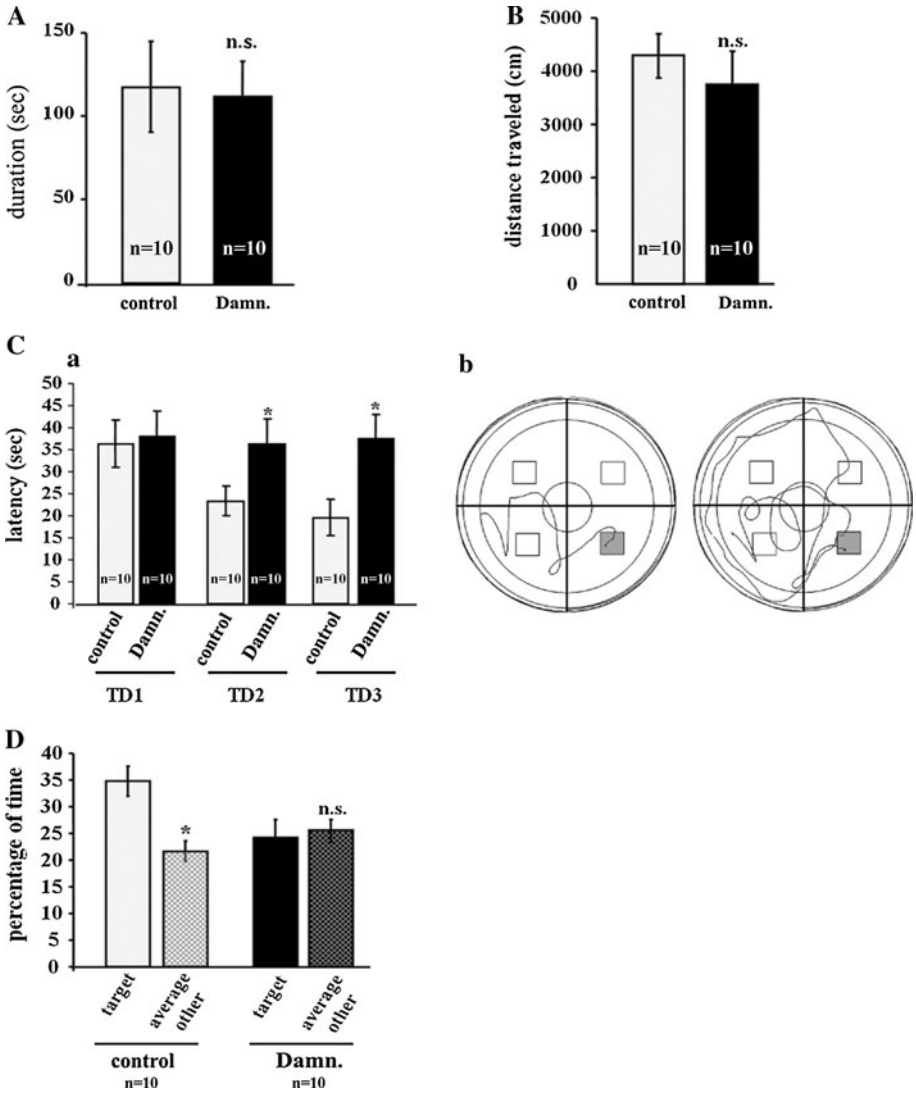



**Fig. 5** The selective inhibition of Lck impairs hippocampus-dependent spatial learning and memory. For a behavioral analysis, 10 animals per experimental group (control vs. damnacanthal) were used. The control mice were injected with a saline/DMSO solution (at a final concentration of 8 % DMSO). The damnacanthal (*Damn.*)-treated mice were injected with damnacanthal/DMSO at a concentration of 5 mg/kg ("Materials and methods"). No differences in motor coordination and balance, as evaluated by the latency on the Rota rod (**A**), or in locomotor and exploratory activities (total distance traveled) in the open field (**B**) were observed in the damnacanthal-treated mice. (**C**) *a* The latency to reach the hidden platform in the Morris Water Maze during 3 days of training (TD1-3) is displayed as the average of three trials per day. The escape latency was significantly longer in the damnacanthal-treated mice compared with that of the saline controls on TD2 and TD3 ($P < 0.05$). *b* Representative swimming path traces of the saline control (*left*) or damnacanthal-treated mice (*right*) during the third training trial (TD3). (**D**) The percentage of time spent in the target quadrant (original platform location during training) versus the time spent in the remaining quadrants during the probe trial in the damnacanthal-treated and saline control mice. The data are displayed as the mean ± SEM. *n.s.* not significant; $^*P < 0.05$

regulation of long-term forms of hippocampus-dependent memory formation and maintenance.

## Inhibition of Lck impairs hippocampus-dependent spatial learning and memory

We next examined the effect of the pharmacological inhibition of Lck on the acquisition and maintenance of hippocampus-dependent spatial learning and memory using the Morris Water Maze ("Materials and methods"). To examine the effect and specificity of damnacanthal in vivo, a dose–response test was first performed by injecting mice (i.p.) with 2.5, 5, or 10 mg/kg of damnacanthal. Subsequently, the levels of the total and phosphorylated forms of Lck and related Src kinases (Src and Fyn) were determined by western blotting using hippocampal protein extracts from damnacanthal-treated and control subjects. The damnacanthal treatment had no effect on the levels of total

Src, Fyn or Lck, and no changes were observed for the 505-phosphorylated form of Lck at any concentration used; however, very slight effects on the levels of the phosphorylated forms of Src and Fyn were observed (Fig. 4). In contrast, a substantial reduction in the levels of Lck that were phosphorylated at residue 394, which constitutes the active form of the protein, was observed in the hippocampal tissue of damnacanthal-treated mice at all three different concentrations used (Fig. 4). Based on the selective and pronounced inhibition of damnacanthal on the phospho-Lck-394, we decided to use a Lck-inhibitory concentration of damnacanthal of 5 mg/kg for the subsequent behavioral experiments (Fig. 4). This concentration had no apparent effects on the levels of the phosphorylated forms of Src and Fyn.

Next, the effect of Lck-inhibition on hippocampus-dependent spatial learning and memory was tested. Damnacanthal was injected i.p. at a dose 5 mg/kg, which did not alter the basic neurological functions, as evaluated by the Irwin [41] observational battery (Table 1), but still led to a significant reduction in Tyr 394 phosphorylation of Lck in the hippocampus (Fig. 4). To exclude a potentially confounding effect of damnacanthal on locomotor and exploratory behaviors, the performance of the damnacanthal-injected mice was evaluated using the open field test. The potential effects of damnacanthal treatment on motor coordination were further evaluated using the Rota Rod. Treatment with 5 mg/kg i.p. damnacanthal ($n = 10$ per group) did not affect either the locomotor or the exploratory activity, as evaluated by the total distance travelled in the open field [$P > 0.05$ (n.s.)] (Fig. 5B), or motor coordination, as measured by the latency to fall off the Rota Rod [$P > 0.05$ (n.s.)] (Fig. 5A).

Mice of both groups were subjected to a learning-acquisition behavioral training in the Morris Water Maze 48 h after the open field test ("Materials and methods"). The damnacanthal-treated mice exhibited significant deficits in the spatial learning task, as evaluated by the latency to reach the hidden platform. The results were averaged over three daily acquisition trials and compared with those of the control animals (Fig. 5C, a–b). Twenty-four hours after the last training trial, a probe trial was conducted in which the time spent in the quadrant that originally contained the platform was used as an indicator of hippocampus-dependent spatial memory. In contrast to the control mice, which displayed a significant preference for the target quadrant, the damnacanthal-treated mice did not spend significantly more time swimming inside the target quadrant than in the remaining three quadrants (Fig. 5D). Collectively, these data indicate that the phosphorylated active forms of Lck play critical roles during both the acquisition and the retention phases of hippocampus-dependent spatial learning and memory.

## Discussion

We explored the involvement of the brain protein Lck, a non-receptor tyrosine kinase also known as p56Lck [6], in the regulation of neuronal morphology, memory-related hippocampal synaptic plasticity and in vivo memory acquisition and long-term maintenance. Pharmacological and genetic loss-of-function approaches, which were previously shown to be effective for the inhibition of Lck, were combined here to examine the role of Lck in the mouse hippocampus. The pharmacological experiments were performed using damnacanthal, a selective inhibitor of Lck tyrosine kinase activity [48, 49] that is broadly used as a specific Lck inhibitor in a wide range of cells and tissues [50–54]. We observed using a western blotting analysis that 10 μM damnacanthal inhibited Lck activity in cultured hippocampal neurons. This concentration (10 μM) is significantly lower than those previously reported to inhibit Lck in other cell types [51, 54]. Damnacanthal is over 100 times more selective for Lck than for other serine/threonine kinases (including PKA and PKC), and selectivity for Lck over other members of its kinase-family including Src and Fyn is statistically significant [48, 49]. Differences in the dose-responses and/or in the molecular mechanisms associated with the Lck function might, therefore, vary depending on the cell types, tissues and regions under study or on the preparation types (i.e., primary dissociated cultures vs. sliced brain tissue or in vivo injections, as also reported here) [64]. We also used the Lck inhibitor 4A6H [6, 55] and corroborated the functional involvement of Lck in hippocampal neuritic outgrowth.

Genetically induced hampering of Lck was based on the neuronal transfection of previously characterized specific murine Lck-inhibitory shRNAs that have been reported to be effective for specific Lck silencing [32, 48]. Proof-of-principle qRT-PCR experiments in PC12 cells further demonstrated that this shRNA-based loss-of-function strategy is an effective method for reducing the levels of the Lck protein. The pharmacological and genetic approaches both confirmed the relevance of Lck for neuronal outgrowth, suggesting that Lck might act as regulator of the plasticity-related growth of synaptic structures in the hippocampus during memory formation and maintenance [46].

Neuronal outgrowth is known to be dysregulated in several neuropathologies that affect memory function, some of which could potentially be related to Lck. For instance, exacerbation of Tau phosphorylation results in anomalously sustained Tau-microtubule interactions that contribute to the formation of insoluble Tau aggregates in Alzheimer's disease [65]. Interestingly, Lck, which is a subfamily-B member of the Src-family [3, 4], can phosphorylate several residues of the Tau protein, including

Tyr18 [5]. Fyn, a subfamily-A member of the Src-family [3, 4], has also been proposed to interact with Tau and to be related to the pathogenesis of AD [66, 67]. However, recent experimental evidence indicates that whereas both Lck and Fyn efficiently phosphorylate Tyr18 of Tau, Lck is more effective than Fyn in phosphorylating several other tyrosine residues of Tau [5]. Moreover, moderate levels of A$\beta$ induced an increase in the expression of Fyn, resulting in marked neuronal deficits [14]. In contrast, significantly lower levels of Lck have been observed in the brains of AD patients [19]. These observations therefore suggest the possibility of a differential involvement of Fyn and Lck in the neuronal function and in the molecular mechanisms underlying or mediating Alzheimer's disease. Lck could thus mediate the modulation of the memory-related synaptic function via its demonstrated interaction with the Tau protein, which is not only critical for neuritic outgrowth and axonal development but is further related to Alzheimer's disease, the most prevalent human neuropathology linked to the loss of memory function [5]. More studies are necessary to better understand the physiological significance of Lck as a regulator of neuritic outgrowth and the possible relationship of Lck to neuropathology.

In the current work, we also observed that treatment with the selective Lck-inhibitor damnacanthal had no effect on several primarily AMPAR-related processes (Figs. 2, 3A–C). Glutamate regulates memory formation and maintenance and mediates memory-related synaptic strengthening via the fine-tuned function of both AMPA and NMDA receptors [10, 68–71]. However, the synaptic function can be selectively modulated via NMDAR-mediated synaptic transmission without a need for major changes in the AMPAR-dependent glutamatergic activity [72–76]. Thus, Lck may play a minor role in the regulation of the AMPAR-related presynaptic and postsynaptic mechanisms associated with short-term forms of synaptic strengthening in hippocampal neurons [60–63]. In contrast, our morphological, behavioral, and long-term potentiation experiments suggest that Lck might be specifically involved in the regulation of the long-term forms of memory-related hippocampal synaptic strengthening and in the modulation of neuritic outgrowth, processes that depend profoundly on the activity of *N*-methyl-D-aspartate (NMDA) receptors [46]. Src protein kinase is known to stimulate the activity of the NMDA type of glutamate receptor [8, 77], which has a pivotal role in long-term potentiation (LTP) in the CA1 region of hippocampus. Therefore, our data suggest that Lck can similarly interact with and modulate NMDA receptors, thus playing a causal role in the maintenance of the functional levels of NMDAR-mediated currents that are required for long-term synaptic strengthening, which is also observed for other Src kinase-family members including Src and Fyn [9, 10, 12, 13, 17, 77–79]. Recent studies characterizing the role of other protein kinases in the context of memory-related synaptic plasticity have demonstrated a functional pattern of activity comparable to those presented here for Lck. Kim et al. [80] concluded that both genetic and pharmacological strategies hampering the protein kinase PI3K$\gamma$ result in the selective alteration of only a specific form of NMDAR-mediated long-term synaptic plasticity without affecting the basal synaptic properties, including the features of the mEPSCs or paired-pulse induced synaptic strengthening. Additional functional experiments using genetically engineered mice that are deficient in Lck could therefore contribute significantly to the understanding of the role of Lck in hippocampus-mediated memory acquisition and maintenance.

The observation that the inhibition of Lck leads to significantly impaired performance in the Morris Water Maze task further supports the proposed relevance of Lck as a critical regulator of learning and memory. Reduced Lck activity prevented the acquisition of spatial memory because no decrease in the latency to reach the platform over a period of days during training was observed in damnacanthal-treated mice. These data suggest a deficit in long-term memory because the 24-h interval between training days requires the occurrence of protein synthesis-dependent long-term memory processes [81]. Although these results are consistent with a deficit in the long-term LTP resulting from the application of damnacanthal to hippocampal slices, the escape latency could also potentially be affected by nonspecific effects of the drug treatment on swimming performance. This possible confounding factor can, however, be excluded because the damnacanthal treatment did not affect swimming speeds throughout the entire experiment. Additionally, the unaltered motor coordination during the Rota Rod and exploratory activities that was similar to the control levels in the Open Field further rule out any experimental bias due to the application of damnacanthal in the Morris Water Maze. Thus, these data suggest that Lck activity is required for the acquisition of hippocampus-dependent spatial memory and may be explained by the constraints on the synaptic plasticity-related outgrowth induced by Lck-inhibition [33, 81–85].

Thus, this work provides the first comprehensive structural and functional characterization of Lck including its role in the modulation of synaptic plasticity and hippocampus-dependent long-term memory. Our data also invite future experiments investigating a possible differential contribution of Lck relative to other Src kinase family members to the pathogenesis of Alzheimer's disease and other potential uses of Lck as a pharmacological target.

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# IV. Summary (English)

The work presented in this cumulative thesis reflects my efforts in studying the functional role of brain enriched protein kinases in memory-related functions of the mouse hippocampus.

In the first article, I have found that FAK is an important regulator of hippocampal neuronal growth and that FAK also mediates neurotrophin-induced neuritic outgrowth. I have proven that FAK regulates spontaneous neurotransmitter release of hippocampal neurons and modulates neurotrophin-induced miniature excitatory postsynaptic currents. Moreover, I confirmed the role of FAK as a critical mediator in both activity-dependent hippocampal long-term potentiation and the acquisition and the retention phase of spatial learning and memory. There are several controversial reports indicating that FAK has facilitatory activities and inhibitory activities on neuritic outgrowth. Therefore, these data contribute to the understanding of the role of FAK in regulation of neuritic outgrowth. Furthermore, these data indicate the relevance of FAK for learning and memory *in vivo* which has not been clearly defined yet.

In the second article, I have found that LCK is involved in the regulation of neuritic outgrowth using a pharmacological and a genetic strategy. Also, I have shown that LCK selectively mediates the activity dependent long-term potentiation especially protein-synthesis dependent long-term forms of memory formation and maintenance without affecting spontaneous excitatory synaptic transmission or short-term forms of memory. Furthermore, I have verified that LCK plays a crucial role on hippocampus-dependent spatial learning and memory *in vivo*. There are no reports showing the role of LCK in leaning and memory except the clinical studies reporting the down regulated LCK in the hippocampus from Alzheimer's disease patients.

Therefore, these data firstly propose an essential role of LCK in the brain function of learning and memory and support the evidence of potential involvement of LCK in memory related synaptic plasticity.

Overall, I have characterized the relevance of these two brain-enriched protein kinases, FAK and LCK on synaptic plasticity by examining the involvement of these protein kinases in the regulation of neuritic-outgrowth, functional properties and spatial learning and memory using comprehensive approaches.

These studies performed in this thesis contribute to understand critical relationship of protein kinases with hippocampal synaptic plasticity such as long-term potentiation in the context of learning and memory.

Moreover, these observations add to the knowledge of a pivotal participation of individual protein kinases in learning and memory and suggest future experiments investigating how these protein kinases are involved in the intracellular signalling cascade during long term synaptic plasticity underlying learning and memory.

# V. Zusammenfassung

Die in dieser kumulativen Dissertation vorgestellten Arbeiten spiegeln meine Anstrengungen wieder, die Rolle von (im Hirn angereicherten) Proteinkinasen im Rahmen Gedächtnis-assoziierter Prozesse im Maushippocampus funktionell zu untersuchen.

Im ersten Artikel habe ich beschrieben, dass die Proteinkinase FAK ein entscheidender Regulator hippocampalen, neuronalen Wachstums und Vermittler des Neurotrophin-induzierten nervalen Auswachsens ist. Ich konnte zeigen, dass FAK die spontane Neurotransmitter-Ausschüttung in Hippocampusneuronen reguliert und über Neurotrophin-Induktion kleinste exzitatorische postsynaptische Ströme moduliert. Darüberhinaus bestätigte sich die Rolle von FAK als bedeutender Mediator sowohl bei der aktivitätsabhängigen hippocampalen Langzeitpotenzierung als auch beim räumlichen Lernen und Erinnern in den Phasen des Erlernens und Erhaltens. Über FAK und seine fördernden, wie auch hemmenden Effekte auf das nervale Auswachsen gibt es zahlreiche widersprüchliche Berichte.

Die hier dargestellten Sachverhalte tragen deswegen zum Verständnis der Rolle von FAK auf die Regulation nervalen Auswachsens bei. Darüberhinaus bekräftigen die Daten die Relevanz von FAK im Rahmen des Erlernens und Erinnerns i*n vivo*, was bis jetzt nicht eindeutig bestimmt worden ist.

In der zweiten hier angeführten Arbeit habe ich mittels pharmakologischer und genetischer Methoden herausgefunden, dass LCK in die Regulation nervalen Auswachsens eingebunden ist. Ebenso konnte ich zeigen, dass LCK selektiv die aktivitätsabhängige Langzeitpotenzierung vermittelt, besonders jene Formen der Entstehung und Erhaltung von Gedächtnis, die von der Proteinsynthese abhängig sind, ohne sich auf die spontane exzitatorische synaptische Übertragung oder auf Kurzzeitformen des Gedächtnisses auszuwirken. Außerdem konnte ich beweisen, dass LCK eine äußerst wichtige Rolle in Hippocampus-abhängigem räumlichen Lernen und Erinnern *in vivo* spielt. Bisher gibt es abseits einiger klinischer Studien, welche eine LCK-Herabregulierung im Hippocampus von Alzheimerpatienten beschreiben, keinerlei Hinweise auf einer Verbindung von LCK und Lernen und Gedächtnis.

Die hier beschriebenen Daten bestätigen erstmals die bedeutende Rolle von LCK im Rahmen der Hirnfunktionen Lernen und Gedächtnis und unterstützen auch die Auffassung einer potentiellen Mitwirkung von LCK bei Gedächtnis-assoziierter synaptischer Plastizität.

Insgesamt konnte ich mittels unterschiedlichen methodologischen Zugängen, wie morphologischen Analysen, elektrophysiologischen Experimenten und Verhaltensstudien, zeigen, dass die im Hirn angereicherten Proteinkinasen FAK und LCK die Regulation nervalen Aussprießens beeinflussen, sowie eine bedeutsame Rolle beim Lernen und Gedächtnis spielen.

Diese im Rahmen der Dissertation durchgeführten Studien tragen zum Verständnis von Proteinkinasen und ihren bedeutenden Funktionen für die hippocampale synaptische Plastizität im Kontext Lernen und Gedächtnis bei.

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## VII. Curriculum vitae

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**PERSONAL INFORMATION**

Date of Birth June 20, 1980
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**EDUCATION**

2009-2013 Ph.D., Department of Biotechnology, Prof. Hermann Katinger
University of Natural Resources and Applied Life Sciences

2005-2007 Master of Health Science, Department of Clinical laboratory of Sciences, Department of Biomedical laboratory science, Prof. Park Yong-Seok
Yonsei University

2001-2005 Bachelor of Health Science, Department of Biomedical laboratory science
Catholic University of Pusan

**PROFESSIONAL EXPERIENCE:**

2008.3- 2008.11
- English language course in New Zealand

2007.8 -2008.3
- Research technician, ProCell Biopharmaceutical Company

2007.3- 2007.8
- Research Technician, department of Nuclear medicine, Yonsei University Medical Center

2005 (1, 2 semester)
- Cell biology Assistant teaching, department of biomedical laboratory science, Yonsei University

**CERTIFICATES AND AWARDS**

2005. 3. - 2007. 2.

- Brain Korea 21 Predoctoral Fellowship from Korean Ministry of Education
2005.3 – 2007. 2
- Research Associate Scholarship for graduate student from Yonsei University
2008.8
- Certificate of TESOL (Teaching English to Speakers of Other Language) from New Zealand
2005.2.
- Certificate of Clinical Medical Laboratory Science from Korea Government

**LANGUAGE SKILLS**

Korean (mother tongue), English

**COMPREHENSIVE LIST OF PUBLICATIONS**

Article: Alzheimer's disease risk factor lymphocyte-specific protein tyrosine kinase regulates long-term synaptic strengthening, spatial learning and memory
*Eun-Jung Kim*, Francisco J. Monje*, Lin Li, Harald Höger, Daniela D. Pollak, Gert Lubec, Cellular and Molecular Life Sciences,* 70(4): 7 43-59. (2012)

Article: Focal Adhesion Kinase Regulates Neuronal Growth, Synaptic plasticity and Hippocampus-Dependent Spatial Learning and Memory
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