Cátia Sofia Pereira Frias

Licenciatura em Biologia

Dissecting neuronal development deficits by inflammation: from morphology to cytoskeleton dynamics

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Dora Maria Tuna de Oliveira Brites,
Investigadora Coordenadora e
Professora Catedrática Convidada,
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Part of the results discussed in this thesis was presented in the following meetings:

Cátia Frias, Adelaide Fernandes, Lorene Lanier, Dora Brites. *Changes in Neuronal Development by Inflammation: from Neuritic Outgrowth to Synaptogenesis*. 12th Meeting of the Portuguese Neuroscience Society. Lisboa, Portugal, May 26th-28th 2011 [Poster];

Adelaide Fernandes, Cátia Frias, Lorene Lanier, Dora Brites. *Acute-phase cytokines IL-1beta and TNF-alpha alters neuronal neuritic outgrowth and synaptogenesis*. 10th European Meeting on Glial Cells in Health and Disease. Prague, Czech Republic, September 13th-17th 2011 [Poster].

AGRADECIMENTOS

Quero agradecer, primeiramente, à Professora Catedrática Dora Brites, orientadora desta dissertação. Agradeço a oportunidade de realizar o meu projecto de mestrado no grupo *Neuron Glia Biology in Health and Disease*, por me ter recebido de forma calorosa, abrindo-me as portas ao mundo científico, e por se ter interessado sempre por este trabalho. O seu rigor científico e espírito crítico, aliados ao seu elevado grau de exigência, aos seus vastos conhecimentos e à sua inteligência, são deveras inspiradores, tendo sido fulcrais para o desenvolvimento desta dissertação e para o meu desenvolvimento enquanto investigadora. Trabalhar consigo foi um privilégio e, mais uma vez, obrigada.

À Professora Doutora Adelaide Fernandes, co-orientadora deste trabalho, agradeço todo o apoio, toda a disponibilidade, encorajamento e dedicação demonstrados ao longo deste ano. És um exemplo, pelas tuas capacidades científicas e pela tua inteligência. Agradeço a tua paciência, as tuas sugestões, o teu acompanhamento e a tua compreensão, que foram essenciais para que este trabalho fosse finalizado. Obrigada por me teres recebido de forma tão calorosa e simpática, mas mais ainda por me teres ensinado tanta coisa.

À Professora Doutora Margarida Castro Caldas, orientadora interna desta dissertação, e ao Professor Doutor José Paulo Sampaio, coordenador do Mestrado em Genética Molecular e Biomedicina, agradeço a prontidão e a eficiência com que sempre esclareceram as minhas dúvidas.

À Professora Doutora Maria Alexandra Brito e ao Professor Doutor Rui Silva, agradeço a forma como me receberam no grupo. Para além da inteligência e conhecimentos científicos, saliento o espírito crítico e o rigor tão patentes em todas as discussões.

Às Doutoras Sofia Falcão e Ana Rita Vaz agradeço a simpatia com que me receberam. Agradeço, também, as sugestões feitas, as mensagens de ânimo e os conhecimentos partilhados ao longo deste ano.

Às meninas doutorandas, Andreia, Inês, Filipa e Ema, agradeço a vossa alegria, o vosso apoio, os vossos conhecimentos e a vossa maturidade. Vocês são todas um exemplo a seguir, pela vossa inteligência e garra! Espero poder ver-vos a conquistar este “monstrinho” do vosso percurso académico, os vossos doutoramentos. Tenho a certeza que vão dominá-lo com a vossa perspicácia, inteligência e espírito crítico. Agradeço-vos todas as gargalhadas e a partilha da cave escura, agora excessivamente quente. Andreia, desejo o melhor neste teu último ano que se inicia e que o teu sucesso seja grandioso. Inês, agradeço toda a tua paciência e por me teres ajudado sempre que necessitei. Filipa, agradeço a tua leveza de espírito, todas as boleias e conversas, e, principalmente, a tua alegria. Somos, de facto, muito

parecidas! Ema, agradeço-te toda a ajuda que me deste ao longo deste ano, todas as tuas sugestões e a tua enorme simpatia. Agora, toca a aproveitar o sol carioca, cara!

Às minhas companheiras de viagem, Ana Filipa e Inês, agradeço o vosso apoio, amizade e a disponibilidade que sempre tiveram para os meus desabafos. Foi um longo caminho, com alguns buracos pelo meio, mas que conseguimos transpor de forma meritória. Espero que o vosso futuro esteja pintado com muito sucesso, muita alegria e muitas concretizações, tanto profissionais como pessoais. Finalmente, conquistámos esta batalha, meninas!

Às novas meninas de mestrado, dou-vos as boas vindas e espero que esta jornada que agora se inicia seja timbrada pelo crescimento pessoal e científico, terminando num grande sucesso.

A todas as pessoas com quem partilhei a Cave escura, muito obrigada pelo vosso companheirismo (mesmo quando pedia para desligar o ar condicionado) e por me terem recebido tão bem. Aproveito para desejar a maior das sortes ao André e ao Miguel para a fase seguinte: o doutoramento.

Ao meu grupinho de mestrado, Ana Filipa, Pedro, Francisco e Marcos, agradeço a paciência que tiveram ao longo daquelas viagens intermináveis para a FCT, no meio de senhores a cantar Queen ou de muitos “Ayo, I’m tired of using technology” e “Fireflies”. Vocês fizeram-me rir durante meses a fio, o que ajudou a enfrentar os dias longos na sala 101. Desejo-vos a maior das sortes nesta fase que termina e que o vosso futuro seja brilhante.

Aos meus grandes amigos, André, Inês, João, João, José e Andreia, agradeço a vossa constante preocupação e o vosso ombro amigo. Foram fundamentais para o meu bem-estar, para o meu conforto. Momentos com vocês são da mais pura alegria. Vocês fazem todos parte da pessoa que sou hoje. André, meu padrinho, uma palavra especial para ti por seres quem és e por sentires orgulho naquilo em que me tornei. Espero retribuir a todos o carinho, o companheirismo, a alegria, a amizade.

À minha enorme família, agradeço o apoio e toda a preocupação. Sinto um enorme orgulho em ter-vos a todos na minha vida. Novecentos quilómetros não são suficientes para apagar este carinho especial que nutro por todos vocês! Somos quem somos por causa da nossa família e vocês ajudaram-me a crescer sempre. Uma especial palavra à minha prima Carla, a minha “senhoria”, agradeço-te todo o apoio, todo o carinho e toda a preocupação durante estes dois anos em que vivemos juntas. És uma chata, mas lá no fundo gosto imenso de ti! Quero também deixar uma palavra à minha tia Goretti. Kika, a garra e a força que saltam do seu ser são contagiantes e fazem-me querer lutar sempre por mais. Querer é poder! Por último, quero agradecer à minha avó Isabel, por ser uma pessoa tão especial. A sua doença

fez-me seguir este caminho das Neurociências e a avó sempre me fez acreditar que devemos seguir os nossos sonhos, tendo-me mostrado que a determinação e a força de vontade levam-nos onde ambicionamos ir.

Aos meus pais, os meus fantásticos pais, João e Maria, agradeço por me terem tornado na pessoa que sou hoje. Sou o que sou graças a vocês. Mantivemo-nos sempre unidos, independentemente de todos os obstáculos que apareceram no nosso caminho. Tenho imenso orgulho em ser vossa filha. Pai, somos muito parecidos e revejo-me em muita coisa do que o pai representa para mim, justiça, persistência, lealdade, força de vontade. Mãe, muito da minha personalidade vem de si, a sensibilidade, a amabilidade, a alegria, a gentileza. Obrigada por terem sempre acreditado em mim e pelo apoio incondicional.

Ao Miguel, o meu Miguel, o homem que me dá sempre força, ânimo, alento. Obrigada pelo teu apoio incondicional, pela tua amizade, pela tua compreensão, pelo teu carinho, pela tua paciência, pelo teu amor. És o companheiro que muitos gostavam de encontrar para partilhar esta viagem. Miguel, ouvir-te dizer que tens orgulho em mim torna a minha vida mais brilhante. Agradeço-te por nunca me teres deixado ir abaixo nos piores momentos, por me fazeres sentir uma pessoa especial, por todo o amor que me dás e continuas a dar ao fim de quase quatro anos. O amor está em cada sorriso, em cada palavra, em cada adversidade. Agora é a minha vez de te apoiar nesta fase que se inicia. Sabes que tentarei retribuir da melhor forma humanamente possível tudo o que sempre fizeste por mim.

Por último, à minha avó, Zita, pela pessoa que foi, pelo que representa para mim. Avó, sei que algures está a ver-me neste momento e sei que está com um sorriso nos lábios, a ver-me terminar mais esta etapa. Perdê-la foi a maior dor da minha vida, mas sei que foi o melhor para si. Lembro-me de si, do enorme carinho que partilhámos, de todos os bons momentos que nos unem e que nunca se irão dissipar da minha memória. Avó, obrigada por tudo.

ABSTRACT

Neuroinflammation, a response of the nervous system to injury, results in the release of pro-inflammatory mediators, as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α). Exposure of nerve cells to a neuroinflammatory environment was shown to change the normal neurodevelopment, which can be linked to the appearance of neurological disabilities. In this work, we aimed to assess the effects of moderate levels of IL-1β and TNF-α in the establishment of neuronal arborization, growth cone morphology and synaptogenesis.

An early exposure of embryonic hippocampal neurons to cytokines delay neuronal development, with an increase in the number of non-polarized cells, stage 2 of development. When analyzing stage 3 neurons, IL-1β showed to decrease total arborization, in particular at the axonal level, while TNF-α increased dendritic arborization. In fact, IL-1β reduces dendritic and axonal length and the number of axonal branches, whereas it increases the extent of dendritic and axonal branches, probably to compensate the other effects. In contrast, TNF-α increases the number of primary dendrites and dendritic branches, as well as their length. By next analyzing microtubule dynamics as the ratio of acetylated- (old) *vs.* tyrosinated-tubulin (newly-formed), we found that IL-1β and TNF-α induce microtubule stabilization, which may be related to a deficient axonal outgrowth. In addition, both cytokines reduced the area of growth cones, with an increase in the immunofluorescence of F-actin, indicating alterations at the cytoskeleton which may compromise axonal elongation and branching. Regarding neuronal connectivity, we demonstrated that both cytokines not only reduced the density of dendritic spines and synapses, but also the maturity of dendritic spines, suggesting a reduction in the synaptic strength.

These findings establish a relation between neuroinflammation in fetal life and the emergence of neuronal damage, similar to those observed in neurodevelopmental disorders, as schizophrenia.

Keywords: Neuroinflammation; hippocampal neurons; neuronal arborization; growth cone; synaptogenesis; cytoskeleton.

RESUMO

A neuroinflamação, resposta do sistema nervoso a danos, resulta na libertação de mediadores pró-inflamatórios, como a interleucina (IL)-1β e o factor de necrose tumoral (TNF)-α. A exposição de células nervosas a ambientes neuroinflamatórios induz mudanças no normal neuro-desenvolvimento, podendo levar ao aparecimento de incapacidades neurológicas. Assim, neste trabalho pretendemos elucidar os efeitos de níveis moderados de IL-1β e TNF-α na formação da arborização neuronal, morfologia do cone de crescimento e sinaptogénese.

Uma exposição inicial dos neurónios de hipocampo às citocinas atrasa o seu desenvolvimento, aumentando a percentagem de neurónios não polarizados, estadio 2 de desenvolvimento. A análise de neurónios já polarizados, estadio 3, demonstrou que a IL-1β diminui a arborização total, em particular no axónio, enquanto que o TNF-α aumenta a arborização dendrítica. De facto, a IL-1β reduz o comprimento das dendrites e do axónio, bem como o número de ramificações axonais, embora aumente a extensão dos ramos axonais e dendríticos, provavelmente para compensar os outros efeitos. Relativamente ao TNF-α, este aumenta o número de dendrites primárias, suas ramificações e extensão. Analisando a nível axonal a dinâmica dos microtúbulos (rácio tubulina-acetilada, antiga *vs.* tubulina-tirosinada, recém-formada), observámos que ambas as citocinas induzem uma estabilização selectiva dos microtúbulos, o que pode originar deficiências no crescimento axonal. A IL-1β e o TNF-α induzem ainda uma redução da área do cone de crescimento, estrutura que direcciona o movimento axonal, em paralelo com um aumento da F-actina, indicando alterações do citoesqueleto e possível comprometimento da arborização axonal. Relativamente à conectividade neuronal, as citocinas reduzem o número de espículas dendríticas e de sinapses, atrasando a maturação das espículas, o que sugere uma redução na força sináptica.

Assim, os nossos dados apontam para uma relação entre a neuroinflamação no período embrionário e o estabelecimento de danos neuronais semelhantes aos observados em doenças do neuro-desenvolvimento, como a esquizofrenia.

Termos-chave: Neuroinflamação; neurónios do hipocampo; arborização neuronal; cone de crescimento; sinaptogénse; citoesqueleto.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMCA	amino-methyl-coumarin-acetate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
Cdc42	cell division cycle 42
CNS	central nervous system
CO_2	carbon dioxide
C-domain	central domain
DIV	days in vitro
ECM	extracellular matrix
EGTA	ethylene glycol tetraacetic acid
E16	embryonic day 16
FBS	fetal bovine serum
Fig.	figure
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
G-actin	globular actin
GTP	guanosine triphosphate
HBSS	Hanks' balanced salt solution
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HSA	human serum albumin
IL-1	interleukin-1
IL-1ra	interleukin-1 receptor antagonist
IL-1α	interleukin-1alpha
IL-1β	interleukin-1beta
LPS	lipopolysaccharide
LTP	long-term potentiation
MAPs	microtubule-associated proteins
MAP2	microtubule-associated protein 2
MEM	minimum essential medium
miRNAs	microRNAs
$MgCl_2$	magnesium chloride
PBS	phosphate buffer saline
pH	negative logarithm for hydrogen ion
PHEM	PIPES, HEPES, EGTA, $MgCl_2$ buffer
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid

PPS	paraformaldehyde in PHEM buffer with sucrose;
P-domain	peripheral domain
PSD	postsynaptic density
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family member A
SEM	standard error of the mean
SV2	synaptic vesicles 2
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor-alpha
TRITC	tetramethylrhodamine isothiocyanate
T-domain	transition domain

I. INTRODUCTION

I. INTRODUCTION

The formation of a functional nervous system, in which neurons are accurately connected to each other, is one of the major steps of embryogenesis. This defined process involves a sophisticated neuronal polarization and the appropriate extension of axons and dendrites directed by external guidance cues and intracellular signalling pathways, with the ultimate goal of synapse formation. Hence, understanding how the neuronal development can be negatively affected by perinatal risk factors has a captivating importance, since changes in this early neurodevelopment have been linked with several detrimental outcomes, as mental disorders and cognitive deficits.

1. Development of Hippocampal Neurons

A mature neuron is a highly polarized and specialized cell characterized by elongated protrusions named dendrites, which can appear in a variable number, and a single axon. Two neurons and their interconnecting dendritic-synaptic-dendritic field are considered to be the basic functional unit in the brain involved in information processing (Baslow, 2011). Dendrites receive electrochemical signals at postsynaptic sites. Then, the electrochemical signals are transmitted to the axon, through the cell body or soma that contains the nucleus and the majority of organelles. The axon is responsible for the transport of the electrical signal from the cell body to the presynaptic terminal, which then will transmit the signal to the postsynaptic partner at the synapse. The process that leads to a fully developed and polarized neuron follows an intrinsic program of various steps with great morphological changes critical for neuronal network assembly and signal propagation (Brandt, 1998; Tahirovic and Bradke, 2009). Furthermore, in order to form the correct connection between neurons, axons and dendrites grow as a response to molecular signals, encompassing one of the major steps of embryogenesis, besides the maintenance of neuronal polarization (Geraldo and Gordon-Weeks, 2009).

One of the earliest and best studied *in vitro* systems to evaluate the development and maturation of nerve cells uses the hippocampal neuronal culture from rodent embryonic brain. This system has shown that neurons develop their characteristic morphology through a stereotypic sequence of events with distinct intermediate steps (Fig. I.1.). In fact, hippocampal neuronal cultures allow the study of neuronal development and synaptogenesis, enabling the comprehension of the events that lead to the differentiation of both pre- and postsynaptic compartments (Verderio *et al.*, 1999). Hippocampus has a central role on learning and memory processes, and several cognitive impairments have been linked to lesions in this cerebral region (Chauvière *et al.*, 2009; Finke *et al.*, 2011). Indeed, oxidative damage in rat hippocampus after injection of corticosterone induced marked deficits in memory processes (Sato *et al.*, 2010). Furthermore, neuronal network dysfunction has been observed in hippocampal slice cultures after traumatic brain injury, leading to deficits in electrophysiological function (Yu and Morrison III, 2010)

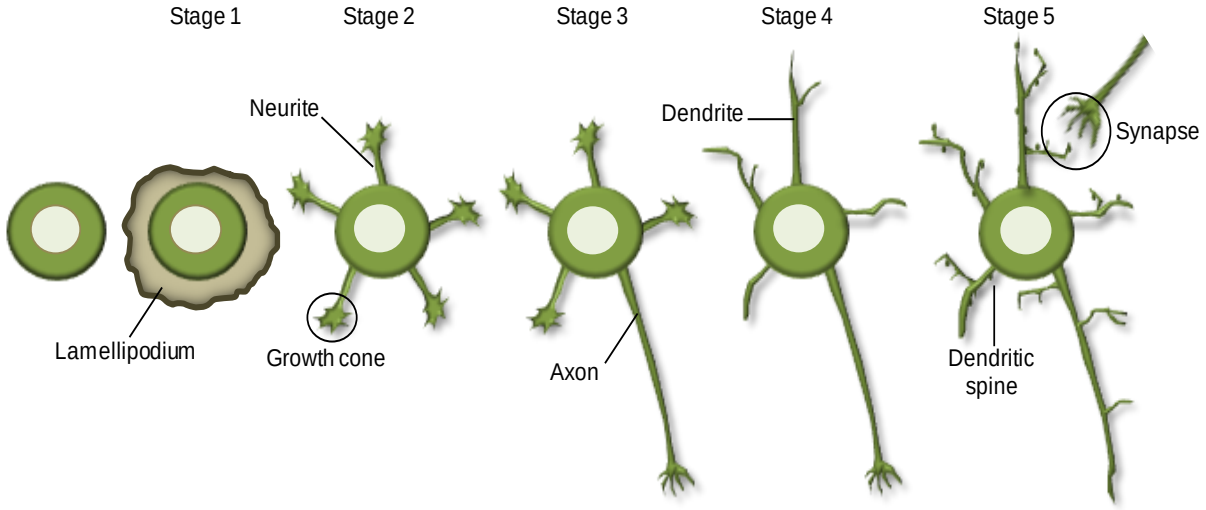

Fig. I.1. Developmental stages of mouse hippocampal cultured neurons. Initially, neurons are round cells that extend lamellipodia (stage 1) and develop minor processes called neurites (stage 2). One of these neurites starts to grow more rapidly and becomes the axon (stage 3), while the remaining neurites will develop into dendrites (stage 4). Finally, dendritic spines develop and mature at dendritic shafts leading to the establishment of synapses with the axonal presynaptic partner (stage 5), originating a mature neuron.

Upon seeding, hippocampal neurons start as simple and symmetric spheres that spread lamellipodia all around the cell body. The lamellipodia stably attach to the substrate and this is considered the stage 1 of neuronal development (Tahirovic and Bradke, 2009). Next, these round cells start to acquire several minor processes with similar lengths, named neurites, which are formed by an active outgrowth from the cell body (Verderio *et al.*, 1999; Tahirovic and Bradke, 2009). Neurites exhibit a dynamic behavior and frequently elongate in a saltatory manner, with periods of sudden advances and retractions or pausing, also displaying turning and branching (Dehmelt and Halpain, 2004a; Tahirovic and Bradke, 2009). These neurites are decorated with dynamic growth cones at their tips (stage 2), which are bulbous and highly motile structures responsible for sensing the surrounding environment. At this early developmental state, all neurites are assumed to be similar and the regulation of polarity *in vivo* is induced by extracellular signals (*e.g.* nerve growth factor), which trigger intracellular signaling events (Tahirovic and Bradke, 2009). In the absence of external cues, as in the culture condition, all neurites compete to become the axon (Craig and Banker, 1994). As the polarization of a neuron is deeply associated with intense cytoskeleton rearrangements, alterations in its dynamics occur prior to morphological changes and polarization, which become retained in the future axon. There is also a cytoplasmic flow of cargos containing limiting factors for axonal growth (as cytoskeleton regulators) into the future axon preceding the beginning of neuronal polarization (Witte and Bradke, 2008). These events are required for the formation of the axon, the initial step in the establishment of neuronal polarization and, subsequently, in the loss of the cellular symmetry. The symmetry breakage has to be precisely regulated, since only one of the neurites is selected to become the axon, while the remaining processes develop into dendrites. Once the selected neurite starts to grow more rapidly and to develop into an axon, its growth is reinforced and internal inhibitory cues prevent the growth of the other neurites, being the stage 3 of development achieved (Andersen and Bi, 2000; Tahirovic and Bradke, 2009). Indeed, several intra- and extracellular signals have been

shown to be involved in the selection of the future axon, as small GTPases, and netrins and brain-derived neurotrophic factor (BDNF), respectively (Polleux and Snider, 2010). After the establishment of neuronal polarity, the axon can extend considerably and start branching. Next, the remaining shorter neurites start to grow and acquire the morphology of typical dendrites, characteristic of the stage 4 of neuronal development (Witte and Bradke, 2008). As neuronal development proceeds, neurons become connected due to synapses formation (stage 5) with the purpose of establishing a proper neuronal circuitry. In this final stage, presynaptic axonal tips connect with postsynaptic partners at the dendritic trees in order to transmit the electrochemical signal from one neuron to the other. After neuronal polarization and maturation, axonal and somatodendritic compartments display distinct patterns of protein segregation, due to the proteins sorting into different vesicles at the trans-Golgi (Song *et al.,* 2009). Nevertheless, a physical barrier exists at the initial segment of the axon and it is important to maintain the differential molecular segregation of both membrane and cytoplasmic proteins (Song *et al.,* 2009).

Thereby, during neuronal development, several signals and pathways converge aiming the formation of a functional nervous system.

1.1. Neuronal Growth Cones

Growth cones are motile structures, composed by microtubules and microfilaments that incorporate the information of the molecules present in the extracellular milieu, which is then translated into cytoskeletal rearrangements. It is generally believed that these guidance molecules interact with membrane receptors and induce intracellular signaling cascades, thus targeting the growth cone cytoskeleton (Suter and Forscher, 2000; Gordon-Weeks, 2004). The rearrangements of the cytoskeleton ultimately determine an appropriate movement, either toward the guidance cue (attraction) or away from it (repulsion) (Gordon-Weeks, 2004). Growth cones are divided in three domains according to its cytoskeletal composition, as displayed in Figure I.2: the central domain (C-domain), majorly composed by the distal-ends of axonal microtubules; the transition domain (T-domain), consisting in a dense network of actin filaments; and the peripheral domain (P-domain), a protrusive region enriched in actin filaments (Geraldo and Gordon-Weeks, 2009). The P-domain exhibits lamellipodia, sheet-like cellular protrusions, and filopodia, which consists of finger-like dynamic and transient plasma-membrane protrusions. Morphologically, growth cones can vary in size, being large during the quiescence or pausing phases and small during the active or growth phases. In the quiescence phase, the growth cones become stalled and their microtubules achieve an inactive state, forming a loop in the C-domain. As the quiescence phase ends, the active state leads to the breakage of the loop by microtubules, which start to invade the P-domain, allowing the protrusion growth (Dent *et al.*, 1999; Dent *et al.*, 2011a).

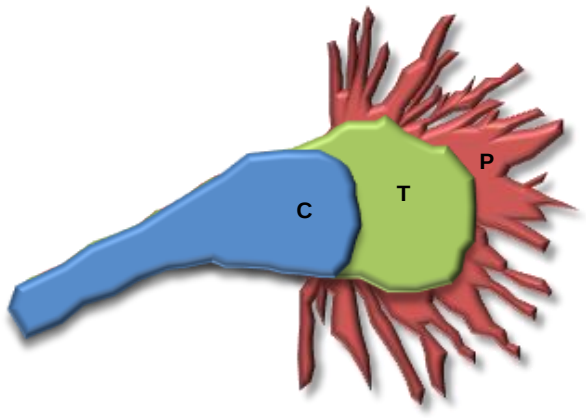

Fig. I.2. The growth cone cytoskeleton in primary embryonic hippocampal neurons. The cytoskeletal filaments are organized into three different regions. The microtubules emerge from the axon shaft and splay out into the central domain (C; blue). Here, there is a relatively sparse network of actin filaments of unknown organization. The transition region (T; green) contains a dense meshwork of cross linked actin filaments, while the peripheral domain (P; red) is dominated by filopodia with bundle actin filaments oriented with their polymerizing ends at the distal tips.

Filopodia are probably the first to detect the guidance cues, since their dynamic expansion and retraction places them in the front line to sense the surrounding environment (Gallo and Letourneau, 2004; Gordon-Weeks, 2004). Moreover, filopodia, as well as the surface of the growth cone, express several receptors that bind to extracellular molecular signals, ultimately eliciting the guided axonal outgrowth (Myers *et al.*, 2011). Therefore, filopodial length is important in determining the environmental area that a growth cone can directly probe. As a transient structure, the growth cone is only present during the time elapsed between the beginning of neuronal polarization and synaptogenesis, as well as during axon sprouting and regeneration following axonal injury (Avwenagha *et al.*, 2003). Interestingly, during neuronal polarization cytoskeleton dynamics at the growth cone plays an important role. Indeed, the actin cytoskeleton becomes more dynamic and less stable in the growth cone of one neurite and the microtubules at the neuritic shaft become stabilized and aligned to form a bundle, initiating the future axon (Dehmelt and Halpain, 2004a; Witte and Bradke, 2008). By contrast, the growth cones of the remaining neurites are static and exhibit a rigid actin cytoskeleton, which blocks the microtubules protrusion (Tahirovic and Bradke, 2009).

In order to achieve a directional growth, extracellular molecules, as the ones present in the extracellular matrix (ECM), are thought to induce a selective stabilization of actin cytoskeleton in both lamellipodia and filopodia, since actin destabilization alters growth cone turning (Challacombe *et al.*, 1996). In fact, ECM proteins can provide a cellular substratum to the axon outgrowth and they can also bind to soluble molecules at the growth cones, influencing intracellular signaling pathways and, consequently, the axonal outgrowth (Myers *et al.*, 2011). Also, in the developing axon, specific micro RNAs (miRNAs), non-coding oligoribonucleotides that regulate the gene expression at the post-transcriptional level, are translated in response to extracellular guidance cues at growth cones, influencing the axonal pathfinding (Hengst and Jaffrey, 2007). Furthermore, microtubule dynamic instability is also required for growth cone turning in response to guidance cues. Indeed, attenuation of microtubule dynamics blocks *in vitro* growth cone turning due to microtubule restriction to C-domain, which decreases or abolishes the interaction with actin network (Williamson *et al.*, 1996; Challacombe *et al.*, 1997). Additionally, the release of the microtubule-stabilizing drug taxol in one side of the growth cone induces attraction and turning toward the site of the cue, while microtubule-destabilizing drug nocodazole induces repulsion, steering the growth cone away from that side (Buck and Zheng, 2002).

Thus, there is dynamic microtubule reorganization in the growth cone in the direction of a guidance cue and microtubule stabilization in the direction of the turn (Gordon-Weeks, 2004).

1.2. Neuritic Arborization

Both axonal and dendritic development and stabilization is due to several complex processes extremely organized at the molecular level. Indeed, there is an intricate network of molecules involved in various processes as signal transduction, synthesis of macromolecules, cytoskeleton rearrangements and protein intracellular trafficking, all of them regulated by both intrinsic programs and extracellular cues (Urbanska *et al.*, 2008). Furthermore, the neuronal development and maturation are also controlled by miRNAs, since they are able to regulate the expression of proteins involved in the extension of neuritic processes and dendritogenesis (Loohuis *et al.*, 2011). The axonal arborization provides neurons with the ability to make synaptic contacts with a multitude of targets, being a decisive factor for the interconnection, a characteristic of the nervous system (Hall and Lalli, 2010). For an interstitial axonal branch to emerge, the primary axonal growth cone should pause, becoming larger. After the pausing period, a new primary growth cone then emerges to direct the growing axon, whereas the pausing one remains behind (Szebenyi *et al.*, 1998; Kalil *et al.*, 2000). The formation of axonal branches is characterized by the local splaying of the bundled microtubules and the breakdown of longer microtubules (Kalil *et al.*, 2000). These shorter microtubules start to explore new directions and to invade the branches formed from the paused growth cone, allowing the ramification development from the axonal shaft (Kalil *et al.*, 2000). Thus, cytoskeleton, specially the microtubule compartment, assumes an important role in the development of axonal complexity.

On the other hand, dendrites receive electrochemical signals transmitted by axons from other neurons, being the preferential localization of postsynaptical sites. Hence, dendrites and their branching complexity are intimately related with synaptic integration (Häusser *et al.*, 2000). The development of the dendritic tree is associated with high rates of branch addition and retraction, but the mature dendritic arborization is less plastic and has lower rates of branching (Wu *et al.*, 1999). Nevertheless, the dendritic trees preserve some degree of plasticity in the mature nervous system (Urbanska *et al.*, 2008). In this context, the contribution of extracellular signals, as BDNF and semaphorins, may be critical to direct dendritic arborization development, stability and plasticity (Urbanska *et al.*, 2008). Moreover, neuronal activity may count to the growth of dendritic trees. Indeed, modification of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activity decreases the complexity of the dendritic arborization and the lifetime of the dendritic branches (Haas *et al.*, 2006). Curiously, anomalies in dendritic arborization structure are related with several mental retardation syndromes and even neurodegenerative conditions such as Alzheimer's disease (Kaufmann and Moser, 2000; Couch *et al.* 2010).

1.3. Synapse

Synapses are highly specialized and asymmetrical junctions responsible for the directional transfer of electrochemical signals from a presynaptic neuron to a postsynaptic cell. In vertebrates, synapse formation, or synaptogenesis, essentially occurs from embryonic development to early postnatal life, characterized by its complex nature, involving a myriad of hierarchical signals (Waites *et al.*, 2005). However, the formation of new synapses also occurs throughout adulthood and is thought to contribute to learning and memory, despite the progressive decline of synapses with age (Waites *et al.*, 2005; Harms and Dunaevsky, 2007).

Synapse formation engages coordinated changes due to ligand-receptor interactions, intracellular signaling cascades and subsequent cytoskeletal rearrangements, and intrinsic processes that contribute to the pre-establishment of synaptic machinery prior to synaptic contacts (Shen and Cowan, 2010). A multitude of signaling molecules, including secreted factors and cell-adhesion molecules, are involved in synaptogenesis specificity. Prior to synapse formation, secreted molecules involved in growth cone guidance act diffusely from local sources and guide axons to their targets, which are normally dynamic dendritic filopodia that extend from the dendritic shaft (Shen and Cowan, 2010). Upon approach to the target site, a presynaptic growth cone slows its advance, makes a physical contact and transforms itself into a rudimentary synaptic ending (Munno and Syed, 2003). Then, priming factors derived from surrounding glia and neurons promote the maturation of both target neurons and innervating axons, as well as the competence of dendrites and axons to undergo synaptogenesis (Waites *et al.*, 2005). The premature contacts between axons and dendrites are allowed by adhesive factors, being then stabilized by the cooperative action of adhesive and inductive factors, which are involved in the specialization of both pre- and postsynaptical compartments (Waites *et al.*, 2005). So, several mechanisms permit the establishment of proper connections, upon growth cone guidance, allowing axons and dendrites to find their appropriate synaptic targets (Fig. I.3.).

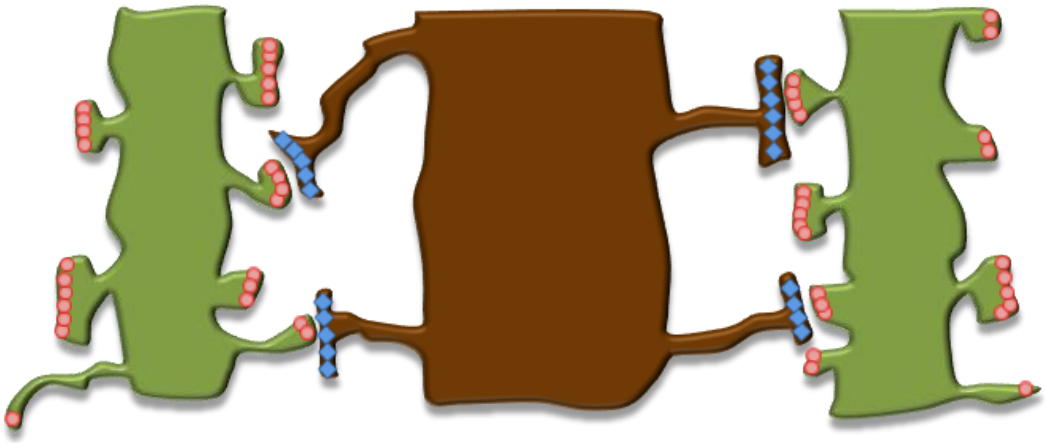

Fig. I.3. Synapses allow the directional connection of neurons. Axonal boutons (brown) can form synapses with different dendrites (green). Axons are guided towards their targets, normally dendritic specializations, as dendritic spines, and both pre- and postsynaptical compartments suffer maturation (blue and red, respectively), allowing the propagation of the electrochemical signal from the axon to the dendrites.

Synaptic development is characterized by a prolonged maturation phase with the purpose of properly integrate individual neurons into a functional network. Perhaps the most dramatic change in the postsynaptic side is the appearance and maturation of dendritic spines. Dendritic spines are small protrusions emerging from dendrites and the postsynaptic regions of most excitatory neuronal synapses, exerting an important role in several brain functions, such as memory and learning at the hippocampus (Mattila and Lappalainen, 2008). The development of these spines usually involves filopodia-like precursors that dynamically grow, with the intention of reaching the presynaptic partner, being very abundant during early development, but decreasing as neurons maturate (Fiala *et al.*, 1998). The filopodial precursor stabilizes and matures into a dendritic spine if the signal is proper, whereas it shrinks back to the dendritic backbone in the absence of an appropriate signal (Sekino *et al.*, 2007). Generally, dendritic spines consist of a head linked to the dendrite by a stalk or neck and have been divided into several categories of shapes that vary from filopodium-like (more prominent on younger dendrites), to mushroom-shaped (in more mature ones) (Fig. I.4. A). The size of the head correlates with the synaptic force since the smaller spine head corresponds to a smaller associated postsynaptic density (PSD) (McKinney, 2005). Thus, mushroom-shaped spines, consisting of a short neck and a large mushroom-shaped head, bear larger PSD, oppositely to elongated and thin filopodial spines. PSD is a marker of the contact between a presynaptical terminal and a dendritic spine, containing the machinery that links the synaptic transmission to various signaling cascades and cytoskeletal components (Kennedy, 2000). Indeed, head spines are enriched in actin filaments, which modulates the high degree of spines plasticity and motility, allowing the dynamic behavior of the spines, as well as their ability to continuously change their morphology (Lippman and Dunaevsky, 2005; Mattila and Lappalainen, 2008). Thus, regulators of the cytoskeleton may exert effects in dendritic spines number and shape, which alterations are often correlated with changes in neuronal activity (Lippman and Dunaevsky, 2005). Synaptic dysfunction and deficits in cognitive function can be associated with irregular shapes and densities of dendritic spines (McKinney, 2005). Actually, abnormal alterations in dendritic spines are often seen in cases of mental retardation, such as fragile X syndrome (Fig. I.4. C) and neurological disorders including schizophrenia (Fig. I.4. D) (von Bohlen und Balbach, 2010; Faludi and Mirnics, 2011).

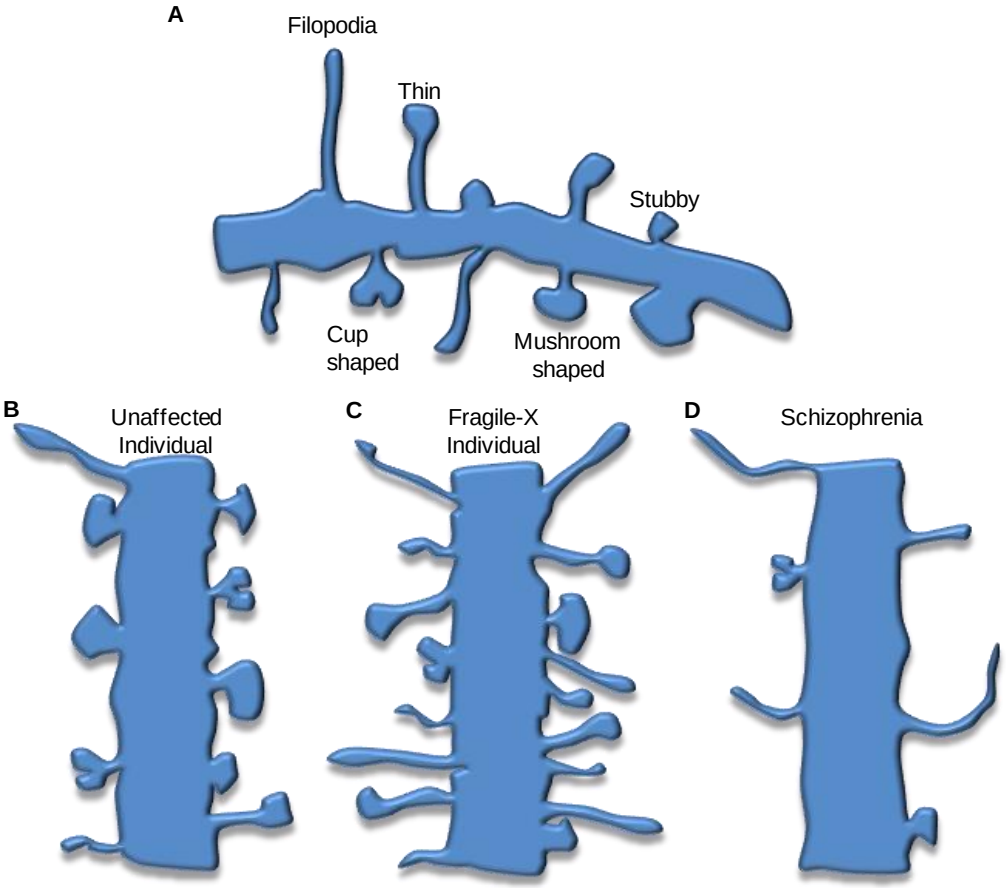

Fig. I.4. Schematic representation of different dendritic spine morphologies. The immature morphologies are characterized by longer necks and smaller heads (*e.g.* filopodia and thin shapes), whereas the mature forms have lower height and larger heads (*e.g.* cup and mushroom shapes) (A). Dendritic shafts usually present several types of dendritic spines which may suffer morphological changes during plasticity (B). Spine density and morphology is altered in cases of mental retardation, as fragile-X syndrome (C). In such case, increased immature spines and high density appear along the dendritic shaft of neurons from both temporal and visual cortices. On the other hand, schizophrenic patients exhibit lower spine density along the dendritic shaft, but the dendritic spines are also more immature (D). The alterations observed in both conditions may trigger the cognitive deficits verified in patients. (Based in Irwin et al., 2001; von Bohlen und Halbach, 2010 and Faludi and Mirnics, 2011).

Interestingly, a high degree of neuronal plasticity does not end during the early neuronal development and persist even after neuronal polarization and synaptogenesis. As Gomis-Rüth and colleagues (2008) have demonstrated, neurons fully integrated in a neuronal network can convert a mature dendrite to a functional axon upon a proximal axon cut, whereas a more distal injury leads to the re-growth of the axon (Gomis-Rüth *et al.*, 2008). Additionally, synaptic plasticity, characterized by reconfigurations in the structure and functionality of synapses, is important in normal brain function and induces changes in synapse number, shape and strength and, ultimately, in neuronal connectivity. In fact, the inability to undergo these plastic changes can be a cause for several neurodegenerative and psychological disorders (Munno and Syed, 2003; von Bohlen und Halbach, 2010; Faludi and Mirnics, 2011). However, neuronal plasticity may not only depend on neurons. Indeed, glial cells have been indicated as active controllers of dendritic outgrowth and dendritic spine morphologies, therefore contributing for the non-stationary state of the central nervous system (CNS) (Procko and Shaham,

2010). Moreover, the development of synapses is facilitated by the presence of glial cells, as they help the guidance and the growth of both axons and dendrites in the CNS (Pfrieger, 2009).

2. Cytoskeleton Dynamics in Neuronal Development

Cytoskeleton has a vital role in the normal cell function and takes part in several activities from cell shape and locomotion to intracellular organelle transport. Within eukaryotic cells, the cytoskeleton is composed by three organized types of polymeric protein filaments: microtubules, intermediate filaments and microfilaments. These long protein polymers, that exhibit distinct properties, interact with each other and are dynamic structures. The regulation of cytoskeleton filaments is achieved by several proteins, such as microtubule-associated proteins (MAPs), actin-binding proteins and motor proteins. In neurons, all cytoskeletal components are crucial to provide structural organization, to establish and maintain neuronal polarity, to serve as tracks for intracellular transport and to allow different cellular morphologies (Tahirovic and Bradke, 2009; Polleux and Snider, 2010; Dent *et al.*, 2011a). In fact, the formation of a polarized neuron is essential for the integration and proper propagation of synaptic information in the adult CNS, which is dependent on cytoskeleton rearrangements (Witte and Bradke, 2008; Barnes *et al.*, 2008; Tahirovic and Bradke, 2009). Moreover, cytoskeleton has also a substantial role in neurite elongation, growth cone turning, advance and branching, and all of these processes are involved in neuronal development (Dehmelt and Halpain, 2004a; Gallo and Letourneau, 2004). So, an intricate interplay between the different compartments of cytoskeleton and their respective proteins exists during neuronal development.

From a classic point of view, the microtubule cytoskeleton has been thought to be important for cell division and organelle trafficking, while the dynamic actin cytoskeleton was known to generate force during cell contraction and dispersion. In more recent times, this classic vision has been contested and a cooperative relation between these two cytoskeleton complexes has emerged, as actin filaments have been implied in both cell division and trafficking, as well as microtubules have been addressed with roles in cellular morphology generation and plasticity (Dehmelt and Halpain, 2004a; Dent *et al.*, 2011b). Furthermore, the precise control of neuronal morphogenetic program involves the regulation of cytoskeleton dynamics, which can be achieved by several intracellular signaling pathways, as the Rho family of small GTPases. This family possesses several members, as Ras homolog gene family member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42). Rho GTPases cycle between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state, which interacts with specific effectors to propagate downstream signaling events (Hall and Lalli, 2010). Indeed, the conserved and major role of Rho GTPases is the control of the dynamic rearrangements of both microtubules and microfilaments, therefore playing an influential in the neurodevelopment (Hall and Lalli, 2010).

2.1. Microtubules

Microtubules are formed by polymers of α- and β-tubulin heterodimers associated head-to-tail to form a hollow and elongated cylinder normally composed by 13 protofilaments (Lodish *et al.*, 2004). The microtubules are polar structures given the intrinsic polarity of the tubulin heterodimers and their linearly disposition. These polymers undergo post-translational modifications, as tyrosination, present in recently synthesized and dynamic microtubules, and acetylation, which occurs in more elderly and stable microtubules (Fukushima *et al.*, 2009). These structures have the ability to grow and shrink through end-polymerization of heterodimer subunits using the energy derived from the hydrolysis of GTP bound to β-tubulin (Gordon-Weeks, 2004). Therefore, the rate of growth or shrinkage depends on the kinetics of subunit addition or subtraction, which is different in the two ends. The microtubule's fast-growing end, with the β-subunit exposed, is named the "plus end" and it is the preferential local to tubulin assembly, while the slow-growing end, with the α-tubulin uncovered, is denominated the "minus end" and is usually attached to a microtubule organizing center or capped (Gordon-Weeks, 2004). Microtubule cytoskeleton *in vitro* cultured cells suffers a dynamic instability characterized by cycles of relative slow and continuous growth interrupted by catastrophes, phases of rapid shortening due to heterodimer dissociation probably caused by the loss of the GTP-β-tubulin cap at the end of the microtubule (Kirschner and Mitchinson, 1986; Cassimeris *et al.*, 1988). However, this rapid length loss does not lead to microtubule complete depolymerization because it occurs in parallel to a phase of sustained growth (Gordon-Weeks, 2004, Geraldo and Gordon-Weeks, 2009).

Microtubules exhibit a typical compartment-specific distribution in the axons of developing neurons with their plus-ends oriented toward the distal end (Fig. I.5.). In contrast, dendrites display a mixed microtubules orientation, *i.e.* some have their plus-ends toward the cell soma, while others have their plus-ends pointing toward the peripheral zone. The uniform axonal distribution is thought to be the default state in neurites, as it exists when minor neurites start to emerge, where they are usually found in bundles (Brandt, 1998; Gordon-Weeks, 2004). As one of the principal cytoskeletal components, microtubules are involved in the maintenance of neuronal morphology and in the establishment of both dendritic and axonal arborizations. In neurites, a gradient of microtubule instability is observed, with distal microtubules undergoing a higher turnover than the microtubules in the middle of the neurite or near the cell body (Bamburg *et al.*, 1986). However, studies with taxol and nocodazole have shown that the spontaneous neurite initiation depends on both the presence and dynamic properties of microtubules (Letourneau and Ressler, 1984; Witte *et al.*, 2008). Therefore, changes in microtubule dynamics are sufficient to induce modifications in normal neuronal development. Indeed, stabilization of microtubules is required to the formation of the axon and, consequently, to neuronal polarization rising. Witte and colleagues (2008) have shown that low doses of taxol lead to the formation of multiple axons, while local microtubules stabilization, by UV-mediated photoactivation of caged taxol at the tip of one random minor neurite, promotes axon formation (Witte *et al.*, 2008). This stabilization favors microtubules to distally advance with their dynamic plus-ends, promoting axon formation (Witte and Bradke, 2008). Also, microtubule cytoskeleton confers a structural support for dendrites and its depolymerization leads to the invasion of filopodia and subsequent filopodial stabilization, which will form a dendritic branch (Georges *et al.*, 2008).

At both dendritic and axonal growth cones, microtubules are disposed in a similar way with their plus ends oriented distally towards the P-domain (Heidemann *et al.*, 1981; Baas *et al.*, 1988). Here, as microtubules enter the C-domain, they defasciculate and some can even cross this domain as single microtubules (Gordon-Weeks, 2004). Furthermore, dynamically unstable microtubules invade the P-domain and explore the actin network, where they become stabilized, leading to axon specification (Geraldo and Gordon-Weeks, 2009; Dent *et al.*, 2011a). In response to guidance cues, microtubule dynamic instability is required for growth cone turning and consequently to the directional axonal outgrowth (Tanaka *et al.*, 1995; Challacombe *et al.*, 1997) Moreover, in the absence of dynamic microtubules, the remaining microtubules do not suffer the typical cycle of bundling and splaying, what is deeply associated with the forward movement of the growth cone (Tanaka *et al.*, 1995). In addition, the attenuation of microtubule dynamics in growth cones has also shown to impair lamellipodial protrusion (Gallo, 1998).

Microtubules are also important to dendritic spine morphology and function, even though the central role of the actin filaments in these excitatory postsynaptical sites (Dent *et al.*, 2011b). Thus, modifications in microtubule dynamics are related to changes in the normal dendritic spine formation and further maturation (Gu *et al.*, 2008; Jaworski *et al.*, 2009). Moreover, the polymerization and depolymerization of microtubules is coincident with the extension and retraction of transient spine heads protrusions, respectively (Hu *et al.*, 2008). This study has also shown that an increase of neuronal activity is related to a higher number of spines with a longer-invasion by microtubules (Hu *et al.*, 2008). Additionally to structural support, microtubules are important to cellular trafficking, because they provide tracks for motor proteins, as kinesin and dynein, allowing the cargo transportation to specific cellular parts, as dendritic spines and synaptic terminals. This transportation to and from pre- and post-synaptical sites is critical for synaptic function, as synapses are highly vulnerable to transport impairments (Gendron and Petrucelli, 2009).

2.2. Microtubule-associated proteins

Microtubule associated proteins (MAPs) are a family of proteins known by their role in regulation of microtubules polymerization, stability and organization (Gendron and Petrucelli, 2009). However, besides the regulation of microtubule networks in the axons and dendrites, there are evidences for a larger range of functions for MAPs, including binding to F-actin, recruitment of signaling proteins and regulation of microtubule-mediated transport (Dehmelt and Halpain, 2004b). Microtubule associated protein 2 (MAP2) and Tau are structural MAPs, *i.e.* have the ability to alter microtubule structures, acting to reduce catastrophe periods and to increase rescue phases upon binding along the outer ridges of the protofilaments (Al-Bassam *et al.*, 2002; Dehmelt and Halpain, 2004a). Hence, they do not prevent microtubule dynamic instability. Instead, they simply alter the dynamic behavior of this cytoskeleton compartment, creating a partial stable but dynamic state important for cell growth and transport.

MAP2 and Tau seem to have essential and distinct roles in the early stages of neuromorphogenesis. In cultured neurons, Tau protein is related to the polarity development and is

disposed in a proximo-distal gradient in the process that will develop into the axon, with the higher concentration at the transition from the axonal shaft to the growth cone (Kempf *et al.*, 1996). Thus, Tau is gradually segregated into the future axon and is able to adjust microtubules assembly and stabilization in this process (Fig. I.5.) (Dehmelt and Halpain, 2004a). The distinctive distribution of Tau in neurons is regulated by its degree of phosphorylation, as this post-translational modification of certain residues detaches Tau from microtubules, decreasing its affinity for them. Therefore, phosphatases (e.g. tyrosine phosphatases) are implicated in the complex regulation of the intracellular function and localization of this MAP (Mandell and Baker, 1996). In contrast, MAP2 is essential for initiation of neurites and is segregated into the emerging dendrites, exhibiting a somatodendritic distribution. In fact, the suppression of MAP2 synthesis inhibits the initial formation of neurites in cultured neurons (Cáceres *et al.*, 1992). The differential distribution of these two MAPs is maintained in the mature neuron, *i.e.* MAP2 is exclusively present in the somatodendritic region, whereas Tau only exists in the axon (Dehmelt and Halpain, 2004a).

MAPs were also found to interact with both actin filaments and microtubules. While actin filaments and tubulin polymers do not interact without MAPs, their presence is sufficient to induce interactions between the two cytoskeletal compartments (Griffith and Pollard, 1978). Thus, MAPs might mediate the crosstalk between microtubules and actin filaments. However, it is suggested that both microtubules and microfilaments bind to the same domain in MAP2 and Tau proteins, because microtubules exclude the binding of microfilaments and vice-versa (Correas *et al.*, 1990).

2.3. Microfilaments

Microfilaments consist in two helical and separated strands of filamentous actin (F-actin), each one a polymer of globular-actin (G-actin). As microtubules, microfilaments are intrinsically polar because all actin subunits are directed toward the same end of the filament. Conventionally, the terminal actin subunit exposing adenosine diphosphate (ADP) -G-actin is designated the minus-end or the pointed-end, while the opposite end, the plus-end or the barbed-end, is the fast-growing end of the filament and the local for the binding of adenosine triphosphate (ATP)-G-actin (Lodish *et al.*, 2004). Hence, the barbed-end is the preferential local for actin subunits addition, whereas the pointed-end is the local for actin subunit dissociation. Microfilaments are highly dynamic structures that can rapidly suffer cycles of assembly and disassembly and the actin polymerization is favored by stimulation of ADP/ATP exchange of G-actin (Witte and Bradke, 2008). Actually, actin filaments undergo a process called treadmiling, by which they are able to exert net movement, consisting in the assembly of filaments at their fast-growing ends and disassembly at their slow-growing ends (Brandt, 1998).

Actin cytoskeleton is deeply involved in neurite development, breakage of the initial symmetry in neurons, *i.e.* neuronal polarization, and alterations in actin dynamics are also associated with changes in neuronal morphology. Initiation of neurites is induced by a reduction in the tensile forces mediated by actin cytoskeleton (Georges *et al.*, 2008). Indeed, the use of cytochalasin B, which reduces tensile forces by depolymerization of actin filaments, increases the length of both axons and dendrites (Lafont *et al.*, 1993). On the other hand, jasplakinolide, a macrocyclic peptide that inhibits F-actin turnover,

causes axonal retraction, showing that the turnover of microfilaments is required for axonal extension (Gallo *et al.*, 2002). One feature of the future axonal growth cone in immature neurons is a lower actin cytoskeleton stability, that can be the cause of microtubule protrusion and, consequently, of neurite outgrowth (Bradke and Dotti, 1999). Furthermore, the disruption of the actin network of one individual growth cone is sufficient to induce its neurite to develop as an axon, and the application of actin depolymerizing drugs is enough to produce neurons with multiple axons (Bradke and Dotti, 1999).

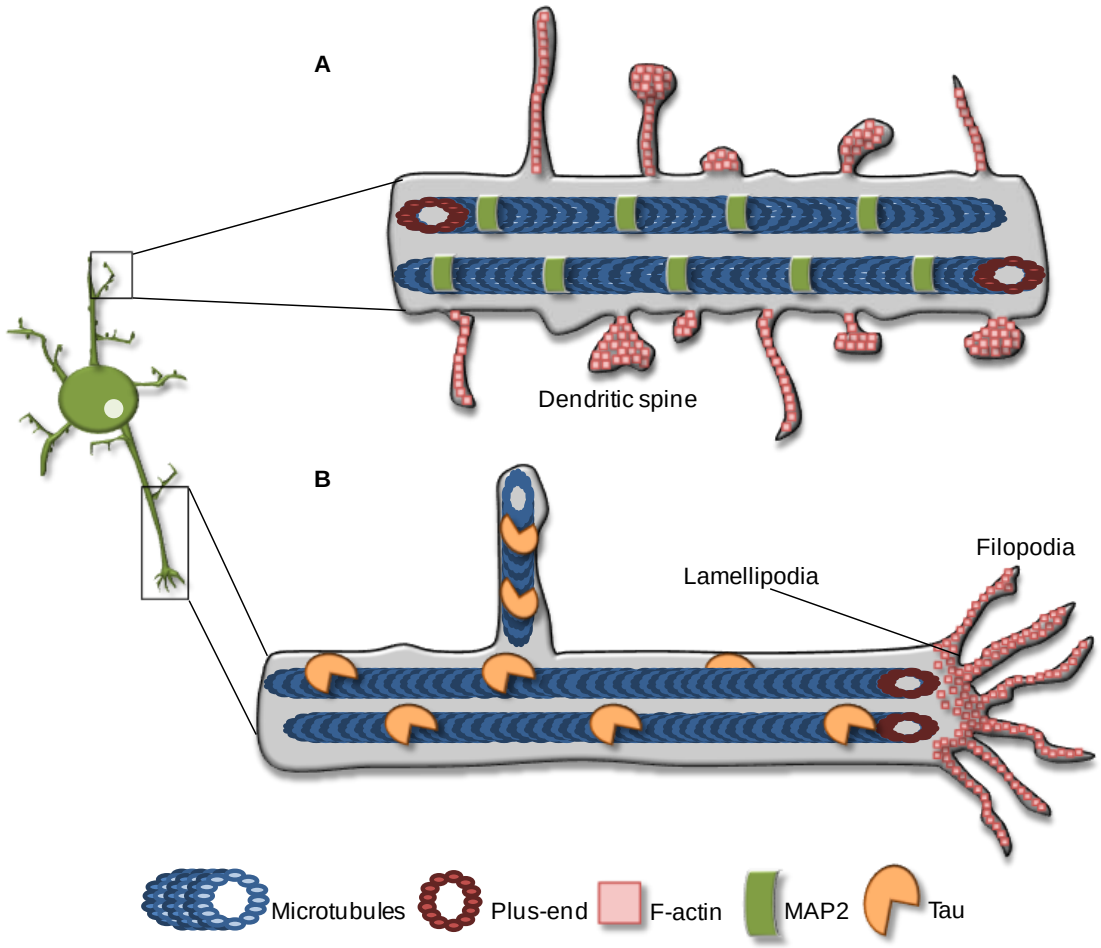

Fig. I.5. Schematic representation of dendritic and axonal cytoskeletons. (A) Dendrites display mixed microtubules orientations, i.e. some microtubules have their plus-end directed to the cell soma, whereas others exhibit their plus-end toward the distal end of the dendrite. The dendritic microtubules are stabilized by MAP2. The dendritic spines are, essentially, composed by actin filaments, which contribute to their dynamics. (B) In the axon, microtubules are stabilized by Tau and disposed in a regular and uniform way, with their plus-ends pointing to the distal end. Usually, microtubules are retained in the C-domain of the growth cone and actin filaments occupy the P-domain, with both filopodia and lamellipodia.

In order to achieve a directional growth, extracellular cues are thought to induce a selectively stabilization of actin cytoskeleton in both lamellipodia and filopodia, the preferential locals for actin polymerization (Fig. I.5.) (Gallo and Letourneau, 2004). Actually, the extension of filopodial tips is determined by the rate of F-actin polymerization and the retrograde transport of the polymerized filaments on the way to the bottom of the filopodium (Mallavarapu and Mitchinson, 1999). During axon guidance, actin polymerization is favored in areas contacting with positive or attractive guidance cues,

inducing growth cone turning (Shen and Cowan, 2010). Moreover, actin structures redirect and stabilize microtubules in the direction of turning, since actin destabilization by low concentrations of cytochalasin B alters growth cone turning and microtubules fail to retract from the side in contact with an inhibitory signal (Challacombe *et al.*, 1997). Furthermore, the loss of actin filaments at the leading edge of the growth cones is related to growth cone collapse (Fan *et al.*, 1993; Fournier *et al.*, 2000; Avwenagha *et al.*, 2003). Hence, F-actin can be a major intracellular target of extracellular guidance cues that modulate growth cone behavior.

The heads of dendritic spines, mainly the postsynaptic density zone, are enriched in actin cytoskeleton, which is important for both formation and motility of dendritic spines (Fig. I.5.) (Dunaevsky *et al.*, 1999; Capani *et al.*, 2001). Moreover, the high level of plasticity observed in spines may be due to the rapid turnover rates of actin cytoskeleton. Concerning synaptogenesis, the use of the actin-depolymerizing agent latrunculin A leads to an almost complete loss of synapses in the first week of hippocampal neuronal cultures, as well as to a decrease in the synaptophysin clusters number and size, revealing an important role of F-actin in the development and maintenance of young synapses (Zhang and Benson, 2001).

Despite the morphological and plastic roles of actin cytoskeleton, these filaments are also involved in the transportation of certain cargos, because myosins, a family of motor proteins, directly bind to actin filaments and transport a cargo in their tails (Lodish *et al.*, 2004). Actin cytoskeleton can even serve as a substrate for the microtubule anterograde movement (from the cell soma to the presynaptic terminal), when there are no longer microtubules available to play this role (Hasaka *et al.*, 2004). Indeed, the transport of microtubules at the growth cone may be dependent on actin based-mechanisms (Myers *et al.*, 2006).

3. Neuroinflammation: from a beneficial to a detrimental effect

For a long time, the CNS was considered an immune privileged site, by the absence of a typical and classical immune response (Di Filippo *et al.*, 2008). Likewise, it was also thought that the brain was not largely affected by systemic or immune inflammatory reactions (Lucas *et al.*, 2006). Nowadays, it is widely accepted that an interaction between immune and nervous systems exists, involving a bidirectional cross talk. Indeed, the CNS is provided with an active immune surveillance and it can be the host of inflammatory responses to different types of injuries (Di Filippo *et al.*, 2008). Neuroinflammation, the inflammation of the CNS, consists in a set of events, including the activation of the resident neural cells, as microglia and astrocytes, which together with neurons release a panoply of inflammatory molecules (Fig. I.6.) (Chavarria and Alcocer-Varela, 2004; Infante-Duarte *et al.*, 2008; Whitney *et al.*, 2009).

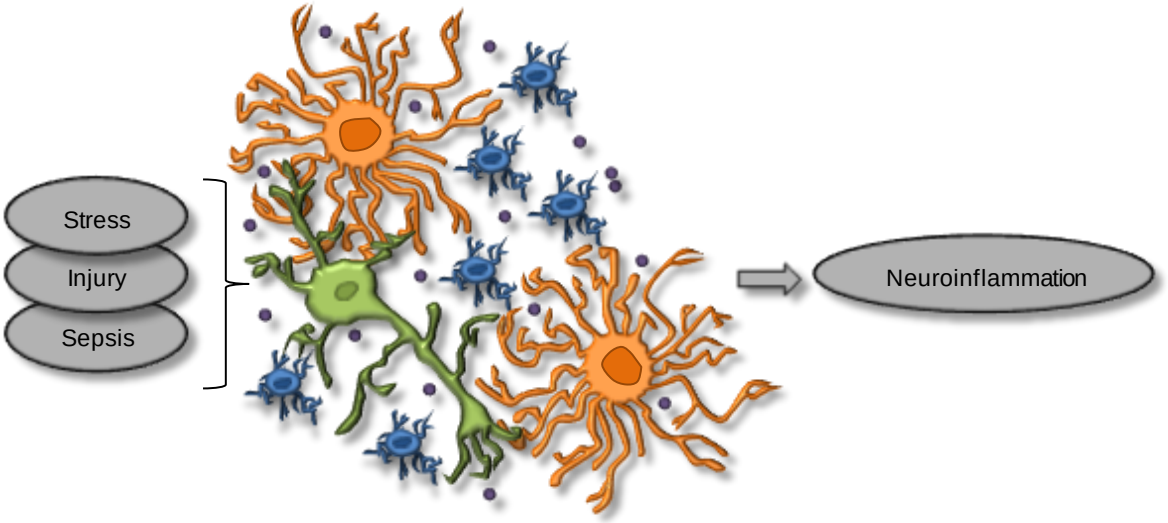

Fig. I.6. Neuroinflammation is a complex response of neural cells to a detrimental stimulus. Upon stress, injury or sepsis, the resident neural cells, as microglia (blue), astrocytes (orange) and neurons (green) produce inflammatory mediators (purple). These molecules influence the normal state of the CNS and are capable of recruiting circulating immune cells, leading to neuroinflammation.

The segregated inflammatory mediators are responsible for the expression of chemokines and adhesion molecules, the activation of endogenous glial cells and the stimulation of astrogliosis, in addition to the recruitment of immune cells to the site of inflammation (Rothwell and Luheshi, 2000; Whitney *et al.*, 2009). Indeed, the CNS can be invaded by circulating immune cells, which contribute to the brain inflammatory state. Hence, neuroinflammation is seen as a complex cellular and molecular response of neural cells to a detrimental stimulus, such as injury, stress or sepsis (Semmler *et al.*, 2008; Whitney *et al.*, 2009). The main goals of neuroinflammation are the defense against these insults, the clearance of dead and damaged neurons, as well as the return of the CNS to homeostasis (Whitney *et al.*, 2009). However, when neuroinflammation is not controlled, its beneficial role is overcome and may then turn into damage. In that case, the recruited cells become activated and release even more inflammatory mediators, establishing a positive feedback and leading to neuronal injury with changes in neurogenesis (Das and Basu, 2008; Whitney *et al.*, 2009). Therefore, neuroinflammation, if in excess, may contribute to neurodevelopmental impairments. In addition, oligodendrocytes are extremely vulnerable to inflammatory molecules which may result in white matter damage and the emergence of long-term neuromotor, cognitive, and behavioral limitations, or to degenerative disorders, such as Alzheimer's and Parkinson's diseases (Lucas *et al.*, 2006; Aktas *et al.*, 2007).

Most attractive is the recent hypothesis that an early infection could produce a latent or hidden change in the immune system that could be unmasked by a second immune challenge. This "two-hit hypothesis" was first proposed for schizophrenia etiology, suggesting that the first hit, which may occur during the embryonic life, "primes" the nervous system for the second one, accelerating the disease symptoms (Maynard *et al.*, 2001). Therefore, the concept of "glial priming" has been proposed to account for the "two-hit hypothesis" (Bilbo and Schwarz, 2009). The authors describe that, after a first insult in the early-life, a subset of glial cells become primed and display an activated morphology.

Hence, when facing a second immune challenge in later-life, the primed glia, which do not chronically produce inflammatory mediators, over-release cytokines within the brain exaggerating the immune response and producing damaging consequences (Bilbo and Schwarz, 2009).

3.1. Cytokines as neuroinflammation mediators

Cytokines are secreted polypeptides with several addressed roles in immune responses. Previously, cytokines were thought to only act in the peripheral system, but nowadays a crescent number of data have found that these molecules can exert several actions in the brain, including in the development of neurons. Pro-inflammatory cytokines, as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), are synthesized by neural cells and take part in the normal intercellular communication, assuming an important role for the maintenance of homeostasis. The pro-inflammatory cytokines can act as neurotrophic factors during the normal development of the brain, *e.g.* IL-1 is regarded as an important growth factor in brain formation (Giulian *et al.*, 1988; Zhao and Schwartz, 1998). Additionally, these cytokines can induce the production of anti-inflammatory mediators, therefore creating a feedback loop, in order to control the immunologic state (Morganti-Kossman *et al.*, 2002). Normally, the levels of such cytokines are low, but their expression is rapid and radically increased in situations of neuroinflammation. When this elevation of expression is sustained, pro-inflammatory cytokines can become pathological, deregulating cytokine release, and can even cause neuron and oligodendrocyte death (Donnelly and Popovich, 2008). In reality, when neuroinflammation happens in the perinatal period during the development of the nervous system, the intrinsic developmental program of the brain can be altered by the exposure of the fetus to inflammatory mediators, what may eventually result in lasting neuronal disorders (Degos *et al.*, 2010). Yet, the detrimental effect of cytokines may rely on the type and level of cytokines produced. In other words, the low physiological rates of cytokine expression may be important for the cross-talk between the neural cells during development, but the overexpression observed during the neuroinflammatory stage may compromise neuronal survival and plasticity.

IL-1 is a potent pro-inflammatory cytokine with the capability to orchestrate inflammatory responses, since it can induce the expression of several inflammatory molecules that intensify the immune response. Moreover, IL-1 has the ability to enhance responses in cells with low levels of its own receptor, for the reason that it activates a complex signaling cascade that amplifies the initial signal (Rothwell and Luheshi, 2000). This cytokine exists in two partially homologous isoforms, IL-1α and IL-1β, both translated as a proform that it is further cleaved by cell-surface-bound calpain and caspase-1 or IL-1β converting enzyme, respectively, in order to produce the mature forms (Mrak and Griffin, 2001; Basu *et al.*, 2004). The two isoforms bind with high affinity to a specific membrane receptor, the IL-1 receptor type I, which contains a cytoplasmic domain engaging intracellular signaling pathways (Di Filippo *et al.*, 2008; Spulber and Schultzberg, 2010). It is described that the binding of IL-1β to its receptor may induce modifications at the cytoskeleton level, through the activation of p38 mitogen-activated protein kinases (Temporin *et al.*, 2008). However, an endogenous receptor antagonist (IL-1ra) can prevent the activation of IL-1 receptor upon IL-1 binding, thus blocking the

actions of the cytokine (Lucas *et al.*, 2006). All these components are present within the brain, albeit at low rates in the normal and healthy CNS (Basu *et al.*, 2004). Following CNS damage, IL-1 is regarded as an important initiator of the immune response and, consequently, is rapidly produced and released specially by activated microglia (Rothwell and Luheshi, 2000). There is accumulating evidence suggesting a detrimental role of high levels of IL-1β in neuronal function following an *in vivo* insult. Indeed, it was reported that IL-1β may even exacerbate the initial damaging stimulus of conditions such as ischemia and/or excitotoxicity (Loddick and Rothwell, 1996; Allan *et al.*, 2000; Boutin *et al.*, 2001).

Other important pro-inflammatory cytokine, TNF-α, is predominantly produced by glial cells and in a lower rate by neuronal cells, also having an important role in the initiation and regulation of the inflammatory process (Whitney *et al.*, 2009; Ziebell and Morganti-Kossmann, 2010). This cytokine is synthesized as a membrane-bound precursor that is subsequently cleaved by the TNF converting enzyme, in order to produce the active cytokine, which levels are quickly increased in response to CNS damage (Perry *et al.*, 2001). TNF-α can bind to two different cell-surface receptors, p55 (tumor necrosis factor receptor-1; TNFR1) and p75 (TNFR2) (Lucas *et al.*, 2006; Whitney *et al.*, 2009). The cytotoxic actions leading to neuronal apoptosis exerted by TNF-α are mediated through p55, while the actions upon binding to p75 are generally proliferative and may involve anti-apoptotic signals (Muñoz-Fernández *et al.*, 1998). Therefore, opposing and different effects of TNF-α may be due to the distinct signaling pathways triggered by the different receptors. Nevertheless, as IL-1β, TNF-α was shown to mediate neuronal damage after an insult, and exert a variety of effects, from neuronal loss to neurotoxicity and brain edema (Shohami *et al.*, 1997; Ådén *et al.*, 2010; Kendall *et al.*, 2011). Besides, TNF-α was also demonstrated to act synergistically with IL-1β, enhancing neuronal injury and neurotoxicity (Chao *et al.*, 1995).

3.2. Neuroinflammation during development

During pregnancy, the inflammatory state of the fetus may be influenced by the substances derived from the uterine environment that can cross the placenta. Indeed, the injection of lipopolysaccharide (LPS) to pregnant rats at day 18 of gestation, mimicking a pre-natal infection condition, triggers the production of cytokines in the fetal brain (Cai *et al.*, 2000). These fetuses present elevated mRNA levels for pro-inflammatory cytokines such as TNF-α and IL-1β in a dose-dependent manner (Cai *et al.*, 2000). Consequently, intrauterine infection can have a significant impact on the proper development of the brain. Indeed, several works have confirmed that maternal infection may influence the neurodevelopment of the fetus due to alterations in pro-inflammatory cytokine levels (Ling *et al.*, 2002; Bell *et al.*, 2004; Burd *et al.*, 2010). Furthermore, neuroinflammation in the fetal brain has been associated with neurological *sequelae*. High levels of pro-inflammatory cytokines in the amniotic fluid or umbilical blood increase the risk for white matter damage and/or cerebral palsy (Yoon *et al.*, 1996; Yoon *et al.*, 2000). Moreover, antenatal inflammatory events have been linked to the permanent loss of dopaminergic neurons in parallel with the rise in TNF-α levels (Ling *et al*., 2002). Therefore, antenatal infections are considered to be a potential risk factor for the

development of Parkinson's disease. In the neonatal rat brain, IL-1β is able to induce apoptosis 24h after its intracerebral injection, whereas TNF-α exhibits a less evasive role in brain injury, inducing a lower number of apoptotic cells (Cai *et al.*, 2004). In addition, co-administration of IL-1ra and LPS decreases caspase-3 activity and white matter injury, suggesting that IL-1β may mediate brain injury in the neonatal rat brain (Cai *et al.*, 2003). Conversely, IL-1β was shown to promote cell survival in primary neuronal cultures from fetal rat cortices after exposure to excitatory amino acids, whereas the co-administration of IL-1ra failed to prevent neuronal cell death (Strijbos and Rothwell, 1995). Nonetheless, the deletion of the TNF cluster can abolish the detrimental effects induced by LPS, as neuronal loss in mouse hippocampus, after an hypoxic ischemic insult in postnatal day 7 (Kendall *et al.*, 2011). In addition to neuronal cell death, inflammation can affect not only the neuronal function and cytoskeleton, but also the nervous system development, which may have important implications for neurological outcome in preterm infants. In fact, the increased levels of pro-inflammatory cytokines mRNA together with the disruption of neuronal morphology were observed in an inflammation-induced preterm birth model (Burd *et al.*, 2010). It was also reported that TNF-α was able to promote the activation of small GTPase RhoA which is an important modulator of neurite outgrowth and branching (Neumann *et al*., 2002). Nevertheless, the exact mechanisms by which IL-1β and TNF-α act on the formation of neuronal arborization are not clarified.

Synaptic strength and plasticity may also be modulated by the presence of pro-inflammatory cytokines. The injection of LPS to pregnant mice at day 15 of gestation causes alterations in glutamatergic synapses in the adult offspring, showing that the prenatal inflammatory environment in the developing brain is able to induce modifications in the normal synaptic functionality (Roumier *et al.*, 2008). These data suggest that a prenatal brain inflammation can have long-lasting impacts in neuronal and synaptic dysfunction, perhaps instigating cognitive disabilities. It is described that during long-term potentiation (LTP) of synaptic transmission, *IL-1β* gene expression is induced and the administration of IL-1ra inhibits the maintenance phase of LTP (Schneider *et al.*, 1998). On the other hand, Bellinger and collaborators (1993) demonstrated that low levels of IL-1β have an inhibitory effect on the induction of hippocampal LTP, reducing the strength of synapses (Bellinger *et al.*, 1993). Therefore, IL-1β seems to modulate the synaptic transmission in the hippocampus, potentially with a concentration dependent mechanism. However, this effect may result in an impairment of the main forms of neuroplasticity and in the destabilization of neuronal circuits, which do occur in several neurodegenerative conditions, as Alzheimer's and Parkinson's diseases (Di Filippo *et al.*, 2008). The cytokine TNF-α raises the number of the glutamate receptor AMPA on neuronal cell membranes, rendering these cells more sensible to extracellular glutamate which can contribute to excitotoxicity (Stellwagen *et al.*, 2005). Besides, the same molecule has a crucial role in some forms of synaptic plasticity, as synaptic scaling, which is responsible for adjustments in synaptic strength, promoting stability in neuronal firing rates (Turrigiano and Nelson, 2004). Thus, it seems that TNF-α has the ability to influence synaptic strength, since changes in AMPA receptors number in the synaptic membrane are often correlated with the ability of synaptic scaling to modify synaptic strength (Wierenga et al., 2005). Overall, cytokines are not only regulatory proteins of the immune responses,

but can also act as neuromodulators. Nevertheless, cytokine role in synaptogenesis during and/or following an inflammatory stimulus is less well described.

4. Aims

Fetal neuroinflammation assumes an important role in neurodevelopment, as it affects neuronal survival, morphology, plasticity and functionality, often leading to damage and to several neurodevelopment *sequelae* in the adult offspring. Therefore, it is fundamental to understand how the inflammatory environment induces alterations in neuronal architecture and inter-neurons communication during the development of the nervous system, in order to identify determinants and novel target-directed therapies.

Hence, the main goal of the present project was to investigate the effects of the pro-inflammatory cytokines TNF-α and IL-1β on neuronal development by assessing alterations on:

(i) neuronal morphology, focusing on the measurement of neuronal arborization and evaluation of cytoskeleton changes, to perceive cytokine influence on the extension and branching of both dendrites and axons;

(ii) growth cone morphology and the presence of cytoskeleton proteins due to its influence on active and sustained growth of the neuronal protrusions;

(iii) synaptogenesis by measuring dendritic spine and synapse densities, that are involved in the communication between neurons and maturation of synapses.

Overall, clarification of the key events involved in alterations of neurite morphology and synaptic plasticity that accompanies CNS inflammation will be vital to achieve neurite regeneration, synaptic reorganization and a successful rehabilitation

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II. MATERIALS AND METHODS

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1. Materials

Neurobasal media, B27 supplement, Hanks' Balanced Salt Solution (HBSS), and Tripsin 2.5% were purchased from Gibco® (Grand Island, NY, USA). Poly-D-lysine, human serum albumin (HSA) and bovine serum albumin (BSA) (fraction V, fatty acid free), were acquired from Sigma-Aldrich®. Laminin was from Invitrogen Corporation™ (Carlsbad, CA, USA). Minimum essential medium (MEM) with Earle's salts, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer, sodium pyruvate, fetal bovine serum (FBS) and glutamine were purchased from Biochrom AG (Berlin, Germany). Recombinant mouse IL-1β and TNF-α were bought to R&D Systems® (Minneapolis, MN, USA). Triton X-100 was obtained from Roche Diagnostics Deutschland GmbH (Mannhein, Germany). Primary specific monoclonal antibodies were mouse anti-Tau1 and rat anti-tyrosinated-tubulin from Chemicon® (Billerica, MA, USA), rabbit anti-Map2 from Covance (Princeton, NJ, USA), rabbit anti-α-tubulin from Santa Cruz Biotechnology® (Santa Cruz, CA, USA), mouse anti-acetylated-tubulin from Sigma-Aldrich® and mouse anti-synaptic vesicles 2 (SV2) from Developmental Studies Hybridoma Bank at the University of Iowa. Alexa 594 phalloidin was purchased from Molecular Probes® (Carlsbad, CA, USA). Secondary antibodies were Alexa 488 anti-rabbit from Molecular Probes®, and FITC anti-rabbit and anti-rat, TRITC anti-mouse and AMCA anti-mouse from Chemicon®.

2. Animals

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering.

3. Hippocampal neuronal cell culture and treatment

Primary cultures for hippocampal neurons were prepared from embryonic day 16 (E16) mice as previously described (Lanier *et al.*, 1999). E16 correlates with the middle of the second trimester in humans, which is preceded by the neurogenesis of hippocampal pyramidal cells (Fatemi *et al.*, 2009). Pregnant mice were euthanized by asphyxiation with CO_2 and the fetuses were removed from uterus. The fetuses were decapitated and their heads placed in a Petri dish with HBSS solution. Mice brains were carefully removed, cerebral hemispheres were dissected and meninges were removed. Then, the dissected hippocampi were placed in a microcentrifuge tube with HBSS solution and dissociated with 2.5 % trypsin for 15 minutes at 37ºC, followed by the mechanical dissociation with a Pasteur pipette. Approximately 2 x 10^4 cells were plated on each 12 mm coverslip, which were coated with poly-D-lysine (100 µg/mL) and laminin (4 µg/mL), in plating medium (MEM with Earle's salts

supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 0.5 mM glutamine, 12.5 µM glutamate, 10% FBS, and 0.6% glucose). Three hours later, the plating medium was replaced with neuronal growth medium (Neurobasal media supplemented with B27 and 0.5 mM glutamine). After 1 day *in vitro* (DIV), cells were incubated with 1 or 10 ng/mL IL-1β, TNF-α or with vehicle (culture medium without cytokines), for 24 hours at 37ºC, as illustrated in Fig. II.1. Following inflammatory exposure, the incubation medium was replaced by conditioned medium from a parallel set of dishes with cultured hippocampal neurons at the same stage of differentiation. The growth medium was changed twice a week. Neurons were analyzed at 3 or 21 DIV.

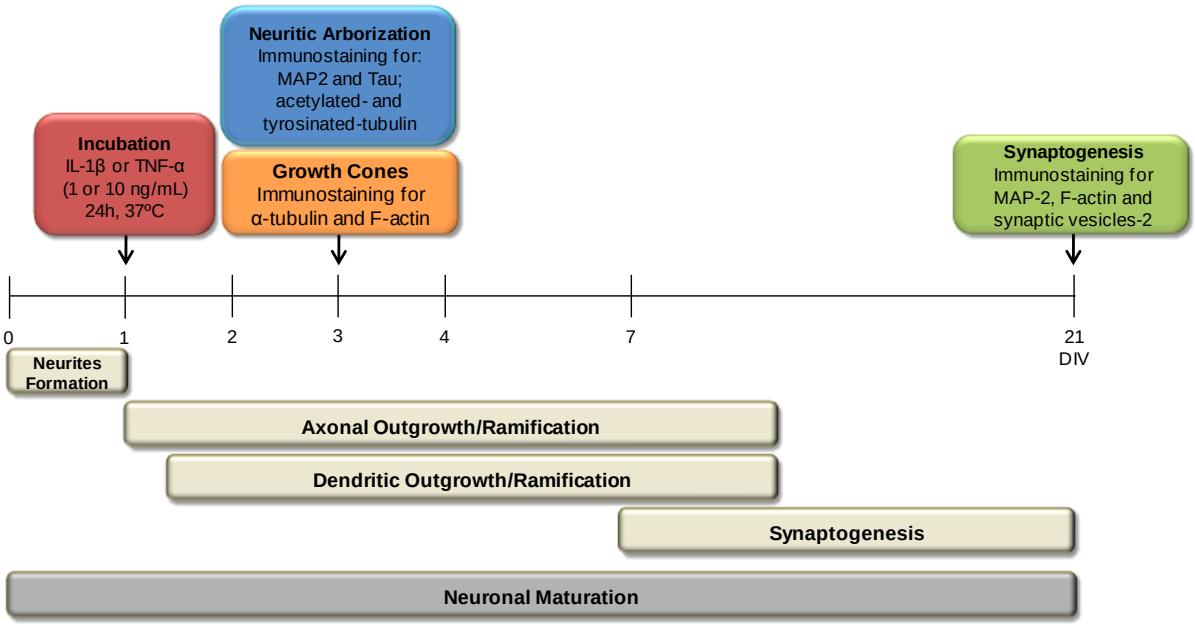

Fig. II.1. Schematic representation of the experimental model. Hippocampal neurons from E16 mice were plated in 12 mm coverslips coated with poly-D-lysine and laminin. At 1 DIV, cells were incubated with 1 or 10 ng/mL IL-1β, TNF-α or with vehicle, for 24h at 37ºC. After the incubation period, media was replaced with conditioned neuronal growth media without cytokines and neurons were analyzed at 3 DIV for neuronal arborizations/growth cone morphology and at 21 DIV for synaptogenesis.

4. Immunocytochemistry

Neuronal cells were fixed at 3 and 21 DIV with PPS (4% paraformaldehyde in PHEM buffer [60 mM PIPES (pH 7.0), 25 mM HEPES (pH 7.0), 10 mM EGTA, 2 mM $MgCl_2$] with 0.12 M sucrose) for 30 minutes at room temperature. After rinsing in phosphate buffer saline (PBS), coverslips were blocked in 3% fatty acid free BSA in PBS for 30 minutes, permeabilized for 10 minutes in 0.2% Triton X-100/PBS, rinsed and reblocked in 3% BSA/PBS for 30 minutes. Incubations with primary and secondary antisera were done in the presence of 1% BSA/PBS, and coverslips mounted with glycerol-based mountant (2.5% 1,4-Diazabicyclo-[2.2.2]Octane, 150 mM Tris (pH 8.0), 30% glycerol) to reduce photo bleaching. Images for neuritic and synaptogenesis analysis were captured on an Axiocam HR adapted to an Axiovert® 200 microscope (Zeiss, Göttingen, Germany) using Openlab software (Improvision). Images of the growth cones were captured using a Leica DC 100 camera model DFC490 (Leica, Wetzlar, Germany) adapted to an AxioSkope® microscope model Scope.A1 (Zeiss,

Göttingen, Germany). F-actin was identified using Alexa 594 phalloidin (1:50) and for the other makers the following antisera were used: anti-Tau1 (1:200), anti-MAP2 (1:1000), anti-acetylated-tubulin (1:1000), anti-tyrosinated tubulin (1:1000), anti-α-tubulin (1:100) and anti-SV2 (1:100).

5. Analysis of neurites

The measurement of dendritic and axonal lengths was performed on stage three neurons, *i.e.* neurons with a single neurite (the axon) that was at least twice as long as all the other neurites (Strasser *et al.*, 2004). Cells were fixed at 3 DIV and neurons were imaged using a 10X plan-neofluar objective. The identification of dendrites and axons were confirmed by the immunostaining of neurons with anti-MAP2 for dendrites and anti-Tau1 for the axon. Neuritic arborization was manually traced using ImageJ v1.43 software (National Institutes of Health) and the NeuronJ plugin v1.4.1 (Meijering *et al.*, 2004). NeuronJ allows a semi-automated tracing of individual neurons, generating a text file with the neuritic length/number and branching measurements. This file was then converted to an Excel file using a Java-based program, the XL-Calculations, as previously described by us (Popko *et al.*, 2008).

6. Analysis of axonal microtubules

Neurons were fixed at 3 DIV and immunostained for anti-acetylated-tubulin and anti-tyrosinated-tubulin, in order to identify the older and the newer microtubules, respectively. Cells were imaged using 10X plan-neofluar objective and axons were manually traced using ImageJ v1.43 software.

7. Analysis of growth cones

The analysis of the growth cones was performed in cells fixed at 3 DIV, stained with anti-α-tubulin, to visualize the axonal shaft, and phalloidin, in order to identify the P-domain cytoskeleton by the visualization of F-actin. Cells were fixed at 3 DIV and images were obtained using a 63X plan-neofluar objective. The areas of axonal growth cones were manually traced and fluorescence was measured using ImageJ v1.43.

8. Analysis of dendritic spines and synapses

Dendritic spine and synapse determinations were performed in cells fixed at 21 DIV, which were stained with anti-MAP2 to identify the dendritic shaft, phalloidin to visualize F-actin at the dendritic spine and anti-SV2 to detect the presynaptic vesicles. Images were taken using a 100X plan-neofluar objective, and the number of dendritic spines and synapses were counted along the dendritic shafts and expressed as the number of spines/synapses per 10 µm of dendrite. Here, all spine-like protrusions on dendritic shafts were counted as dendritic spines, and a synapse was strictly defined as a proximity between the presynaptic protein SV2 and the postsynaptic dendritic spines. To determine the rate of spine maturation, the dendritic spine morphology was also evaluated by the ratio of the

dendritic spine neck height *versus* spine head width, measured from the base of dendritic shaft to the tip of the head.

9. Statistical analysis

Results are presented as mean ± SEM. Differences between groups were determined by one-way ANOVA with Dunnett's multiple comparisons post test, using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $p < 0.05$ was accepted as statistically significant. On each experiment at least 50 neurons were sampled per condition and each experiment was repeated at least three times.

III. RESULTS

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1. Treatment of Immature Hippocampal Neurons With Pro-Inflammatory Cytokines Alters Neuritic Output

Hippocampal neurons acquire their unique and polarized morphology after undergoing dramatic and well-defined morphological changes. However, such characterized development can be altered by the presence of extracellular signals (Tahirovic and Bradke, 2009). Therefore, to characterize the role of a pro-inflammatory environment in the total neuronal arborization, embryonic hippocampal neurons were treated with vehicle and pro-inflammatory cytokines IL-1β (1 or 10 ng/mL) or TNF-α (1 or 10 ng/mL) at 1 DIV, as illustrated in Fig. II.1. After a 24h treatment, the incubation medium was replaced by conditioned growth medium without cytokines and cells were fixed and immunostained with MAP2 for dendritic identification and Tau1 to identify the axon at 3 DIV. It is known that hippocampal neurons suffer polarization within the first 24h after seeding passing from stage 2 neurons with emerging neurites to stage 3 neurons displaying the axon that corresponds to one neurite that is twice longer than the remaining neurites (Strasser *et al.*, 2004). In vehicle-treated neurons, we observed that the proportion between stage 2 and stage 3 neurons was similar (≈ 49% and 51%, respectively). However, the incubation with pro-inflammatory cytokines seems to delay neuronal development, since the treatment with either IL-1β and TNF-α led to an increase in neurons at stage 2 of development (Table III.1).

Table III.1. Number of stage 2 and stage 3 hippocampal neurons following pro-inflammatory cytokine treatment.

	Control	IL-1β 1ng/mL	IL-1β 10ng/mL	TNF-α 1ng/mL	TNF-α 10ng/mL
Stage2	48.92% (± 3.01)	54.08% (± 5.93)	63.53% (± 9.41)	57.67% (± 3.45)	60.18% (± 14.30)
Stage3	51.08% (± 3.01)	45.93% (± 5.93)	36.47% (± 9.41	42.34% (± 3.45)	39.83% (± 14.30)

For the evaluation of cytokine action on neuronal arborization, only stage 3 neurons were analyzed. At 3 DIV, treatment with IL-1β reduced total neuronal arborization, characterized by a more marked effect in axonal arborization (Fig. III.1. A). In addition, this effect also showed to be concentration dependent with significant changes only for the higher concentration (Fig. III.1. B). Indeed, total neuronal output decreased by 17% ($p<0.05$) at 3 DIV after treatment with 10 ng/mL IL-1β, while axonal arborization was reduced by approximately 30% ($p<0.01$) upon the same treatment. On the other hand, the dendritic arborization was not significantly affected by the incubation with IL-1β. In contrast, neurons treated with TNF-α only showed significant alterations at the dendritic level when analyzed at 3 DIV (Fig. III.1. A). In these case, dendritic arborization increased by 20% ($p<0.05$) after treatment with 1 and 10 ng/mL of TNF-α (Fig. III.1. C). Therefore, it seems that IL-1β mainly affects the

axonal compartment, decreasing the axonal arborization and, consequently, the total neuronal output, whereas TNF-α has a more marked effect at the dendritic compartment by increasing the dendritic arborization.

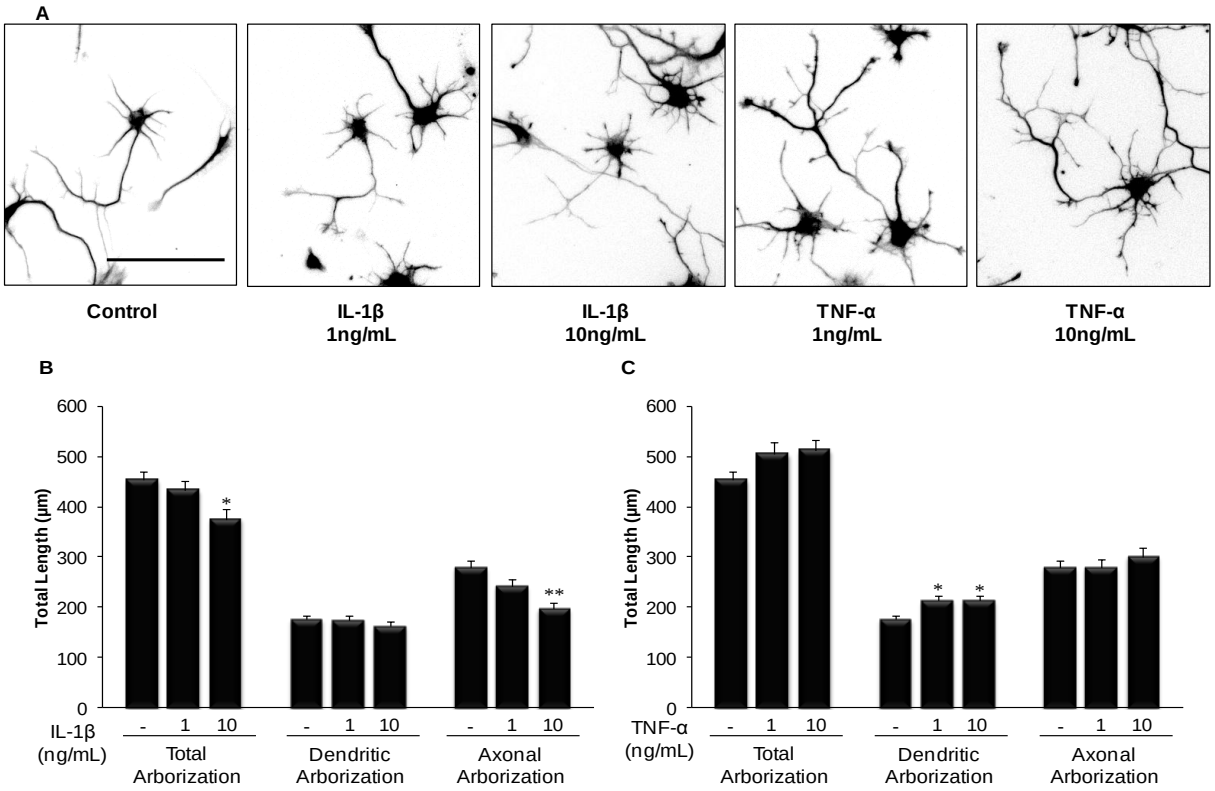

Fig. III.1. Treatment of immature hippocampal neurons with pro-inflammatory cytokines alters the neuritic output. Embryonic hippocampal neurons were treated with vehicle, IL-1β (1 or 10 ng/mL) and TNF-α (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 3 DIV. (A) Representative images of hippocampal neurons immunostained for MAP2, to identify the cell soma and dendrites, and Tau1, to identify the axon (shown in black and white and inverted such that merged MAP2 and Tau1 fluorescence appears black). Cells were visualized by fluorescence and the neuronal arborization was manually traced and measured using ImageJ v1.43 and NeuronJ plugin v1.4.1. Graph bars represent the effect (±SEM) of pro-inflammatory cytokines IL-1β (B) and TNF-α (C) on total, dendritic and axonal arborizations at 3 DIV. *p<0.05 and **p< 0.01 vs. vehicle. Scale bar equals 100 µm.

2. TNF-α Has a Marked Effect at the Dendritic Level of Immature Hippocampal Neurons, while IL-1β Only Slightly Alters Dendritic and Branches Length

Since we observed distinct modifications at the dendritic *versus* axonal level upon cytokines incubation, we next evaluated the capability of pro-inflammatory cytokines to alter different aspects of the dendritic compartment. For this purpose, neurons were incubated with vehicle, IL-1β (1 or 10 ng/mL) and TNF-α (1 or 10 ng/mL) as shown in Fig. II.1. Then, at 3 DIV, cells were fixed and immunostained for MAP2 and Tau1 to identify the dendrites and the axon, respectively. Primary dendrites were identified as the ones emerging directly from the cell soma, while dendritic branches were defined as ramifications arising from both primary dendrites and from the ramifications themselves.

Although the number of dendrites and their branches were not significantly altered by IL-1β at 3 DIV (Fig. III.2. A and C), IL-1β-treated neurons exhibit a decline in the length of the primary dendrites (Fig. III.2. B), but an increase in the length of their branches (Fig. III.2. D). Indeed, the length of primary dendrites was reduced by 11 and 13% after treatment with 1 and 10 ng/mL IL-1β, respectively (n.s.), while the higher concentration of this cytokine increased the length of the dendritic branches by 17% (n.s.).

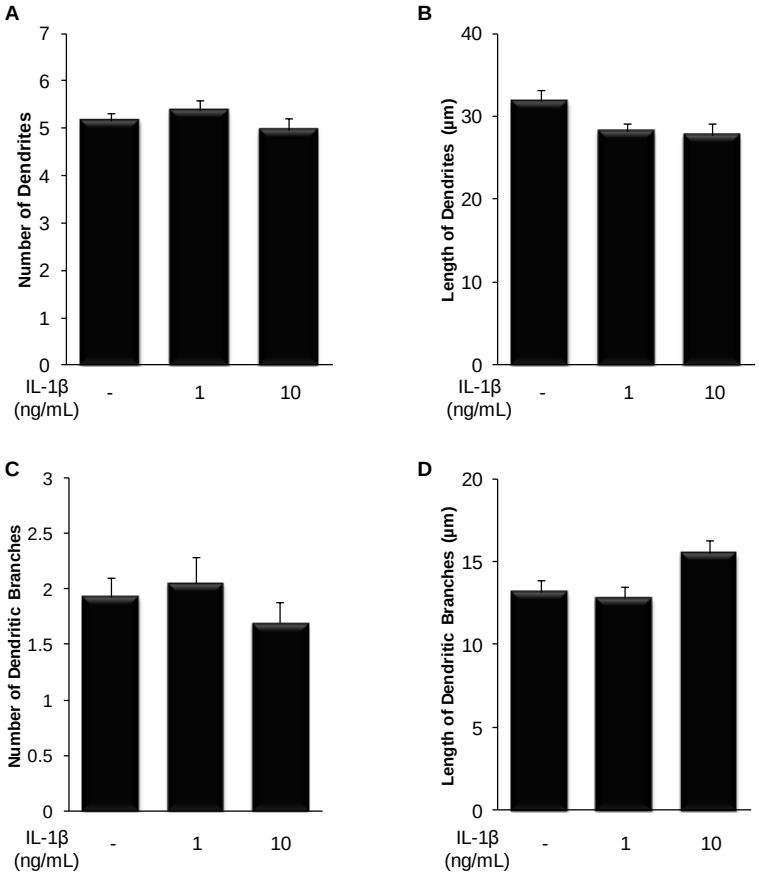

Fig. III.2. Treatment of immature hippocampal neurons with IL-1β reduces the length of primary dendrites, but increases the extent of their branches. Embryonic hippocampal neurons were treated with vehicle and IL-1β (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 3 DIV. Then, neurons were immunostained for MAP2 and Tau1 and visualized by fluorescence. Dendrites were manually traced and measured using ImageJ v1.43 and NeuronJ plugin v1.4.1. Graph bars show the effect (±SEM) of IL-1β on the number (A) and the length (B) of primary dendrites, as well as the number (C) and the length (D) of the dendritic branches at 3 DIV.

On the other hand, embryonic hippocampal neurons incubated with TNF-α showed a marked effect at the dendritic compartment (Fig. III.3.). Indeed, a slightly increase is seen at the number of primary dendrites following TNF-α exposure (Fig. III.3. A), without affecting their length (Fig. III.3. B). In addition, TNF-α treatment induced the formation of more dendritic branches when compared to vehicle-treated neurons (Fig. III.3. C). More importantly, TNF-α exposure led to a significant increase in the length of the dendritic branches at 3 DIV by 28% ($p<0.01$) and by 32% ($p<0.01$) for the lowest and the highest concentration, respectively (Fig. III.3. D).

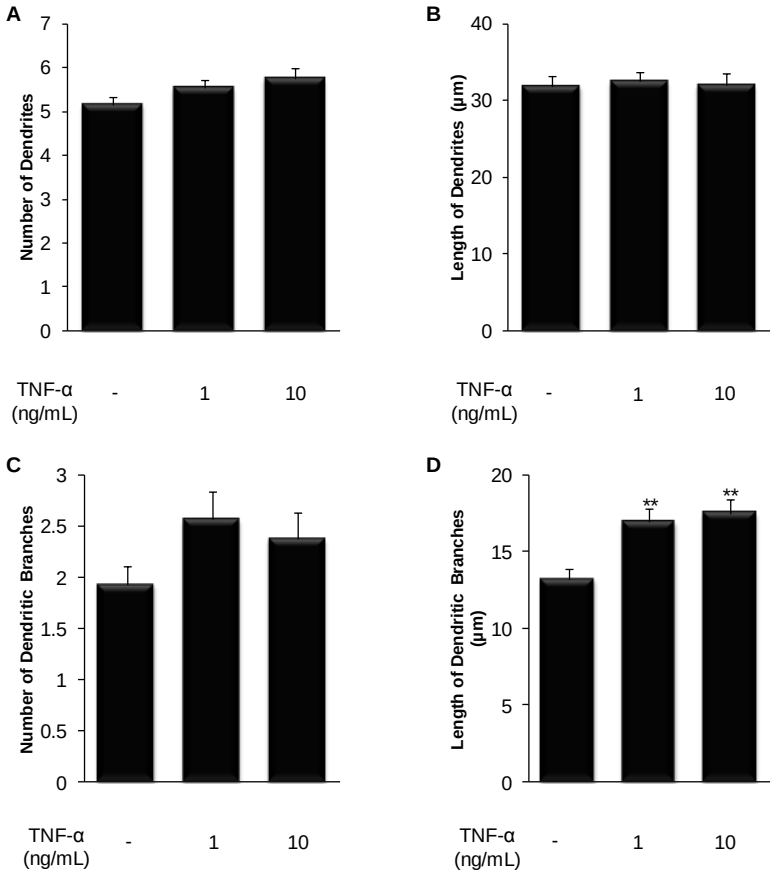

Fig. III.3. Treatment of immature hippocampal neurons with TNF-α has a marked effect at the dendritic level. Embryonic hippocampal neurons were treated with vehicle and TNF-α (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 3 DIV. Then, neurons were immunostained for MAP2 and Tau1, visualized by fluorescence and dendrites were manually traced and measured using ImageJ v1.43 and NeuronJ plugin v1.4.1. Graph bars show the effect (±SEM) of TNF-α on the number (A) and the length (B) of primary dendrites, as well as the number (C) and length (D) of dendritic branches at 3 DIV. **p< 0.01 *vs.* vehicle.

Overall, TNF-α appeared to affect more appreciably the dendritic compartment at 3 DIV than IL-1β, by increasing the number of dendrites and dendritic branches, as well as the length of the branches, confirming the previous data. Alternatively, the length of the primary dendrites is noticeably reduced in neurons incubated with the pro-inflammatory cytokine IL-1β, which is possibly compensated by the increase in the length of their branches.

3. IL-1β Has a Higher Impact at the Axonal Level of Immature Hippocampal Neurons, while TNF-α Only Increases Axonal Branches Length

Next, we also assessed how pro-inflammatory cytokines could modify different aspects of the axonal arborization. For that, embryonic hippocampal neurons were treated as explained in Fig. II.1. and at 3 DIV, cells were fixed and immunostained for MAP2 and Tau1 to distinguish the dendrites and the axon, respectively. The axon was identified by the longest neurite staining for Tau1 and axonal branches were defined as the ramifications rising from the axon or from the ramifications themselves.

At 3 DIV, the treatment with IL-1β drastically reduces the length of the axon, plus the number of its branches (Fig. III.4. A and B, respectively) in a concentration dependent manner. Indeed, the axonal length decreased by 17% ($p<0.01$) and 28% ($p<0.01$) at 3 DIV in neurons treated with 1 and 10 ng/mL IL-1β, respectively, while the number of branches was reduced by 23% (n.s.) and 38% ($p<0.01$) for 1 and 10 ng/mL IL-1β, respectively. In contrast, the lowest concentration of IL-1β induced longer axonal branches (~20%, n.s.) (Fig. III.4. C).

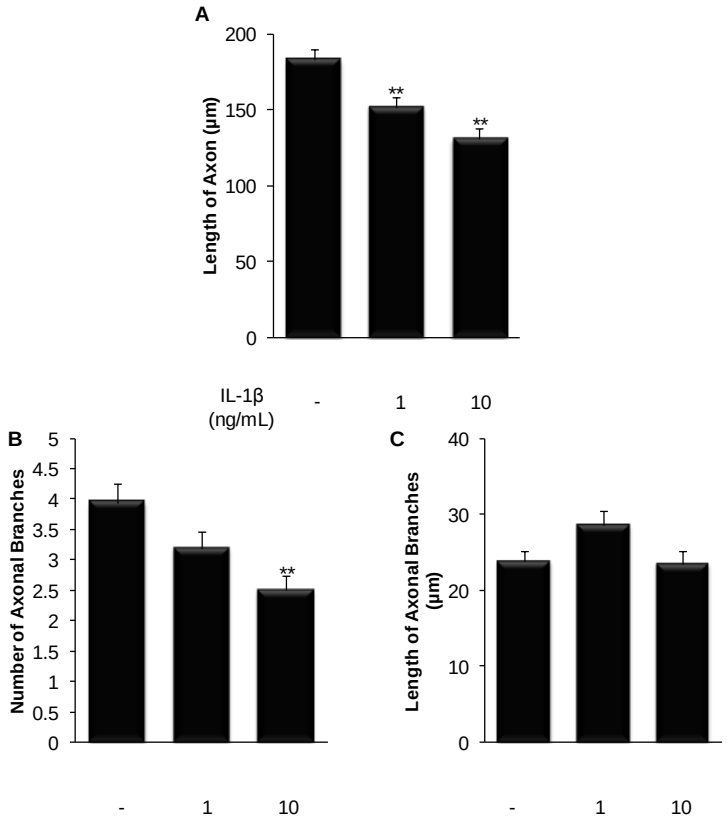

Fig. III.4. Treatment of immature hippocampal neurons with IL-1β has a marked impact at the axonal level. Embryonic neurons from hippocampus were treated with vehicle and IL-1β (1 or 10 ng/mL) 24h at 1 DIV and fixed at 3 DIV. Then, neurons were immunostained for MAP2 and Tau1, visualized by fluorescence and axonal branching was manually traced and measured using ImageJ v1.43 and NeuronJ plugin v1.4.1. Graph bars show the effect (±SEM) of IL-1β on the length of the axon (A), on the number of axonal branches (B) and their length (C). **p< 0.01 *vs.* vehicle.

After treatment with TNF-α, the length of the axon and the number of its branches showed no significant alterations (Fig. III.5. A and B). Nevertheless, the extension of axonal branches was induced in a concentration dependent manner (Fig. III.5. C). The assay with 1 ng/mL TNF-α increases the length of branches by 19% (n.s.), whereas the highest concentration of TNF-α elongates their length by 32% ($p<0.01$).

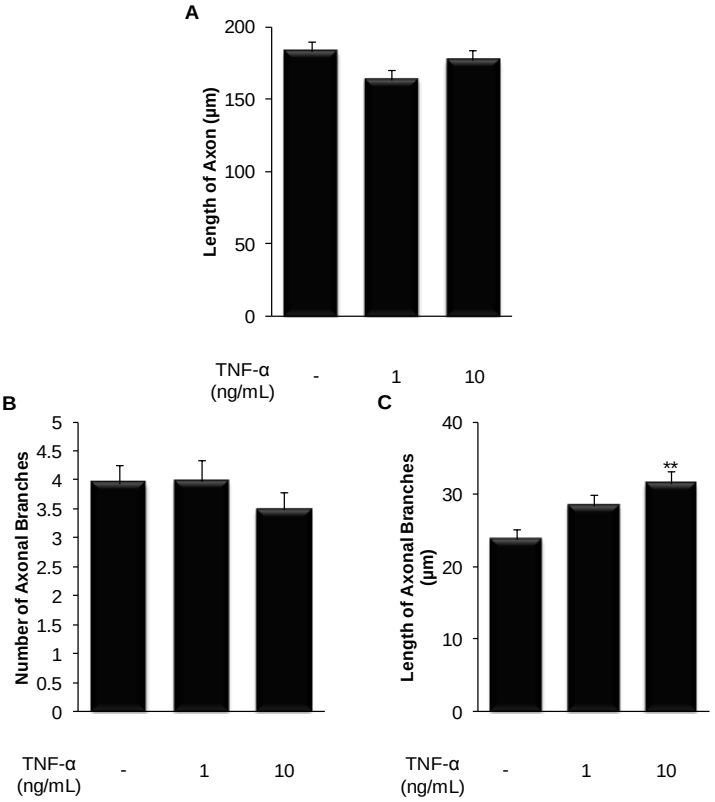

Fig. III.5. Treatment of immature hippocampal neurons with TNF-α only affects the length of axonal branches. Embryonic neurons from hippocampus were treated with vehicle and TNF-α (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 3 DIV. Then, neurons were immunostained for MAP2 and Tau1, visualized by fluorescence and axonal branching was manually traced and measured using ImageJ v1.43 and NeuronJ plugin v1.4.1. Graph bars show the effect (±SEM) of TNF-α on the length of the axon (A), the number of its branches (B), as well as their length (C). *p< 0.05 and **p< 0.01 *vs.* vehicle.

Hence, IL-1β appears to affect the axonal compartment more drastically than TNF-α, as it was able to diminish not only the length of the axon, but also the degree of its branching. However, the length of the axonal branches is increased after incubation with both cytokines, an observation that is in agreement with the results obtained for the dendritic compartment.

4. Cytokines Affect the Distribution of Microtubules Along the Axon of Immature Hippocampal Neurons

Microtubules are polymers of α- and β-tubulin, which suffer diverse post-translational modifications, including tyrosination and acetylation. Tyrosinated tubulin appears in recently synthesized microtubules, whereas acetylated tubulin is found in long-lived and stable microtubules (Fukushima *et al.*, 2009). As cytokines were able to affect the length of the axon, namely IL-1β, we next evaluated the ability of pro-inflammatory cytokines to modify the distribution of microtubules along the axon. For this, embryonic hippocampal neurons were incubated with vehicle and cytokines IL-1β as shown in Fig. II.1. and at 3 DIV, cells were fixed and immunostained for tyrosinated- and acetylated-tubulin to identify the younger and the older microtubules, respectively.

The analysis of the ratio between acetylated- and tyrosinated-tubulin fluorescence along the axon showed that the treatment of immature hippocampal neurons with IL-1β increases the stable and older microtubules along the axon, essentially at its apical portion (Fig. III.6). Indeed, the ratio increased by ~17% in the first quarter of the axon after incubation with 10 ng/mL IL-1β (n.s.).

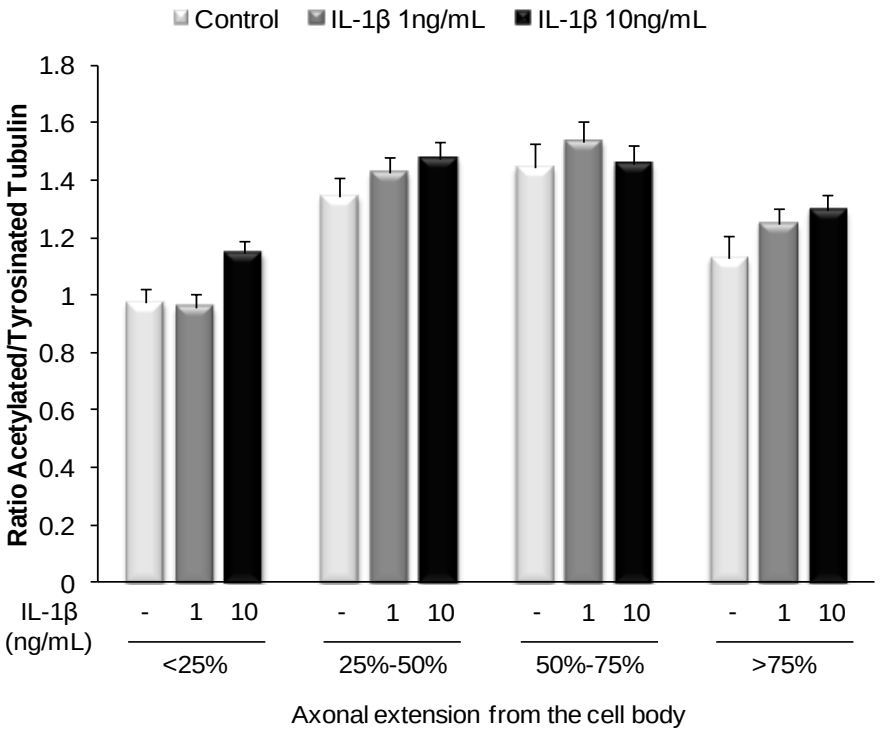

Fig. III.6. Treatment of immature hippocampal neurons with IL-1β affects the distribution of microtubules along the axon. Embryonic neurons from hippocampus were treated with vehicle (light gray) and IL-1β 1 (gray) or 10 ng/mL (black) for 24h at 1 DIV and fixed at 3 DIV. Neurons were immunostained for acetylated-tubulin and tyrosinated-tubulin and visualized by fluorescence. The axons were manually traced and the fluorescence was measured using ImageJ v1.43. Graph bars show the effect (±SEM) of IL-1β on the microtubules post-translational modifications along the axon at 3 DIV.

At 3 DIV, the incubation of immature hippocampal neurons with TNF-α increased the ratio between acetylated- and tyrosinated-tubulin along the axon, mainly at the proximal portion of the axon (Fig. III.7). The ratio significantly increased by 20% ($p<0.05$) and 25% ($p<0.01$) for the first and second quarter of the axon upon treatment with 1 ng/mL TNF-α.

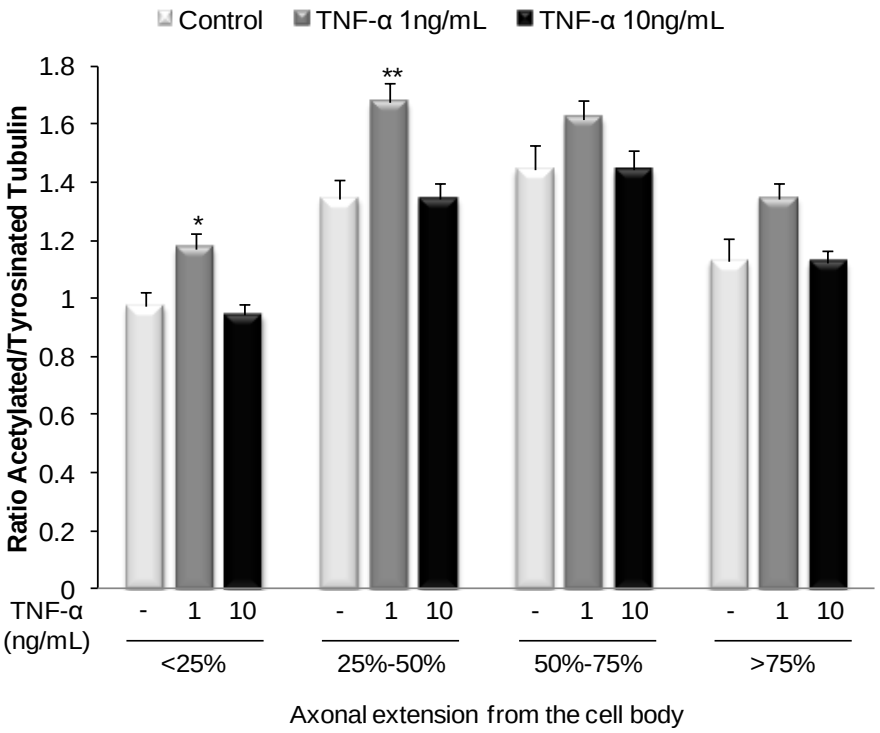

Fig. III.7. Treatment of immature hippocampal neurons with TNF-α affects the distribution of microtubules along the axon. Embryonic hippocampal neurons were treated with vehicle (light gray) and TNF-α 1 (gray) or 10 ng/mL (black) for 24h at 1 DIV and fixed at 3 DIV. Then, neurons were immunostained for acetylated-tubulin and tyrosinated-tubulin and visualized by fluorescence. The axons were manually traced and the fluorescence was measured using ImageJ v1.43. Graph bars show the effect (±SEM) of TNF-α on the microtubules post-translational modifications along the axon at 3 DIV. *p<0.05 and **p<0.01 *vs.* vehicle.

Therefore, IL-1β appears to affect the microtubules from the proximal region of the axon, whereas TNF-α has a higher effect at the distal region of the axon. Overall, cytokines may influence the microtubule dynamics and stabilization, which may therefore account for the reduction of axonal length observed for IL-1β (both 1 and 10 ng/mL) and TNF-α (1 ng/mL) treatment.

5. Exposure of Immature Hippocampal Neurons to Cytokines Decreases Axonal Growth Cone Area and Alters the Composition of Growth Cone Cytoskeleton

The dynamic growth cones at axonal tips are responsible to direct the axons to their synaptic partner, by the incorporation of the extracellular signals present in the surrounding environment, which induce intracellular signaling cascades (Gordon-Weeks, 2004). Szebenyi and colleagues (2001) have shown that alterations of axonal growth cone behavior are correlated with alterations in the normal development of cortical axonal branches. In particular, larger and pausing growth cones determine the formation of collateral axon branches (Szebenyi *et al.*, 2001). Since pro-inflammatory cytokines affected axonal extension and branching, we decided to address if IL-1β and TNF-α were also able to alter the dynamics and the area of growth cones. Thus, embryonic hippocampal neurons were treated

at 1 DIV as illustrated in Fig. II.1. and at 3 DIV, neurons were fixed and immunostained with an antibody to α-tubulin and phalloidin, to visualize F-actin. Hence, it is possible to identify different regions of the growth cone, as the distal-ends of microtubules compose the C-domain and the protrusive region of the P-domain is composed by actin filaments.

At 3 DIV, the treatment of embryonic hippocampal neurons with both IL-1β concentrations has a significant detrimental effect in the areas of the axonal growth cones, which appeared to display less filopodia, a characteristic of growth cone collapse (Fig. III.8. A). Indeed, the growth cone areas decreased by ~20% ($p<0.01$) and ~18% ($p<0.05$) after the incubation with 1 and 10 ng/mL IL-1β, respectively (Fig. III.8. B). TNF-α-treated neurons also exhibited smaller growth cones at the tips of their axons at 3 DIV, when compared to vehicle-treated neurons (Fig. III.8. A). Actually, the exposure of neurons to TNF-α decreased the area by ~30% ($p<0.01$) and ~14% (n.s.) for the lower and the highest concentration, respectively (Fig III.8. C).

As the area of growth cones was altered upon cytokines-treatment, we next evaluated how IL-1β and TNF-α affect the normal distribution of microtubules and actin in the growth cones. Upon incubation with IL-1β, the F-actin protein expression of the growth cone is elevated when compared with vehicle-treated neurons (Fig. III.8. A). Furthermore, F-actin seems to accumulate in the C- and T-domain of growth cones, perhaps due to the loss of filopodia in the P-domain (Fig. III.8. A). In fact, immunofluorescence of F-actin was increased in a concentration-dependent manner by ~27% ($p<0.05$) and by ~79% ($p<0.01$) after incubation with 1 ng/ml and 10 ng/mL IL-1β, respectively (Fig. III.8. D). No significant alterations of the α-tubulin immunofluorescence were observed in growth cones after treatment with IL-1β (Fig. III.8. D). At 3 DIV, neurons treated with TNF-α also exhibited a higher protein expression of F-actin on axonal growth cones (Fig. III.8. A). More exactly, TNF-α increased the immunofluoresce of F-actin in a concentration dependent manner, as neurons treated with 1 ng/mL TNF-α have an increase by ~31 % ($p<0.01$), whereas in neurons treated with 10 ng/mL TNF-α the increase was by ~70% ($p<0.01$) (Fig. III.8. E). As well, the immunofluorescence of α-tubulin was higher by ~9% ($p<0.01$) and ~10% ($p<0.01$) in neurons incubated with 1 and 10 ng/mL TNF-α, respectively (Fig. III.8. E).

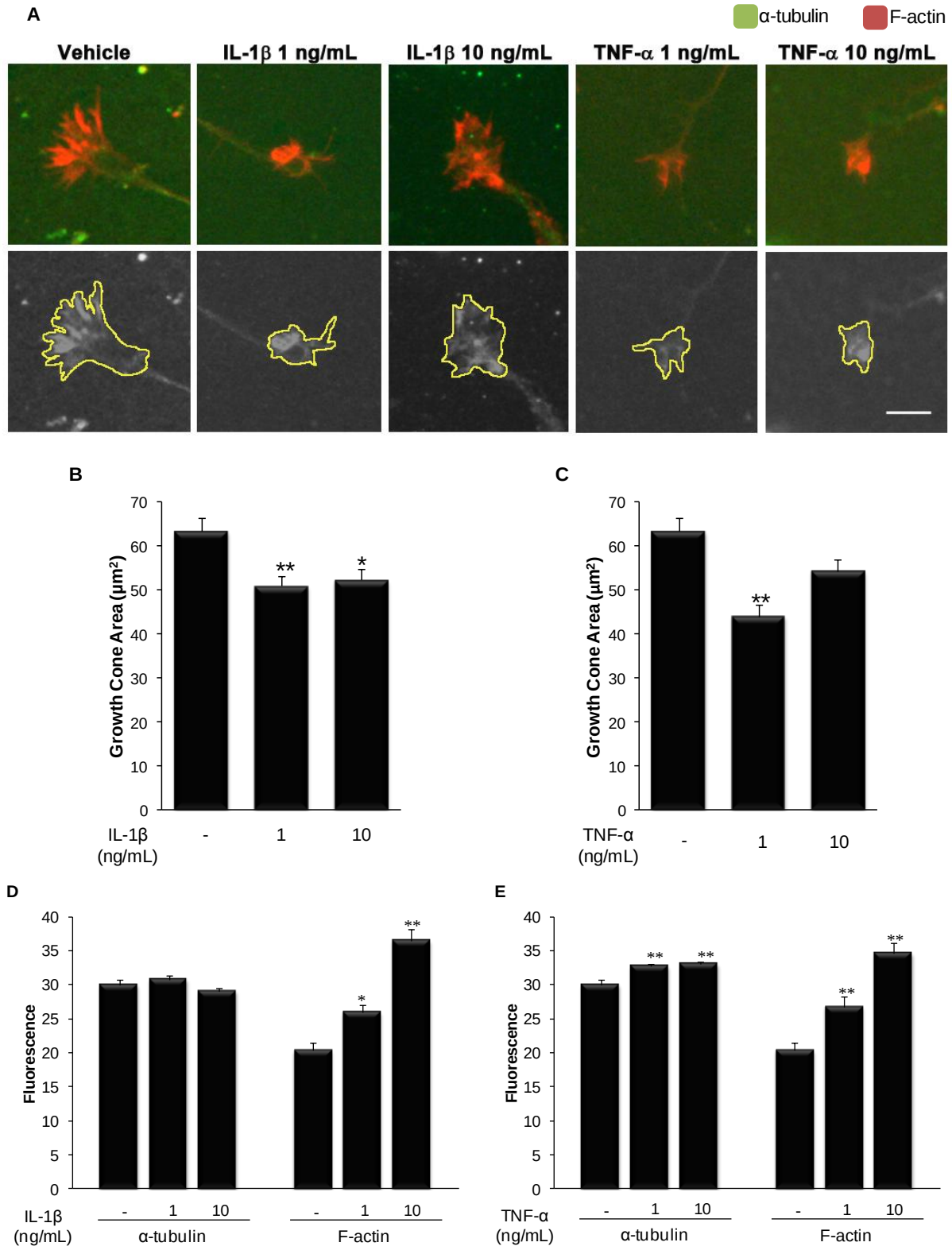

Fig. III.8. Treatment of immature hippocampal neurons with pro-inflammatory cytokines alters the growth cone area and cytoskeleton. Embryonic hippocampal neurons were treated with vehicle, IL-1β (1 or 10 ng/mL) and TNF-α (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 3 DIV. (A) Representative images of axonal growth cones from hippocampal neurons labeled with anti-α-tubulin (green), to identify the microtubules, and phalloidin (red), to visualize F-actin. Cells were visualized by fluorescence, the growth cone area was drawn and measured using ImageJ v1.43, cytoskeleton protein fluorescence was quantified in the same area. Graph bars represent the effect (±SEM) of pro-inflammatory cytokines IL-1β (B) and TNF-α (C) on the areas of growth cones, as well the fluorescence intensity (±SEM) of α-tubulin and F-actin after IL-1β (D) and TNF-α (E) treatment at 3 DIV. *p<0.05 and **p< 0.01 *vs*. vehicle. Scale bar equals 10 µm.

Therefore, neurons incubated with pro-inflammatory cytokines exhibited smaller growth cones, perhaps due to the alteration of the cytoskeleton composition. Indeed, both cytokines increase the actin cytoskeleton, mainly at the C- and T-domain. Overall, the alterations at the growth cone level may contribute to abnormalities in the formation of branches and in the directional growth of the axons, ultimately contributing to deficits on neuronal connectivity.

6. Cytokines Not Only Reduce Spinogenesis and Synaptogenesis, But Also Dendritic Spine Maturation

In vertebrates, the formation of synapses begins in the embryo and extends to the early postnatal period, coupled to the neuronal development (Waites *et al.*, 2005). Moreover, a prolonged maturation phase may occur in synaptic development, with the purpose of promoting the integration of each neuron into a proper and efficient network. In hippocampal neuronal cultures, dendritic spines, the postsynaptic structures with the majority of excitatory synapses, start to emerge by 9-11 DIV and they become mature or stable by 18-21 DIV. During maturation, dendritic spines change their morphology from long and thin immature spines (filopodial-like) to shorter spines ending in a bulbous head (Lippman and Dunaevsky, 2005). To examine the effect of pro-inflammatory cytokines on spinogenesis and synaptogenesis, neurons were treated with vehicle and IL-1β or TNF-α as explained in Fig. II.1. After the treatment, the incubation medium was replaced by conditioned growth medium without cytokines and cells were allowed to develop until 21 DIV. Dendritic spines were visualized by the use of phalloidin to detect F-actin, the dendritic shafts were identified with MAP2 staining and synapses were identified by the proximity of SV2, at the pre-synaptic partner, and phalloidin at the dendritic spine.

The treatment of immature hippocampal neurons with IL-1β led to a significant decline in the density of dendritic spines and synapses along dendrites (Fig. III.9. A). In fact, this cytokine decreases the spinogenesis by ~28% ($p<0.01$) for both concentrations (Fig. III.9. B), and the synaptogenesis by 28% ($p<0.01$) and 30% ($p<0.01$) for 1 and 10 ng/mL of IL-1β, respectively. (Fig. III.9. C). Vehicle-treated neurons exhibited mature shorter spines with a mushroom-shape, on the contrary, IL-1β-treated neurons displayed more long and thin spines, resembling a more immature form (Fig. III.9. A). By analyzing the ratio between the spine neck length and spine head width, these observations were confirmed, as the incubation with IL-1β increased this ratio by 15% ($p<0.01$) and 12% ($p<0.01$) for 1 and 10 ng/mL IL-1β, respectively (Fig. III.9. D).

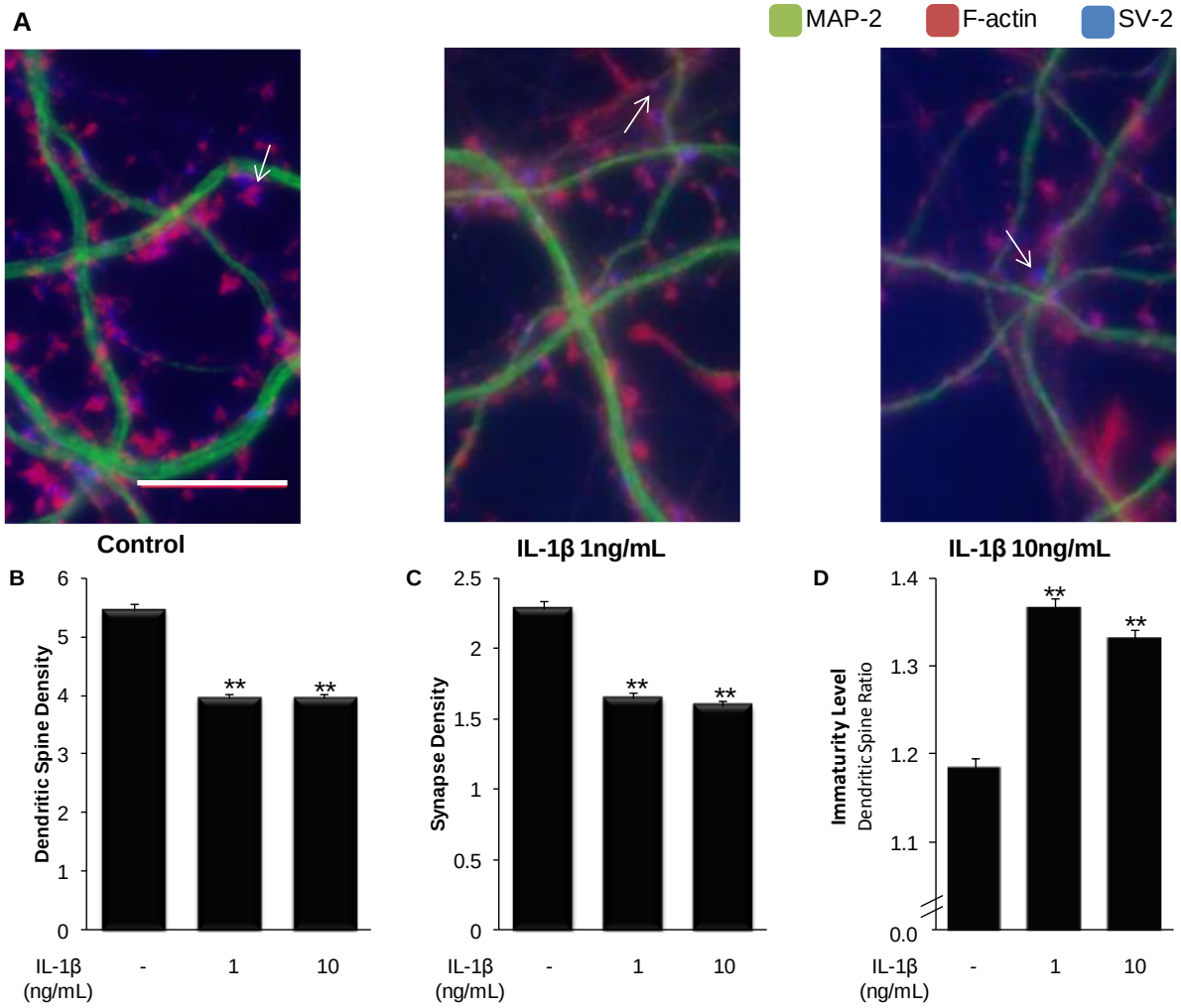

Fig. III.9. Treatment of immature hippocampal neurons with IL-1β decreases the density of dendritic spines and synapses, as well the rate of dendritic spine maturation. Embryonic hippocampal neurons were treated with vehicle and IL-1β (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 21 DIV. (A) Representative images of hippocampal neurons immunostained for MAP2 (green) to identify the dendritic shaft, phalloidin (red) to detect the actin cytoskeleton at the dendritic spines, and SV2 (blue) to visualize the pre-synaptical sites, are shown. Cells were visualized by fluorescence and the synaptic and spine densities were measured using ImageJ v1.43. Arrows identify a synaptic site by the proximity of SV2 and phalloidin. Graph bars represent the effect (±SEM) of IL-1β on the dendritic spine (B) and synapse density (C), as well as on dendritic spine morphology (D). **p< 0.01 *vs.* vehicle. Scale bar equals 10 µm.

TNF-α-treated neurons exhibit a lower density of dendritic spines and synapses all along the dendritic shaft (Fig. III.10. A). Actually, the spine density decreased by, approximately, 25% ($p<0.01$) and 34% ($p<0.01$) for 1 and 10 ng/mL TNF-α, respectively (Fig. III.10. B), while the synaptic density decreased by 30% ($p<0.01$) and 32% ($p<0.01$) for the same treatments (Fig. III.10. C). Moreover, the incubation with this cytokine led to the appearance of longer and thinner dendritic spines, *i.e.* the spines correspond to a more immature pattern (Fig. III.10. A). Indeed, by the calculation of the spine neck length to spine head width ratio, the ratio was increased by ~11% ($p<0.01$) and 12% ($p<0.01$) in neurons incubated with 1 and 10 ng/mL TNF-α, respectively (Fig. III.10. D).

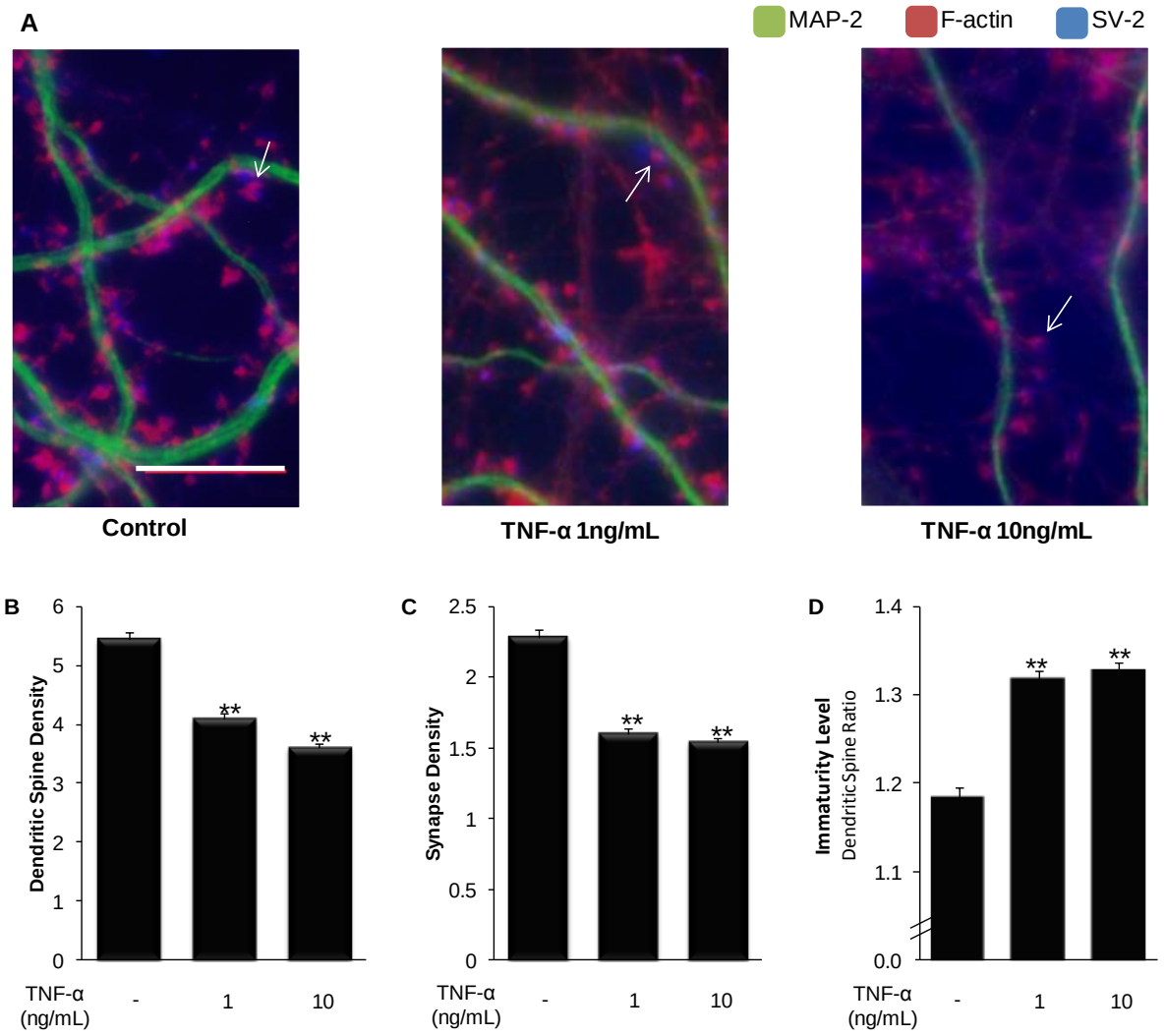

Fig. III.10. Treatment of immature hippocampal neurons with TNF-α decreases the density of dendritic spines and synapses, decreasing the rate of dendritic spine maturation. Embryonic hippocampal neurons were treated with vehicle and TNF-α (1 or 10 ng/mL) for 24h at 1 DIV and fixed at 21 DIV. (A) Representative images of hippocampal neurons immunostained for MAP2 (green) to identify the dendritic shaft, phalloidin (red) to detect the actin cytoskeleton at the dendritic spines, and SV2 (blue) to visualize the pre-synaptical sites, are shown. Cells were visualized by fluorescence and the synaptic and spine densities were measured using ImageJ v1.43. Arrows identify a synaptic local by the proximity of SV2 and phalloidin. Graph bars represent the effect (±SEM) of TNF-α on the dendritic spine (B) and synapse density (C), as well as on dendritic spine morphology (D). **p< 0.01 *vs.* vehicle. Scale bar equals 10 µm.

Altogether, these results suggest that pro-inflammatory cytokines IL-1β and TNF-α may influence the correct formation of neuronal circuits in the hippocampus, since they are able to reduce the formation of synapses, keeping dendritic spines in a more immature state.

IV. DISCUSSION

IV. DISCUSSION

During pregnancy, intrauterine infection was suggested to induce the production of pro-inflammatory cytokines, as IL-1β and TNF-α, in the immature fetal brain (Bell *et al.*, 2004; Huleihel *et al.*, 2004). Several studies have linked the abnormal inflammatory environment with the propensity of the offspring to develop several neurodevelopmental disorders, as autism, schizophrenia, cerebral palsy and periventricular leukomalacia (Huleihel *et al.*, 2004; Fatemi *et al.*, 2008). In addition, high levels of pro-inflammatory cytokines have been associated with alterations in neuronal architecture, synaptic function and cytoskeleton (Roumier *et al.*, 2008; Ådén *et al.*, 2010; Burd *et al.*, 2010; Montgomery and Bowers, 2011). Since the elaboration of the proper neuronal architecture and neuronal connections are the basis of the CNS functionality, in the present study we have further analyzed the effects of a pro-inflammatory environment on neuronal arborization, neuronal cytoskeleton, growth cone morphology and synapse formation. Here, we have demonstrated that cytokines IL-1β and TNF-α differently affect neuronal arborization, but both reduce growth cone area and synaptic connectivity of developing neurons.

Moreover, cytokines affect microtubule dynamic instability necessary for the axonal extension, by increasing the number of acetylated-microtubules. Similarly, in growth cones, cytokines are responsible for an increase in the F-actin immunofluorescence, and TNF-α has also induced an increase in α-tubulin fluorescence, which can be related to detrimental axon guidance and pathfinding. Therefore, we have demonstrated that pro-inflammatory cytokines may influence not only several steps of neuronal development and function, but also the dynamics of neuronal cytoskeleton, crucial for the emergence and maintenance of neuronal polarization. Some of abovementioned effects have yet been described in some neurological disorders, as schizophrenia (Sweet *et al.*, 2009; Faludi and Mirnics, 2011). Hence, our study provides structural evidences for the effect of inflammatory mediators in the neuronal development and its association with long-term disabilities, which can lead to cognitive impairments later in life.

The acquisition of neuronal polarization occurs in the stage 3 of neuronal development, when one of the neurites starts to grow more intensively becoming the axon (Tahirovic and Bradke, 2009). Here, we demonstrated that cytokines delay the progression of the development of hippocampal neurons, since a higher percentage of stage 2 neurons is present after incubation with both IL-1β and TNF-α. This first result may unveil that pro-inflammatory cytokines are able to change the normal development and polarization of embryonic hippocampal neurons. In accordance, others have showed that TNF-α-deficient mice exhibit an accelerated maturation of the neurons on the dentate gyrus (Golan *et al.*, 2004). Importantly, the formation of a functional nervous system depends on the growth and branching of axonal and dendritic processes, as they allow the finding of the proper synaptic partner and, consequently, the formation of the correct neuronal networks. Therefore, the complexity of the neuronal tree is intimately related with the formation of synaptic contacts (Häusser *et al.*, 2000; Hall and Lalli, 2010). We have observed that the axonal arborization is reduced in IL-1β-treated neurons, essentially by impairment in axonal elongation and axonal branches formation in

hippocampal embryonic neurons, and this effect is translated to a less complex neuritic arborization. In contrast, a study with cerebellar and dorsal root ganglion neurons from neonatal Wistar rats incubated with higher levels of IL-1β (50 ng/mL) showed that IL-1β overcomes the neuritic outgrowth inhibition by myelin-associated glycoprotein (Temporin *et al.*, 2008). In this work, IL-1β induces the axonal growth upon nerve injury by the deactivation of the small GTPase RhoA. These results indicate that IL-1β may play a different role on neurite outgrowth depending on the concentrations attained. Furthermore, our data showed that IL-1β also decreases the length of dendrites, thus indicating that IL-1β has a detrimental effect in the elongation of the main neuronal processes, both dendrites and axons. In accordance, another study performed in cortical neurons from prenatal rats showed that moderate levels of IL-1β decrease the extension of dendrites (Gilmore *et al.*, 2004). However, our observations that IL-1β increases the length of axonal and dendritic ramifications may reveal that neurons try to overcome the detrimental effects of IL-1β at both the axonal and dendritic main processes.

In contrast, we observed that TNF-α has an inductor role at the dendritic level, increasing the number of dendrites and their branches, as well as the length of dendritic ramifications, which results in a raise of the dendritic arborization. In contrast, a study in hippocampal neurons from E16 mice co-cultured with glial cells showed that incubation with TNF-α reduces both outgrowth and branching of neurites by the activation of RhoA (Neumann *et al*., 2002). Although the author also use 10 ng/mL TNF-α, we must not forget that in this case neurons were platted on top of a glial cell layer, which can be easily activated by TNF-α to produce a higher amount of cytokines. Indeed, we have previously demonstrated in a bilirubin-induced astroglial inflammatory response model that silencing of TNFR1 reduce the overall production of cytokines including IL-1β, IL-6 and also TNF-α, indicating that either in an autocrine or paracrine fashion TNF-α may elicit a further release of this cytokine by glial cells. (Fernandes *et al.*, 2011). Therefore, it seems that in resemblance with IL-1β, TNF-α may also have a varied role in neuritogenesis based on the concentration that is present. Furthermore, in a model of inflammation-induced preterm birth by intrauterine infusion of LPS, the authors report an increase in the levels of pro-inflammatory cytokines mRNA such as TNF-α, IL-1β, IL-6 and IL-10 in whole fetal brains, in parallel with a reduction in the number of dendrites observed by MAP-2 staining (Burd *et al.*, 2010). Accordingly, in mice autoimmune disease models, the cerebral inflammatory environment causes dendrite atrophy, decreasing the complexity of the dendritic arborization (Zhu et al., 2003), while the same results were obtained in primary cortical neurons from embryonic rats incubated with moderate levels of TNF-α (Gilmore *et al.*, 2004). On the other hand, in a study using a developing visual system of *Xenopus laevis*, TNF-α-treated tectal neurons exhibited enhanced dendritic outgrowth, with larger dendritic arbors (Lee *et al.,* 2010). Furthermore, postnatal mice deficient in *TNF-α* gene exhibited smaller dendritic trees of neurons from hippocampal areas, indicating that TNF-α is involved in the morphogenesis of hippocampal neurons with an inductive role in the development of the dendritic arbor (Golan *et al.*, 2004). However, those mice showed an improvement in the performance on behavioral tasks related to spatial memory (Golan *et al.*, 2004). This result may lead us to hypothesize that the induced outgrowth of dendritic arbor by TNF-α is not related to better cognitive performances. Therefore, no consensus exists concerning the precise effects of cytokines on neuronal arborization, nevertheless this may be a result of different experimental approaches. In

addition, our results also suggests that pro-inflammatory cytokines IL-1β and TNF-α are involved in the elongation and branching of neuronal processes during the development of embryonic hippocampal neurons, possibly by alterations at cytoskeleton dynamics and its regulation pathways, as Rho GTPases. If this proves to be true, disruption of cytoskeleton dynamics may be an indicator of future neuronal damage. Indeed, cytokines effect on dendritic development has been considered a link between prenatal exposure to infection and a risk to suffer schizophrenia and related neurodevelopmental disorders (Gilmore *et al.*, 2004; Meyer *et al.*, 2009).

Microtubule stabilization is required for axonal elongation, but the dynamic properties of microtubules have to be present in order to the axonal process continue to grow (Witte *et al.*, 2008). Here, we present that pro-inflammatory cytokines alter the ratio between acetylated-tubulin, a post-translational modification of tubulin present in older and stable microtubules, and tyrosinated-tubulin, characteristic of recently synthesized and more dynamic microtubules. In reality, the presence of the tubulin tyrosin ligase, the protein responsible for the post-translational modification of the last amino acid of α-tubulin, is necessary for the proper morphogenesis and axonal extension (Erck *et al.*, 2005; Marcos *et al.*, 2009). Thereby, cytokines may influence the axonal growth by inducing changes at the cytoskeleton level, more precisely at the post-translational modifications, as they induce a selective stabilization at the microtubule compartment, altering the normal dynamic instability. In particular, it seems that IL-1β and TNF-α (1 ng/mL) reduce the axonal outgrowth by increasing the stability of axonal microtubules, indicated by an increase in the fluorescence of acetylated-tubulin.

The growth cone, present at the edge of the neuritic processes, commands the guidance of extending axons and dendrites towards the correct partner, since they are highly dynamic and sensible structures (Myers *et al.*, 2011). We observed that both cytokines reduce the growth cone area, consequently reducing the area of the surrounding environment that the axonal growth cone can directly probe. Furthermore, the growth cones appeared to display less filopodia, a characteristic of growth cone collapse. Actually, the growth cone collapse, upon treatment with human semaphorin III, is characterized by a decrease in growth cone area (Fritsche *et al.*, 1999). On the other hand, increases in axonal growth cone size, and consequently pausing in growth, are related to an induction of axonal branching (Szebenyi *et al.*, 2001). Our results showed that while IL-1β reduces axonal branching in a concentration-dependent manner, TNF-α has no effect in this feature, therefore cytokines effect on growth cone appears to be related to other consequences rather than a decrease in axonal branching.

Alterations in the growth cone are also intimately related to a deficient and impaired axonal guidance, since the growth cone pathfinding depends on the protrusive and dynamic P-domain and, ultimately on the cytoskeleton (Geraldo and Gordon-Weeks, 2009; Dent *et al.*, 2011a). Indeed, the extension of filopodial tips is determined by the rate of F-actin polymerization and the retrograde flow of filaments to the bottom of the filopodia (Mallavarapu and Mitchinson, 1999). Particularly, semaphorin3A, a repulsive guidance cue, induces a loss of F-actin meshwork, which is moved to the neurite, during growth cone collapse (Brown *et al.*, 2009). Here, we notice that the cytoskeleton of growth cones is altered after the inflammatory stimulus by IL-1β and TNF-α. In fact, all inflammatory treatments induced an increase in the F-actin fluorescence, in a concentration-dependent manner.

Indeed, our results point to an accumulation of F-actin in the C- and T-domain of growth cones, appearing that F-actin from the leading edges is retrogradely transported from the P-domain, perhaps due to the loss of filopodia. A recent study performed in an immortalized mouse brain endothelial cell line, showed that TNF-α, through the activation of RhoA, is able to induce modifications at the microfilaments level (Peng *et al.*, 2011). In addition, in a human neuroblastoma cell line, the acute exposition to TNF-α or IL-1β induces the loss of lamellipodia and ultimately cellular plasticity, due to oxidative damages in actin cytoskeleton and regulation of the small GTPase Rac1 (Barth *et al.*, 2009). Therefore, it seems that both TNF-α and IL-1β are able to change actin cytoskeleton dynamics at the tip of the axon which may influence growth cone activity. Additional to the effect on microfilaments, TNF-α also raises the immunofluorescence of α-tubulin. The raise in the α-tubulin immunofluorescence may indicate an accumulation of microtubules in the C-domain of the growth cone. In growth cone collapse, the dynamic microtubules, characterized by the presence of tyrosinated-α-tubulin, start to fasciculate and bundle, reducing the distance between the dynamic and the stable microtubules, which are retained in the axonal shaft and the most proximal part of the growth cone (Fritsche *et al.*, 1999). The tyrosination of tubulin was shown to be essential for the normal guidance by growth cones, as it is required for the regulation of the cytoskeleton organization at the growth cone (Marcos *et al.*, 2009). Indeed, the dynamic properties of microtubules were found to be a prerequisite to growth cone guidance and subsequent axonal elongation (Tanaka *et al.*, 1995). Our preceding results showed that 1 ng/mL TNF-α, but not the higher concentration, induced a stabilization of microtubules by an increase in the fluorescence of acetylated-tubulin *vs.* tyrosinated-tubulin. However, the treatment with 10 ng/mL TNF-α, despite of no changes at the axonal elongation, may exert problems in growth cone guidance. Having as basis our results, cytokines may affect deeply the growth cone cytoskeleton, impairing the correct axonal guidance toward its partner, and possibly induce long-term modification in synapse formation.

The extension of axonal and dendritic processes, upon guidance by growth cones, enhances the growth towards the correct partner, allowing the formation of the neuronal networks by synapse formation (Shen and Cowan, 2010). Our data demonstrate that pro-inflammatory cytokines trigger events in neurons at 1 DIV that not only modify the normal axonal guidance, by decreasing the growth cone areas, but also decrease the densities of dendritic spines and synapses at 21 DIV. In rodent models of experimental autoimmune encephalomyelitis, characterized by a chronic neuroinflammation, it is reported a disruption in the neuronal circuits with a reduction in synaptic density in hippocampus (Ziehn *et al.*, 2010). Also, the induction of the pro-inflammatory pathways induced by LPS administrated intraperitoneally was shown to decrease the number of dendritic spines along the dendritic shaft (Kondo *et al.*, 2011). Furthermore, a decreased density of dendritic spines exists in auditory cortex of schizophrenic patients, which can be correlated with the cognitive impairment observed in this disease (Sweet *et al.*, 2009). Therefore, the effect of the inflammatory mediators on the synaptic compartments may result in brain dysfunction due to damage on neuronal networks. In fact, altered neuronal connectivity persists in the progeny upon maternal infection (Roumier *et al.*, 2008). Additionally, several reports have associated modifications at the synaptic functionality, strength and plasticity upon a neuroinflammatory stimulus, which might result in cognitive

impairments. Moreover, morphological alterations of synapses result from synaptic plasticity and pro-inflammatory cytokines have been addressed with contradictory and even opposing roles in some forms of synaptic plasticity, as LTP, which induces an expansion of dendritic spines and an increase of spine volume (Yamagata *et al.*, 2009). Indeed, in what concerns to synaptic plasticity, IL-1β was shown to have both inhibitory and inducing effects on LTP (Bellinger *et al.*, 1993; Schneider *et al.*, 1998; Vereker *et al.*, 2000). The inhibitory effect of IL-1β (10 pg/ml) on LTP is accompanied by stimulation in stress-activated kinases activity, which, in turn, may attenuate the release of glutamate and, consequently, compromise LTP (Vereker *et al.*, 2000). Interestingly, a study performed in an elderly population revealed that genetic variation in the *IL-1β-converting enzyme* gene, responsible for the formation of the active form of IL-1β, is not only related with a better performance in cognitive function, but also with lower levels of IL-1β production (Trompet *et al.*, 2008). On the other hand, TNF-α at 6-60 nM increases the synaptic strength in hippocampal neurons, by inducing the expression of AMPA glutamate receptors, therefore contributing to synaptic plasticity (Beattie *et al.*, 2002). Nevertheless, a more recent report showed that TNF-α at 4.5 ng/ml inhibits LTP in hippocampal slices (Butler *et al.*, 2004). Additionally, chronic neuroinflammation was related to impairments in LTP in rat hippocampus, which may lead to defects in spatial memory (Min *et* al., 2009). Hence, pro-inflammatory cytokines may affect the dendritic spine and synapse densities at embryonic hippocampal neurons by affecting their ability to undergo synaptic plasticity, which may lead to detrimental neurological outcome. Indeed, chronic inflammation induces damaging effects on hippocampus by a disruption in CA3 region networks, decreasing the ability to process spatial information (Rosi *et al.*, 2009). Furthermore, as dendritic spine density is directly related to the diffusion of intracellular chemical signals in hippocampus (Santamaria *et al.*, 2011), the exposure to cytokines at 1 DIV may influence the movement of signaling molecules along the dendrite, thereby reducing dendritic spine density and affecting neuronal connectivity as previously reported.

The strength of a synapse may be regulated through several aspects, as changes in postsynaptic receptor clustering (Turrigiano and Nelson, 2004). Therefore, the size of a dendritic spine may influence the strength of a synapse, since a more mature form, as the mushroom- or cup-shaped spine, can bear a larger PSD and, consequently, a larger synaptic transmission machinery, whereas a more immature form, as filopodia-like spines, are related with a lower synaptic strength (McKinney, 2005). In other words, the size of a spine head is directly proportional to the strength of synapses, possibly by an increase in the number of post-synaptic receptors at the dendritic spine. Our results showed that IL-1β and TNF-α-treated neurons exhibited more immature spines, characterized by a higher ratio between the spine neck height and head width. Thereby, following neuroinflammation, the presence of more immature spines may result in synaptic strength reduction which may compromise cognitive function. In accordance, in some cases of mental retardation, as in Fragile-X Syndrome patients, and several cognitive disorders, as schizophrenia, longer and thinner dendritic spines occur along the dendritic shaft, resembling a more immature morphology (Irwin *et al.*, 2001; Faludi and Mirnics, 2011).

Collectively, our results demonstrate that an early exposure of immature embryonic hippocampal neurons to pro-inflammatory cytokines IL-1β and TNF-α induce modifications in the

normal development by affecting neuronal arborization, increasing microtubule stability in the axon, and by altering growth cone area and the dynamics of growth cone cytoskeleton. Cytokines also promote long-term effects altering the formation of synapses, by decreasing both dendritic spine and synapse densities, and delaying the maturation of dendritic spines. As most of these modifications were described in neurodevelopmental disorders, as schizophrenia, the present work points out a causal relationship between neuroinflammation in embryonic life and subsequent brain damage leading to neurological disabilities such as learning and memory deficits.

Future Perspectives

Our results point to a modification of cytoskeleton dynamics upon IL-1β and TNF-α treatment, hence it would be interesting to evaluate the effect of cytokines in the major regulators of cytoskeleton, *i.e.* small Rho GTPases. Indeed, Rho GTPases regulate several pathways involved in the extension of neuritic processes, therefore controlling the neuronal development (Hall and Lalli, 2010). This could be first addressed by evaluating alterations in the expression of these proteins using Real Time- PCR and the specific primers, and then by assessing the level of these proteins, namely the activated forms GTP-bound, using a Rho active Pull-down assay followed by Western Blot. Upon engagement of specific cell surface receptors, both cytokines can also trigger intracellular pathways, as the translocation of nuclear factor-kappaB (NF-κB) to the nucleus or the activation of mitogen-activated protein kinases (MAPK), which can culminate in the regulation of small Rho GTPases and ultimately their effectors (Temporin *et al.*, 2008; Fernandes and Brites, 2009; Hall and Lalli, 2010). Therefore, the activation of these proteins could also be evaluated in order to elucidate the cascade of events that bridge cytokine cellular interaction with modifications of neuronal development.

The axonal transport is intimately related to the establishment of the pre-synaptic machinery, by promoting the transport of proteins and/or molecules from the cell body to the axonal bouton which are necessary for a proper transmission of the synaptic impulse (Gendron and Petrucelli, 2009). So, it would also be interesting to perceive the role of pro-inflammatory cytokines in the axonal transport process, namely by assessing the expression (Western Blot) and localization (immunocytochemistry) of motor proteins dynein and kinesin.

Glial cells are the principal source of inflammatory mediators in the CNS. Indeed, microglia and/or astrocytes upon stimulation, such as LPS, over-produce several pro-inflammatory cytokines and are then responsible for the levels of cytokines that we have used in our model upon brain injury. Thus, to corroborate our results it would be interesting to incubate hippocampal neurons with conditioned medium from previously activated glia and analyze whether the same effects that we have observed were maintained or even exacerbated. In addition, by using specific antibodies directed to either IL-1β or TNF-α we could certify the role of each cytokine in the neuronal arborization effects.

Although the use of cell cultures give us the possibility to study in a simplified context the mechanism of action of each cytokine, the use of organotypic hippocampal slice cultures incubated

with pro-inflammatory cytokines will provide an *in vivo* approach and a more precise effect of neuroinflammation in the cerebral context. Moreover, incubation with inhibitors of TNF-α and IL-1β, as soluble TNF receptors and IL-1ra respectively, will give us novel cues to achieve a successful recovery upon neuroinflammation. These experimental procedures might unveil new possible targets to ameliorate the detrimental outcomes, as cognitive impairments, and the neurodevelopmental disorders, emerging after a neuroinflammatory episode in the embryonic life.

V. REFERENCES

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