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Effects of Systemic Inflammation on Synaptogenesis
in Developing Mouse Hippocampus

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Slawomir Piotr Sloniowski

December 2011

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DEDICATION

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ABSTRACT OF THE DISSERTATION

Effects of Systemic Inflammation on Synaptogenesis
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by

Slawomir Piotr Sloniowski

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, December 2011
Dr. Iryna M. Ethell, Chairperson

Synapses are specialized points of contact between neurons allowing for rapid transfer of signals in electrical or chemical form. Synaptic transmission and plasticity are integral to information processing and memory functions in any nervous system, regardless of its level of complexity. Chemical synapses can be broadly classified as excitatory or inhibitory, depending on how their activity affects the membrane potential of the postsynaptic neuron. The majority of excitatory synapses are made onto small protrusions in dendrite membrane termed dendritic spines, while inhibitory synaptic terminals contact smooth membrane of dendritic shafts and cell bodies. The initial process of synapse formation, or synaptogenesis, occurs early in animal development and in humans as well as in mice it takes place postnatally over an extended period of time. Many neurodevelopmental as well as neurodegenerative disorders involve synapse and spine abnormalities, suggesting that undisturbed synaptogenesis is important to development of a healthy brain. Astrocytes and microglia are non-neuronal cells which play supportive and protective roles and thus shape the environment within the nervous system. Although their function is critical to proper neuronal operation,

under pathological conditions their activities can have deleterious effects on neurons and their synapses. The purpose of the presented research was to elucidate the potentially detrimental influence of glial activation on developing synapses. Using the lipopolysaccharide model of systemic inflammation we induced glial inflammatory response in mice at several points of postnatal development. Hippocampus, one of the best described brain structures was used as the region of interest. We observed varying levels of microglial activation depending on the age of the animal and similar levels of astrocyte reactivity at all ages. Morphometric analysis of dendritic spines identified a period of vulnerability, manifested as a decrease in spine density in response to inflammation. The density of presynaptic excitatory terminals was similarly affected. When the systemic inflammation was extended from 24h to 8 days, the negative effects on the excitatory terminals were more pronounced and suggested a reduced excitatory drive. The improvement of seizure outcomes confirmed this hypothesis. We also investigated synaptic development in the mouse model of Nasu-Hakola disease, a genetic neurodegenerative disorder characterized by dementia and microglial activation. We found that the mice failed to develop normal levels of excitatory presynaptic terminals, while exhibiting reduced susceptibility to seizures. Furthermore, inducing a systemic inflammation in these mice resulted in a decrease in inhibitory terminal density and higher seizure susceptibility. The results of this study demonstrate a period in postnatal development with elevated sensitivity to immune inflammatory responses. Using a mouse disease model we confirmed the impact of inflammation on synaptic development.

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Abbreviations

AD: Alzheimer's disease

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

APP: Amyloid precursor protein

ATP: adenosine-5'-triphosphate

BBB: blood brain barrier

BDNF: brain-derived neurotrophic factor

bFGF: basic fibroblast growth factor

BNPI: brain-specific Na^+-dependent inorganic phosphate cotransporter

CA: cornu Ammonis

CAM: cell adhesion molecule

CamKII: calcium/calmodulin dependent kinase II

CD14: cluster of differentiation 14

CNS: central nervous system

DAP12: DNAX adaptor protein-12

ERK: extracellular signal-regulated kinase

GABA: γ-aminobutyric acid

GAD: glutamate decarboxylase

GFAP: glial fibrillary acidic protein

GLAST: glutamate aspartate transporter

GLT-1: glutamate transporter 1

GM-CSF: granulocyte/macrophage colony stimulating factor

GSH: glutathione

Hsp60: heat shock protein 60

Iba1: ionized calcium binding adaptor molecule 1

IFN-γ: interferon-γ

IL-1: interleukin-1

IL-1β: interleukin-1β

IP: intraperitoneal

ITAM: immunoreceptor tyrosine-based activation motifs

LBP: LPS-binding protein

LPS: lipopolysaccharide

LTD: long term depression

LTP: long term potentiation

M-CSF: macrophage colony stimulating factor

MD-2: myeloid differentiation 2 receptor

mIPSC: mini inhibitory post-synaptic current

NFκB: nuclear factor κB

NGF: nerve growth factor

NMDA: N-methyl-d-aspartate

NO: nitric oxide

P: postnatal day

PAMP: pathogen associated molecular patterns

PLOSL: lipomembranous osteodysplasia with sclerosing leukoencephalopathy

PSD: post-synaptic density

SER: smooth endoplasmic reticulum

SPARC: secreted protein acidic and rich in cysteine

TGF-β: transforming growth factor-β

TLR: toll-like receptor

TNF: tumor necrosis factor

TREM2: Triggering receptor expressed on myeloid cells-2

TSP: thrombospondin

vGlut: vesicular glutamate transporter

Chapter 1 - Background

INTRODUCTION

Brain, Neurons, Synapses

The brain is widely considered the final frontier of biology and not coincidentally, the most complex structure in the known universe. We have been fascinated by its ability to integrate sensory data and to control animal behavior to ensure its survival. In the current age of robotics, we have realized the difficulty of describing and performing tasks we had not given much consideration to, such as object manipulation or even body movement within the constraints of the surroundings. Still more fascinating are the capabilities of the human brain, which separate us from other primates, such as use of language and complex abstract thought. It has been proposed that the human brain is an anatomically scaled up version of the primate brain (Azevedo et al., 2009) and yet it is capable of creating culture and building a technological civilization. Is there a critical computational mass that gives rise to the emergent properties of the human brain or perhaps it is inherent to the wiring of its neuronal networks? How do the basic building blocks interact to give it the capacity to perform the formidable tasks of daily existence? What are the mechanisms making this magnificent structure assume its fully developed shape from a simple zygote? Only further research can give us a clue to these and other important questions we aspire to solve.

Neuron is the principal brain cell involved in computation of the incoming sensory input, retrieving, comparing and storing relevant memories and producing motor output, whether it is walking, feeding or speech. Even though the number of neurons in the

human brain has been estimated at above 100 billion (Pakkenberg and Gundersen, 1997) it is not the most numerous brain cell type, as it is commonly accepted that neurons constitute only about 10% of all brain cells while glial cells make up the majority (Kandel et al., 2000). Nevertheless it is the neuron that possesses the necessary electrical properties that make brain function possible. Neuronal population is not homogenous and it takes a wide array of neuronal cell types varying in morphological and physiological properties to wire the functioning brain. Separate brain regions are characterized by their unique neuronal composition both in terms of neuronal and non-neuronal cell types (Stevens, 1998; Masland, 2004).

Neurons assemble into intricate networks by forming specialized asymmetrical contact sites termed synapses. These points of contact function to transfer information between the communicating neurons, which can take either electrical or chemical form (López-Muñoz F and Alamo C, 2009). In the vertebrate central nervous system (CNS) chemical synapses predominate and are defined by specific ultrastructural features (Palay, 1956; Gray, 1963). The synapse is formed by a presynaptic neuron which is the source of the signal and the postsynaptic neuron, which is the recipient. The presynaptic side of the synapse takes shape of a synaptic bouton or a varicosity along the length of an axon. It is filled with hundreds to thousands of synaptic vesicles, each about 50nm in diameter, which contain neurotransmitter molecules that constitute the chemical signal in synaptic transmission. Synaptic vesicles that cluster around the active zone are able to release their contents into the synaptic cleft by the process of exocytosis (Schoch and Gundelfinger, 2006). The postsynaptic side of the synapse is separated from the presynaptic terminal by intercellular space termed the synaptic cleft. The hallmark of chemical synapses is the lack of membrane or cytoplasm continuity between

participating neurons, unlike in electrical synapses. The postsynaptic neuron receives signal in the form of neurotransmitter molecules and recognizes them through neurotransmitter receptors. The postsynaptic side of the synapse directly is juxtaposed to the active zone and contains the post-synaptic density (PSD), an electron dense meshwork of proteins involved in the receiving of synaptic transmission, including neurotransmitter receptors. Depending on the type of neurotransmitter, the effect of neurotransmission can be either excitatory or inhibitory, resulting in depolarization or hyperpolarization of the postsynaptic neuron, respectively (Moore, 1993). Synaptic transmission mediated by the amino acid glutamate is typically excitatory, while γ-aminobutyric acid (GABA) mediates inhibitory transmission, although there are well described exceptions to these general rules (Ben-Ari et al., 2011; Fiorillo and Williams, 1998).

When an action potential traveling down the axon of the presynaptic neuron reaches the presynaptic terminal, it triggers calcium ion influx and consequently synaptic vesicle exocytosis and neurotransmitter release, thus initiating synaptic transmission (Neher E and Sakaba T, 2008). The neurotransmitter rapidly diffuses across the synaptic cleft to the postsynaptic side of the synapse, where it binds to appropriate receptors, gating them and thus altering the electrical potential of the postsynaptic neuron. In this manner the electrical signal originating at the presynaptic neuron is converted and transmitted in chemical form, to be received and transformed into electrical input at the postsynaptic neuron.

Hippocampus

Hippocampus is an anatomical structure within the medial temporal lobe of the brain that is involved in memory formation (Battaglia et al., 2011). Hippocampus proper consists of the dentate gyrus (DG) and the cornu Ammonis (CA), which is subdivided into four fields: CA1-CA4. Dentate gyrus is stratified into the polymorphic layer, stratum granulosum and stratum moleculare. CA area is also multi-layered; its layers include: stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum-moleculare (Andersen et al., 2007). In the hippocampal stratum radiatum layer of the field CA1, the primary excitatory neuronal type – the pyramidal neuron, receives excitatory input from CA3 pyramidal neurons via Schaffer collaterals, while the entorhinal afferents and recurrent collaterals terminate on stratum lacunosum-moleculare and stratum oriens, respectively. Inhibitory input to CA1 pyramidal neurons within stratum radiatum is received from local inhibitory neurons, or interneurons – bistratified, horizontal trilaminar and radial trilaminar cells, most of which use GABA as their neurotransmitter (Fig. 1.2A) (Shepherd, 2004).

Dendritic Spines

The majority (90%) of excitatory presynaptic input in the vertebrate CNS terminates onto dendritic spines (Gray, 1959) – small protrusions of dendritic membrane in the micrometer range (Fig. 1.1A, 1.2B). A stereotypical dendritic spine consists of a head connected to the dendrite via a thin neck. However, spines exist over a range of shapes and sizes; not all have a differentiable head and can significantly vary in length. Spines have been classified into three main categories – thin, stubby and mushroom shaped (Peters and Kaiserman-Abramof, 1970). Thin spines have small heads and

narrow necks; stubby spines have uniform width over their length and no discernible heads; mushroom spines have large heads and thin necks. Filopodium is considered a structural precursor to a functional dendritic spine (Fig. 1.1B, C). Spines which are long, thin with smaller head volumes are considered less mature and less stable, while spines which are shorter with large, distinct heads, such as mushroom spines more mature and stable (Ethell and Pasquale, 2005). It ought to be noted that spines exist over a spectrum of morphologies rather than in several discrete states. At the ultrastructural level (Fig. 1.1D), spine shape is supported by an actin cytoskeleton and in some spines microtubules have been detected (Gu et al., 2008), although their function is not yet clear. The PSD is usually located in the head of the dendritic spine and contains the machinery which manages the postsynaptic aspect of excitatory synaptic transmission. It consists of hundreds of proteins, most importantly α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-d-aspartate (NMDA) receptors, as well as scaffolding proteins such as PSD-95 and signaling proteins, for instance, calcium/calmodulin dependent kinase II (CamKII) (Okabe, 2007). Clathrin coated vesicles arise from patches of clathrin lining spine membrane and providing sites for endocytosis (Blanpied et al., 2002; Racz et al., 2004). Recycling endosomes are an important aspect of spine shape regulation (Park et al., 2006). Depending on the brain region and spine size, fewer or more spines may contain the smooth endoplasmic reticulum (SER) (Spacek, 1985; Harris and Stevens, 1988) which acts in calcium storage and regulation. In many large spines (80% of mushroom spines in CA1), a structure called "spine apparatus" consisting of laminated SER may be present (Spacek and Harris, 1997). Polyribosomes reside at the base of dendritic spines and regulate protein synthesis (Steward and Levy, 1982). Mitochondria are largely absent from dendritic

spines, however they may be present in very large, complex spines, for example, the thorny excrescences of the pyramidal cells of hippocampal CA3 (Chicurel and Harris, 1992).

The morphology of dendritic spines is highly related to their function. It is believed that the thin neck of the spine in relation to its head serves to compartmentalize biochemical signaling events taking place postsynaptically and calcium ion transients in particular (Koch and Zador, 1993; Denk et al., 1996; Sabatini et al., 2001). It was originally suggested that spine neck may restrict electrical coupling with dendritic shaft and thus regulate the spread of postsynaptic potentials. This idea has been largely abandoned, although it may apply in certain cell and synapse types (Nimchinsky et al., 2002). Spine morphology also influences synapse strength. Large, mushroom spines have larger PSDs of higher complexity than spines with smaller heads (Harris et al., 1992), which in turn translates to a higher capacity for, and density of glutamate receptors (Matsuzaki et al., 2001; Nicholson et al., 2006). There is a correlation between PSD size, capacity for glutamate receptors and synaptic strength, suggesting that larger spines harbor stronger synapses (Nimchinsky et al., 2004; Sheng and Hoogenraad, 2007). Based on these findings, it follows that changes in synaptic strength may coincide with alterations to dendritic spine morphology. Several studies support this prediction. Induction of long term potentiation (LTP) in hippocampal slices by glutamate uncaging at single spines results in persistent enlargement of smaller spines and transient enlargement of mushroom spines (Matsuzaki et al., 2004; Lang et al., 2004; Fortin et al., 2010). On the other hand, long term depression (LTD) induced by low frequency stimulation results in spine shrinkage (Zhou et al., 2004). *In vitro* studies are corroborated by rat *in vivo* data – a motor learning task resulted in widening of spines in

layer I of primary motor cortex along with an increase of synaptic responses (Harms et al., 2004). Similarly, motor learning in an obstacle course was observed to increase spine length and density on Purkinje neurons (Lee et al., 2007). It must be noted that there are instances where synaptic plasticity also occurs in the absence of morphological plasticity, as shown for hippocampal (Wang et al., 2007) and cerebellar LTD (Sdrulla and Linden, 2007).

Dendritic spines are highly dynamic assemblies and can change their shape on the order of minutes relying on rearrangement of actin cytoskeleton (Dunaevsky et al., 1999). It is thought that they arise primarily from filopodia which are long (up to 10μm) and transient (mean lifetime 9.5min) structures (Ziv and Smith 1996) protruding from dendritic membrane, similarly to spines. Spines can also emerge directly from shaft synapses, without a transitory filopodial stage (Engert and Bonhoeffer, 1999; Marrs et al., 2001). Additionally, filopodia may establish a synaptic contact, retract forming a shaft synapse, which then transforms into a spine synapse (Fiala et al., 1998). Lastly, spines may form without synaptic contact as has been shown for cerebellar Purkinje cells (Takacs et al., 1997). It is therefore suggested that spine synapses form through a combination of several mechanisms that may depend on intrinsic characteristics of different types of neurons (Ethell and Pasquale, 2005).

Dendritic spine formation

During early rat hippocampal development, contribution of dendritic spine synapses to the synapse population as a whole increases from 5% at P1 to 37% at P12 and the overall number of spines doubles from P15 to adulthood (Fiala et al., 1998). The morphological profile of spines also changes, with a four-fold increase in mushroom

spines with perforated PSDs and no change or decrease in other spine types (Harris et al., 1992). Adult animals retain the ability to form new spines, as spine density increases in rodents and primates exposed to enriched environments (Kozorovitskiy et al., 2005; Moser et al., 1997).

As spines assume mature, mushroom shape they become more stable and resilient to turnover. Thin spines are transient, form and retract over a few days in response to varying levels of synaptic activity, while mushroom spines can last for several months (Holtmaat et al., 2005; Zuo et al., 2005). Thin spines have NMDA receptors and low amounts of AMPA receptors and can be strengthen by insertion of additional AMPARs (Matsuzaki et al., 2001; Ganeshina et al., 2004; Ashby et al., 2006). It has been suggested that thin spines are plastic "learning spines", while mushroom spines are established, more stable "memory spines" (Bourne and Harris, 2007; Kasai et al., 2003). Distinct brain regions exhibit different spine turnover rates, as demonstrated in the cortex of juvenile mice – spines of the somatosensory cortex experienced a higher turnover rate than those of auditory and visual cortices (Majewska et al., 2006).

Synaptogenesis

Dendritic spine formation is one of the aspects of the complex process of synaptogenesis, which requires bidirectional communication of the participating pre- and postsynaptic structures (Cohen-Cory, 2002). Axons growing towards their targets are guided by a variety of molecular cues that make correct connectivity possible, even across large distances (Kolodkin et al., 2011). In the early stages of synaptogenesis, growing axons promote filopodia motility and synaptogenesis by localized exocytic glutamate release (Dailey and Smith, 1996; Lendvai et al., 2000). Secreted growth

factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) promote synapse formation in cultured neurons (Vicario-Abejon et al., 1998; Bolton et al., 2000). Wnt proteins also contribute to synapse formation – Wnt7a released from granule cells in the cerebellum induces mossy fiber axon growth cone remodeling and accumulation of synapsin 1 protein, which is associated with synaptic vesicles (Hall et al., 2000). Agrin has also been implicated in synapse formation, although not in an essential, but rather a modulatory role (Bose et al., 2000; Serpinskaya et al., 1999). Synapse specificity is guided by temporally and spatially restricted synaptogenic molecules and their receptors, such as several classes of cell adhesion molecules (CAMs), including cadherins and protocadherins (Shapiro and Colman 1999; Takai et al., 2003). Another group of molecules promoting synaptic differentiations are the β-neurexins and neuroligins (Missler and Sudhof, 1998; Rao et al., 2000). The Eph family of proteins which includes Eph receptors and their ligands ephrins plays a manifold role in synapse formation, from axon guidance and laying down neuronal connectivity (Martinez and Soriano, 2005), to excitatory synaptogenesis (Henkemeyer et al., 2003) and dendritic spine formation (Moeller et al., 2006).

Presynaptic specializations are assembled by vesicular delivery of proteins in small clear-centered vesicles, tubulovesicular structures and 80-nm dense core vesicles (Ahmari et al., 2000). It isn't clear what each vesicle type is composed of, however the small vesicles are thought to be synaptic vesicle precursors that carry synaptic vesicle proteins (Hannah et al., 1999; Huttner et al., 1995). The tubulovesicular structures were suggested to be either post-Golgi membranes and/or endosomal intermediates (Waites et al., 2005). The 80-nm dense core vesicles carry active zone scaffold proteins and the synaptic vesicle excocytotic machinery (Shapira et al., 2003; Zhai et al., 2001).

While the presynaptic portion of the synapse is assembled primarily by vesicular delivery of protein and lipid material, the postsynaptic specialization takes shape by gradual accumulation of receptors (Bresler et al., 2004; Ziv and Garner 2004). The initial events of postsynaptic assembly involve recruitment of PSD-95 family of scaffolding protein, followed by NMDA and AMPA receptors. Similarly, CaMKII and other scaffolding proteins are recruited from the cytosol (Shen and Meyer, 1999; Bresler et al., 2004). Synaptic maturation involves a size increase, two- to threefold increase in the number of synaptic vesicles during the first month of cortical development (Vaughn, 1989) and overall coordination between the growth of pre- and postsynaptic components, including bouton volume, number of synaptic vesicles, PSD area and spine head volume (Harris and Stevens 1989; Pierce and Mendell 1993; Schikorski and Stevens 1997). In the hippocampus, probability of transmitter release decreases as synapses mature (Bolshakov and Siegelbaum, 1995), while quantal size exhibits an increase (De Simoni et al., 2003). Receptor composition also changes, as NR2B subunits of NMDA receptors are replaced with NR2A subunits, decreasing NMDA current durations (Tovar and Westbrook, 1999). Silent synapses which lack surface AMPA receptors are "unsilenced" by NMDA receptor activation and resulting recruitment of AMPA receptors to the postsynaptic membrane (Takumi et al., 1999). Synapse elimination is a critical aspect of development, as synaptic pruning removes excessive or inappropriate connections (Rakic et al., 1986). The ability of the CNS to form and eliminate synapses is retained in adulthood and appears to be a crucial component of general plasticity in the nervous system (Waites et al., 2005).

Synapsins

One of the synaptic protein families conserved in invertebrates and vertebrates are the synapsins. They are a family of phosphoproteins serving multiple functions within the presynaptic terminal, where they are expressed. (Porton et al., 1999; Südhof et al., 1989). Synapsins are expressed in both central and peripheral nervous systems and are virtually absent from non-neuronal cells, or expressed at very low levels (Cesca et al., 2010). Synapsin 1 localizes to the cytoplasmic surface of small synaptic vesicles, which contain classical neurotransmitters (Navone et al., 1984) and is preferentially expressed in mature neurons. The majority of presynaptic terminals express at least one synapsin isoform, however the expression patterns of individual synapses vary greatly (Kielland et al., 2006; Staple et al., 1997). The primary function of synapsins involves trafficking of synaptic vesicles and regulating their availability for release by reversibly crosslinking them to the actin cytoskeleton. This action is thought to be of importance to the formation of the reserve pool of synaptic vesicles and maintenance of balance between the reserve pool and the readily releasable pool (Hackett et al., 1990; Llinas et al., 1985, 1991).

Vesicular Glutamate Transporters

A protein associated with presynaptic terminals of excitatory synapses is the vesicular glutamate transporter (vGlut). The vGlut group of glutamate transporters has been identified with the discovery of the brain-specific Na^+-dependent inorganic phosphate cotransporter (BNPI) (Ni et al., 1994). Once it became apparent that the isolated protein is a glutamate transporter, it was renamed to vGlut1 (Bellocchio et al.,

2000). Subsequently, two more vGluts were discovered, DNPI, later renamed to vGlut2 (Aihara et al.,2000; Bai et al., 2001) and vGlut3 (Fremeau et al., 2002). Vesicular glutamate transporters utilize proton electrochemical gradient across the vesicle membrane generated by H^+-ATPase to transport glutamate molecules into synaptic vesicles. Glutamate exocytosis and hence, excitatory neurotransmission rely on vGlut operation. In agreement with their function, vGlut1 and vGlut2 were found to be expressed in presynaptic terminals of glutamatergic synapses. VGlut1 and vGlut2 have a largely complementary expression pattern with vGlut1 being highly expressed in the cortex, hippocampus and basolateral nuclei and vGlut2 dominating in the thalamus, brainstem and deep cerebellar nuclei (Fremeau et al., 2001; Kaneko et al., 2002). At the level of individual synapses, vGlut expression also appears to be complementary, with vGlut1 and vGlut2 expressed at separate synapses in the cortex, hippocampus, ventral striatum, thalamus, hypothalamus and cerebellum (Fremeau et al., 2004). However, in the early stages of development, vGlut1 and vGlut2 have been found to colocalize in the hippocampus, cerebellum and thalamus within the same synapses and vesicle pools (Herzog et al., 2006). Nevertheless, vGluts are considered to be genuine markers of glutamatergic synapses and are formally used to identify not only glutamatergic neurons, but also to assign glutamatergic phenotype to neurons using a different primary neurotransmitter (Mestikawy et al., 2011).

Glutamate Decarboxylase

GABA is the predominant neurotransmitter mediating inhibitory neurotransmission in the CNS. Inhibitory neurons that use GABA as their neurotransmitter synthesize it from glutamate via action of glutamate decarboxylase

(GAD). Two forms of this enzyme, GAD65 and GAD67, are produced by GABA-ergic neurons and are often coexpressed, but frequently at different relative levels (Esclapez et al., 1994; Feldblum et al., 1993). Furthermore, GAD67 is widely distributed in the cells, while GAD65 is targeted to presynaptic terminals. Additionally, GAD65 is the much more abundant isoform in the dentate gyrus and CA1 field in the hippocampus (Sloviter et al., 1996). It has been suggested that the dissimilar patterns of expression of these enzymes may indicate differing roles. GAD67, being expressed throughout the cells may provide GABA for general metabolic activity and the synaptically expressed GAD65 may play a prominent role in neurotransmission (Martin and Rimvall, 1993). Interestingly, GAD67 can support the needs of GABA-ergic cells for the neurotransmitter, but GAD65 cannot, as demonstrated in the knockout studies of either isoform (Asada et al., 1996; Asada et al., 1997). However, despite normal GABA levels in GAD65 knockout mice, the animals were more susceptible to seizures. GAD67 knockouts had GABA levels reduced to 7% and died of severe cleft palate soon after birth. It has been proposed that GAD65, being associated with axon terminals and synaptic vesicles, serves in vesicular GABA release, in contrast to GAD67 which may be involved in non-vesicular GABA release (Reetz et al., 1991; Soghomonian and Martin, 1998). GABA has been shown to have excitatory action in several contexts, especially during prenatal development. In the mature brain however, its excitatory mode is restricted to a very narrow set of conditions (Stein and Nicoll, 2003), hence GAD65 is an excellent marker for inhibitory presynaptic terminals, especially in hippocampal CA1.

Microglia

Pioneering studies of the CNS distinguished between electrically active neuronal cells and the remaining non-neuronal cells, which due to our lack of understanding of their function, were named glia and thought to serve as merely "brain glue". Initial research efforts concentrated on neuronal physiology, since the significance of neurons was quickly recognized. Glia remained underappreciated, also in large part due to the lack of necessary tools needed to investigate them. Currently we differentiate between several types of glial cells – the class of macroglia which includes astrocytes, oligodendrocytes and recently discovered NG2 glia and the class of microglia (Wigley and Butt, 2009). Microglia are the sole resident macrophages of the CNS and are responsible for generating innate and adaptive immune responses in the immunologically privileged CNS tissue. In the healthy state microglia constantly survey their immediate surroundings while maintaining separate territories (Fetler and Amigorena, 2005). They exhibit highly ramified morphology with numerous fine processes undergoing perpetual remodeling as microglia probe their environment without disturbing neuronal connectivity (Nimmerjahn et al., 2005). This state has been referred to as "resting" or "quiescent" with the original assumption that in the healthy CNS microglia were inactive. It is now clear that these cells constantly patrol brain parenchyma sampling their microenvironment for pathological changes. Microglia can rapidly respond to potential and real threats to normal CNS functioning that may take form of infection, injury or neurodegenerative disease (Garden and Möller, 2006). Disturbances of CNS homeostasis can trigger microglial activation manifesting itself in changes in gene expression, functional behavior and morphology (Colton and Wilcock 2010; Kreutzberg 1996). Upon activation, microglia withdraw and reduce their intricate

protrusions shifting to amoeboid morphology (Fig 1.4). They can proliferate, move to sites of lesion, produce and release proinflammatory and immunoregulatory molecules and cytotoxic compounds to combat invading pathogens.

Microglia respond to a variety of signals representing tissue damage, pathogen invasion or even changes in neuronal activity (Hanisch and Kettenmann, 2007; Wake et al., 2009). They can detect microbial threats as has been demonstrated with lipopolysaccharide (LPS) endotoxin, a gram-negative bacterial cell wall component (Hetier et al., 1988). LPS and stimuli such as lipoteichoic acid, flagellin, single stranded viral RNA and unmethylated CpG DNA of bacteria and viruses are detected by the toll-like receptor (TLR) family (Takeda et al., 2003). Abnormal proteins associated with neurodegenerative disorders such as Alzheimer's disease (AD) or Huntington's disease may also induce microglial activation. β-Amyloid peptides which are derived from Amyloid precursor protein (APP) and form the β-Amyloid plaques in the brains of AD patients can also activate microglial cells (Rogers et al., 2002), possibly through the scavenger receptors (Husemann et al., 2002). Adenosine-5'-triphosphate (ATP) is released from damaged cells and is a potent activator of microglia via P2 purinergic receptors (Inoue 2006). An absence of certain factors may also indicate a disturbance in CNS homeostasis and microglia can activate in response to a lack of normally present stimuli. One example of such behavior is activation of microglia in CD200 knockout mice, a ligand for CD200 receptor. The ligand is expressed on neurons and the receptor on microglia. In the absence of the ligand microglia become activated (Hoek et al., 2000; Masocha 2009).

Neurons are sensitive cells that, for the most part, cannot repopulate after cell loss resulting from trauma or pathogen attack. For this reason any damage resulting

from an immune response must be limited and thus microglial activation is tightly regulated. The generic term "activated microglia" does not reflect the nuances of this state and the processes it involves. Microglial activation is not a binary state with a linear timeline and a predictable outcome (Kettenmann et al., 2011). It can result in significantly different functional states of microglial cells depending on context (Carson et al., 2007; Colton 2009; Schwartz et al., 2006). Microglia that have undergone activation and returned to the resting state may be morphologically indistinguishable from "inexperienced" microglia, yet some changes may persist and the cell may respond differently when challenged again (Hanisch and Kettenmann, 2007). Furthermore, heterogeneity in the activated population may exist, even within the same brain structure (van Weering et al., 2011). These and other factors contribute to the complexity of microglial responses to threats of CNS health.

Early in the activation process microglia begin to migrate to the site of injury or inflammation attracted by a variety of molecules including ATP, ADP, chemokines and trophic factors (Cartier et al., 2005. Forstreuter et al., 2002, Honda et al., 2001). Some conditions, such as neuronal degeneration, produce signals inducing microglial proliferation. Neurons undergoing mechanical injury or seizures upregulate their tumor necrosis factor (TNF) expression which microglia recognize and respond to (Minami et al., 1991). Astrocytes can promote microglia division by secreting macrophage colony stimulating factor (M-CSF) protein (Kim and de Vellis, 2005). As part of pathogen response, microglia can produce nitric oxide (NO) and respiratory burst, generating reactive oxygen species, creating oxidative damage in the invading organisms. However, this defensive mechanism is also toxic to neurons. Microglia is also a

phagocytotic cell, capable of engulfing microbes, pathological proteins, apoptotic cells and cellular debris (Streit et al., 1988).

A major aspect of microglial inflammatory response is secretion of diffusible factors as means of communicating with neuronal cells and invading leukocytes in order to coordinate the overall immune reaction. These factors primarily include cytokines and immunomodulatory peptides, which can be pro- or anti-inflammatory and thus regulate the immune response, chemokines, which act as chemotactic molecules and regulate cell motility, and trophic factors that promote neuronal survival (Garden and Möller, 2006).

Microglia originate from primitive macrophage precursors that arise in the yolk sac and colonize the rudimentary brain through the blood circulation, as soon as it is established. This early population of settlers proliferates during prenatal as well as postnatal development and over 95% of the adult pool of microglia is born within the brain parenchyma after P0 (Alliot et al., 1999; Ginhoux et al., 2010).   It appears that in the adult, healthy CNS microglia form a stable, self-renewing population of cells that do not require turnover from circulating blood progenitors (Chan et al., 2007). During and early after their migration they exhibit ameboid shape before assuming the classical ramified phenotype (Brockhaus et al., 1993). The process of ramification is likely to be induced by astrocytes and to require Cl⁻ channel activity. Astrocytic cytokine candidates for this role include transforming growth factor-β (TGF-β), M-CSF and granulocyte/macrophage colony stimulating factor (GM-CSF) (Eder et al., 1998, Schilling et al., 2001).

The role of microglia in the CNS appears not to be limited to immune functions. Recent studies indicate that microglia participate in synaptic development and plasticity.

During normal postnatal development of mouse hippocampus, microglia have been shown to be involved in synaptic pruning by engulfing synaptic material. In transgenic mice lacking the fractalkine receptor (Cx3cr1) normally expressed on microglia, their numbers were transiently reduced and the synaptic pruning was delayed, suggesting that microglia indeed partake in the process of synaptic development (Paolicelli et al., 2011). During the formation of the retinogeniculate system, retinal ganglion cells form excessive connections with dorsal lateral geniculate nucleus neurons. It has been shown that elimination of inappropriate synapses is mediated by the classical complement cascade and the cells responsible for synaptic pruning are likely to be microglia (Stevens et al., 2007; Chung and Barres 2011).

There is accumulating evidence that microglia may possibly participate in synaptic plasticity. TNF-α, produced by microglia and astrocytes is required for homeostatic synaptic scaling in response to prolonged blockade of neuronal activity (Stellwagen and Malenca, 2006). Changes in neuronal activity induced by application of bicuculline, a $GABA_A$ receptor blocker, increased the volume sampled by quiescent microglia *in vivo* (Nimmerjahn et al., 2005). Additionally, following a cerebral ischemia, the duration of microglia-synapse contacts increased and in some cases the presynaptic boutons disappeared during or after the prolonged microglial contact (Wake et al., 2009). Lastly, altering visual experience by light deprivation and re-exposure has been shown to result in changes in motility of microglial processes and their increased preference for interactions with certain synapses. Under normal conditions microglia contacted small and transient dendritic spines. Light deprivation resulted in changes in microglial morphology and more frequent and extensive contacts with synapses, with increased preference for large and shrinking dendritic spines. This behavior was reversed by re-

exposing the mice to light (Tremblay et al., 2010). One of the proteins expressed by microglia, but not by neurons, astrocytes or oligodendrocytes is ionized calcium binding adaptor molecule 1 (Iba1). It has been widely used as a microglial marker since it is expressed throughout the cell and can visualize fine microglial processes (Ito et al., 1998). However, Iba1 is a macrophage specific protein and labels peripheral macrophages infiltrating the brain during inflammatory events (Imai and Kohsaka, 2002). Currently, there is no method of differentiating between microglia and infiltrating macrophages in immunohistochemical experiments.

Astrocytes

Astrocytes, a subset of macroglia, are the most numerous glial, and by extension, neuronal cell type, constituting nearly half of all brain cells. Yet, even more so than in case of microglia, which were recognized to be immune cells, true roles of astrocytes were not appreciated for decades. Initially they were thought to provide merely structural support for the neural network, but as research progressed, the array of tasks known to be performed by astrocytes grew steadily, culminating with the concept of a "tripartite synapse", where astrocytes play an active role in neuronal communication (Haydon 2001; Volterra and Meldolesi, 2005). The extent to which astrocytes participate in information processing with the use of neurotransmitters, in addition to gliotransmitters, has been debated and remains controversial (Hamilton and Attwell, 2010). However, the significance of astrocytes to brain physiology is broad and well established.

Astrocyte morphology is defined by a dense network of processes branching away from soma into very fine terminal processes which either ensheathe or closely

associate with synapses in addition to lining brain vasculature (Freeman, 2010). Each astrocyte covers a specific territory of thousands of synapses and blood vessel interfaces with no or minimal overlap with its neighbors (Bushong et al., 2002). The territories are divided into microdomains that can be regulated independently of each other (Mulligan and MacVicar, 2004). Astrocytes are excitable in the sense of experiencing calcium transients and oscillations that can spread through networks of astrocytes bridged by gap junctions formed by hexamers of connexin 43 (Volterra and Meldolesi, 2005). The main component of astrocytic cytoskeleton is the intermediate filament network and its primary constituent is the glial fibrillary acidic protein (GFAP). It is a classical marker for astrocytes, although it has been demonstrated to be expressed in other cell types as well. In the brain, most notably, GFAP is expressed in the neural precursor cells of the subventricular zone (Middeldorp and Hol, 2011). Astrocytes are essential to normal functioning of neurons. They provide for energy needs of neurons by acting as intermediaries between them and the circulatory system. Astrocyte endfeet enwrap capillaries and so most nutrients and metabolites reaching neurons pass through astrocytes first (Barros et al., 2005; Rouach et al., 2008). This close relationship with vasculature puts astrocytes in the position to regulate bloodflow (Zonta et al., 2003). Neurons obtain their energy either in form of lactate or glucose from astrocytes (Benaroch 2005; Gandhi et al., 2009). Excitatory neurotransmission involves glutamate exocytosis at synapses; only a small amount is re-uptaken by releasing neurons while the majority is taken up by astrocytic glutamate transporters, primarily glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1). Astrocytes metabolize glutamate into glutamine which they release for uptake by neurons (Anderson and Swanson, 2000). Extracellular ion homeostasis is important in

determining levels of neuronal activity and astrocytes have been shown to maintain normal potassium concentrations (de Lanerolle et al., 2010). Furthermore, astrocytes maintain the glutathione (GSH) system by synthesizing and releasing GSH, which neurons and other brain cells do not secrete. GSH is the main antioxidant thiol which provides for detoxification of reactive oxygen species and neutralization of organic hydroperoxides such as hydrogen peroxide (Dringen and Hirrlinger, 2003).

Astrocytes are crucially involved in synaptogenesis. It has been demonstrated that synapse number is not intrinsic to neurons themselves but is regulated by astrocytes. Initial studies have shown that astrocyte condition media dramatically enhances synapse formation in culture. Further research has been aimed at identifying the specific factors. Both in vitro and in vivo experiments demonstrated that thrombospondin (TSP) 1 and 2 are released by astrocytes to induce functional presynaptic terminals (Christopherson et al., 2005). Estrogen derived from astrocytes also induces synapse formation (Göritz et al., 2002), while cholesterol enhances both synapse formation and synaptic transmission (Hu et al., 2007). Recently two new factors have been identified to regulate excitatory synaptogenesis – hevin and secreted protein acidic and rich in cysteine (SPARC, Kucukdereli et al., 2011). Dendritic spine morphology is maintained by interaction of EphA4 receptor tyrosine kinase expressed on the surface of dendritic spines and ephrin-A3 expressed on astrocyte processes enwrapping the spines. Inhibiting or eliminating signaling stemming from EphA4 activation results in spine shape irregularities (Murai et al., 2003). Not only do astrocytes participate in synaptogenesis, they are also contributors to the regulation of synaptic transmission and plasticity (Achour and Pascual, 2010). D-serine is synthesized primarily by astrocytes and acts as a glutamate coagonist at NMDA receptors. It has

been shown that in the case of CA1 pyramidal neurons, D-serine contributes to LTP induction and strengthens NMDA currents (Henneberger et al., 2010). In the hypothalamic paraventricular nucleus, ATP released by astrocytes acts on postsynaptic neurons of glutamatergic synapses to enhance their responses, thus providing a form of distributed feed-forward synaptic plasticity (Gordon et al., 2009). Astrocytes produce TNF-α and are a likely source of this molecule for mediation of homeostatic synaptic scaling (Stellwagen and Malenka, 2006). Astrocytes also appear to contribute to modulation of glutamatergic transmission by varying expression levels of excitatory amino acid transporters (EAATs) at synapses. The significance of glutamate release by astrocytes is still debated (Paixão and Klein, 2010).

Inflammation

Inflammation is a complex physiological process with markedly different expression in the CNS than in the periphery due to the immune privileged status of the brain. It was initially thought that the brain did not participate in inflammation and was unaffected by immune responses of the periphery. It is now recognized that the opposite is true and that CNS can even regulate some aspects of systemic inflammation (Lucas et al., 2006). It is also know that not only microglia and infiltrating leukocytes actively take part in neuroinflammation, but also astrocytes and neurons themselves. Microglial activation and CNS inflammation in general are aimed at protecting the brain from infection and injury. However, the process itself can be neurotoxic and may have detrimental effects on neuronal cells and therefore brain function (Fig. 1.3). Microglia are rapidly activated by and recruited to sites of injury, whether excitotoxic, ischemic or traumatic (Akiyama et al., 1994; Dusart and Schwab 1994; Kato et al., 1994). They have

the ability to detect invading pathogens through pathogen associated molecular patterns (PAMPs, Crack and Bray, 2007). Importantly, also in the absence of a stimulus within the CNS, microglia can become activated in the event of a systemic inflammation, by inflammatory cytokines released into systemic circulation by immune cells in the periphery (Perry, 2004). There are several ways in which systemic inflammation can penetrate into the brain. Circumventricular organs of the brain act in secretory and sensory manner with respect to the circulatory system and as such, lack a complete blood brain barrier (BBB). By this route, blood cytokines can diffuse into the brain and influence microglial activation states. Even an intact BBB does not insulate the brain from communication with the periphery, as perivascular macrophages can detect endothelial activation and in turn activate microglia. Additionally, cytokines can be actively transported across the BBB (Banks et al., 2002). Lastly, the sensory neurons of the vagal nerve which innervates the abdominal cavity express receptors for the pro-inflammatory cytokine interleukin-1 (IL-1) and can communicate inflammation in the periphery with the brain (Konsman et al., 2002).

Microglial inflammatory response is ultimately aimed at protecting the host and includes release of neurotrophic factors which play neuroprotective roles and enhance neuron survival. However, most pro-inflammatory factors are neurotoxic and can participate in neuron damage and pathogenesis of neurological disorders. Activated microglia release cytokines which include TNF-α, interleukin-1β (IL-1β), interferon-γ (IFN-γ) and prostaglandins which amplify overall inflammatory response and have been implicated in neuronal degeneration (Liu and Hong, 2002). Microglial response can be generally cytotoxic and involves production of free radicals such as superoxide, damaging lipids, DNA and proteins. NO overproduction in particular has been linked to

contributing to cell damage in a variety of conditions (Moro et al., 2004). Dopaminergic neurons have been observed to be particularly sensitive to oxidative damage (Gao et al., 2002). Other substances released by activated microglia which may be detrimental to neurons include fatty acid metabolites such as eicosanoids and quinolinate (Giulian et al., 1995).

Secretion of pro-inflammatory cytokines from microglia induces activation of astrocytes whose actions may exasperate damage to neurons. IL-1 was shown to inhibit glutamate uptake by astrocytes in a dose dependent manner, which puts neurons in risk of over excitation (Hu et al., 2000), in addition to inducing NO release from astrocytes (Thornton et al., 2006). TNF-α and prostaglandins control glutamate release from astrocytes, possibly affecting this aspect of neuronal-glial communication (Vesce et al., 2007). More importantly, glutamate handling by astrocytes under inflammatory conditions may be compromised, leading to potential excitotoxicity resulting from inadequate glutamate clearance (Tilleux and Hermans, 2007). TNF-α and IL-1β treatment of astrocytes triples their glucose utilization, decreases glycogen stores, while simultaneously decreasing lactate release by 45% in response to glutamate stimulation and increasing GSH and hydrogen peroxide efflux (Gavillet et al., 2008). Lipid peroxidation by various oxidants has been shown to inhibit glutamate transporters resulting in neuron death by glutamate excitotoxicity (Choi and Kim, 1998). The same study demonstrated that LPS and IFN-γ are toxic to astrocytes under low energy conditions by reducing intracellular ATP levels. Thus it has been proposed that inflammation and oxidative stress alter astrocyte metabolism, and that astrocytes possibly neglect their normal functions, resulting in less support for neurons (Steele and Robinson, 2011). Furthermore, reactive astrocytes not only synergize with microglia in

release of TNF-α (Bezzi et al., 2001), they also activate distant microglia, by employing Ca^{2+} waves propagating through their networks.

Extensive research has demonstrated significant involvement of inflammatory effects in neurodevelopmental and neurodegenerative diseases. During prenatal as well as postnatal development the forming and immature CNS often experiences short periods of systemic inflammation with potential for contributing to pathogenesis of such disorders as autism, schizophrenia or cerebral palsy (Vargas et al., 2005; Meyer et al., 2011; Bax et al., 2005). In neurodegenerative diseases, inflammation may play a causal role, as is the case in multiple sclerosis (Lucas et al., 2006), or its contribution may be more complex as in Alzheimer's disease (Sheng et al., 1998) or Parkinson's disease (Gao et al., 2003).

The sheer number of people affected directly and indirectly by neurodevelopmental disorders underscore the importance of research into the relationships of CNS developmental processes and the physiology of inflammation.

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Figure 1.1 Dendritic spine morphology, ultrastructure and types.

(A) Fragment of a dendrite of a pyramidal neuron in stratum radiatum of field CA1 in mouse hippocampus is shown labeled with DiI. Scale bar is 20μm. (B) A magnified portion of the dendrite with examples of dendritic spine types indicated by arrows. Scale bar is 2μm. (C) Diagram drawing of dendritic spine categories and a filopodium. (D) Diagram drawing of of the major components of dendritic spine ultrastructure.

Figure 1.1

A

B

Stubby

Thin

Mushroom

C

Dendritic spines

Head area

Length

Filopodium

Thin

Mushroom

Stubby

D

Axon terminal

Glia

Dendritic spine

○ Synaptic vesicle

Glutamate receptors

Post-synaptic density

Smooth Reticulum

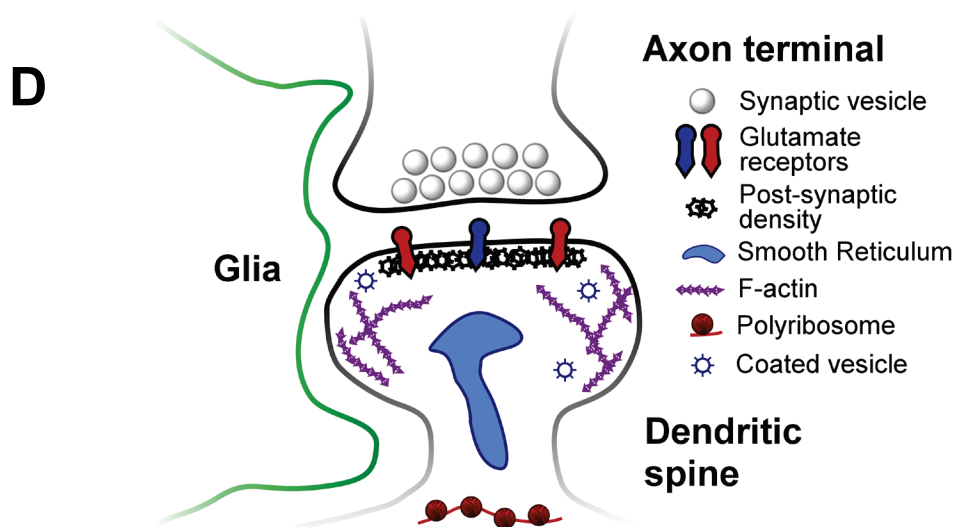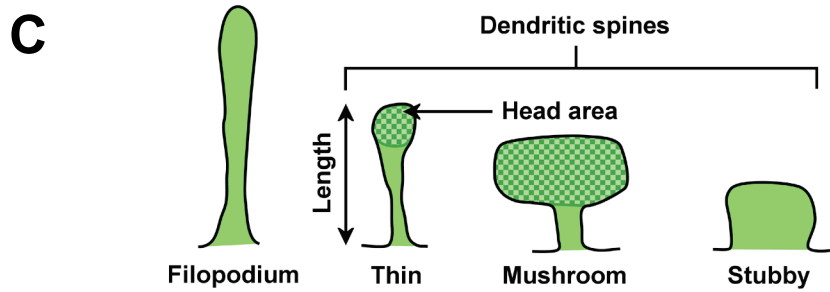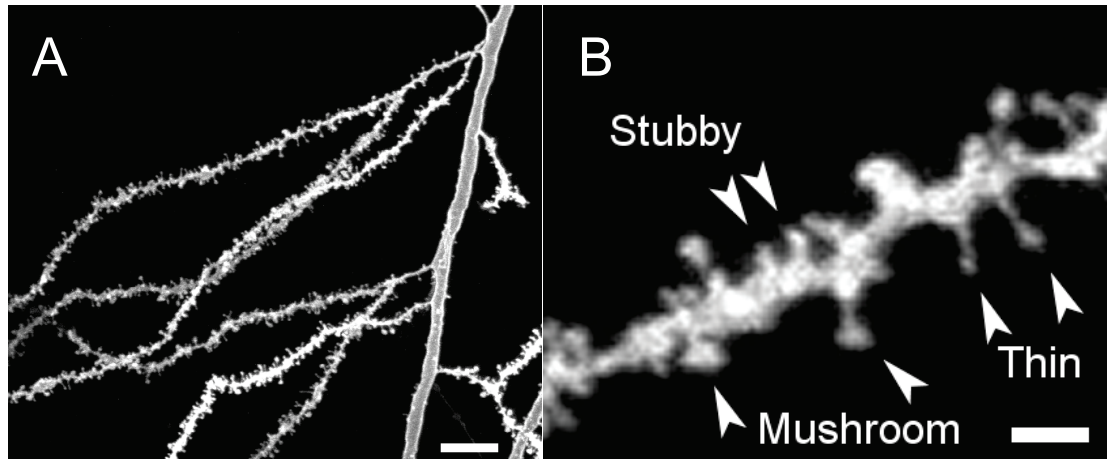F-actin

Polyribosome

☆ Coated vesicle

Figure 1.2 Excitatory and inhibitory inputs of CA1 pyramidal neurons.

(A) A diagram of hippocampal area CA1, indicating sources of excitatory and inhibitory input to pyramidal neurons in stratum radiatum layer. (B) A diagram of primary locations of inhibitory and excitatory synapses on pyramidal neuron dendrites.

Figure 1.2

A

CA1 pyramidal neurons

Excitatory input

Inhibitory input

Schaffer collaterals

Inhibitory interneuron

B

Excitatory input

Dendritic spine

Inhibitory input

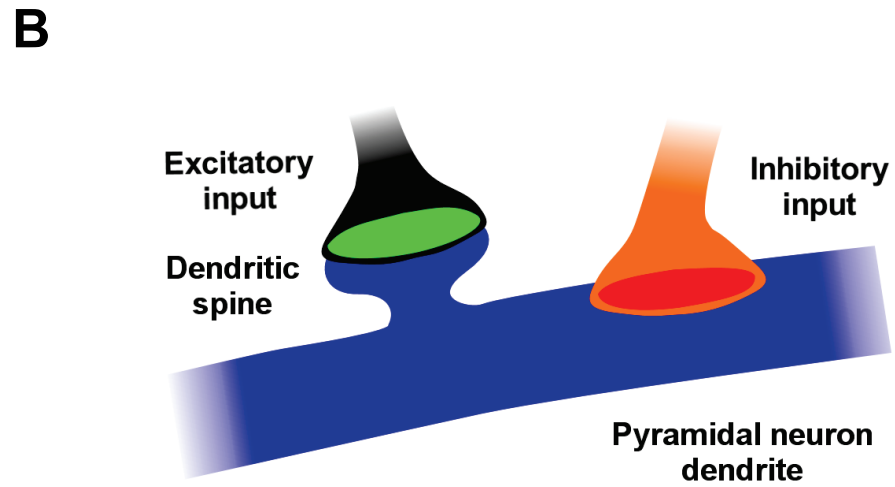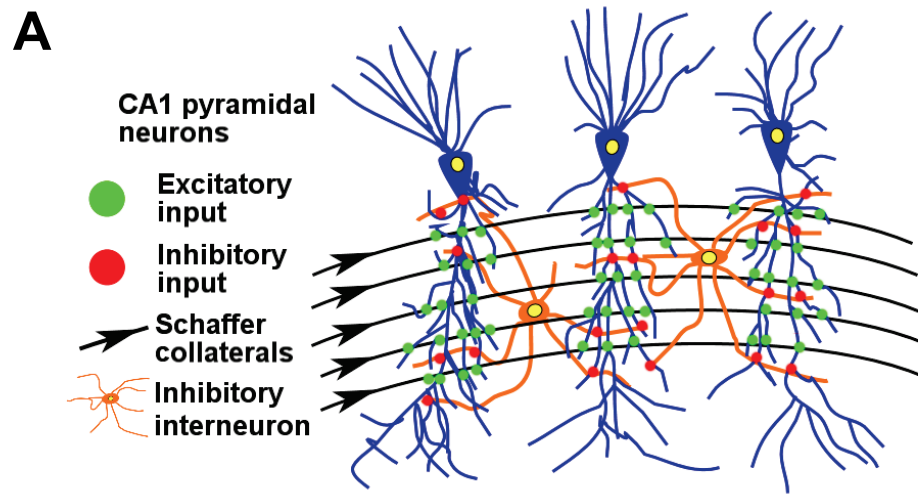Pyramidal neuron dendrite

Figure 1.3 Neuronal-glial interactions under normal and inflammatory conditions.

A diagram showing the activities of resting and activated glial cells in respect to neurons. Systemic inflammation induces microglial activation which promotes astrocyte activation. By becoming activated, glial cells shift from stimulating neuronal health to inducing neurotoxicity.

Figure 1.3
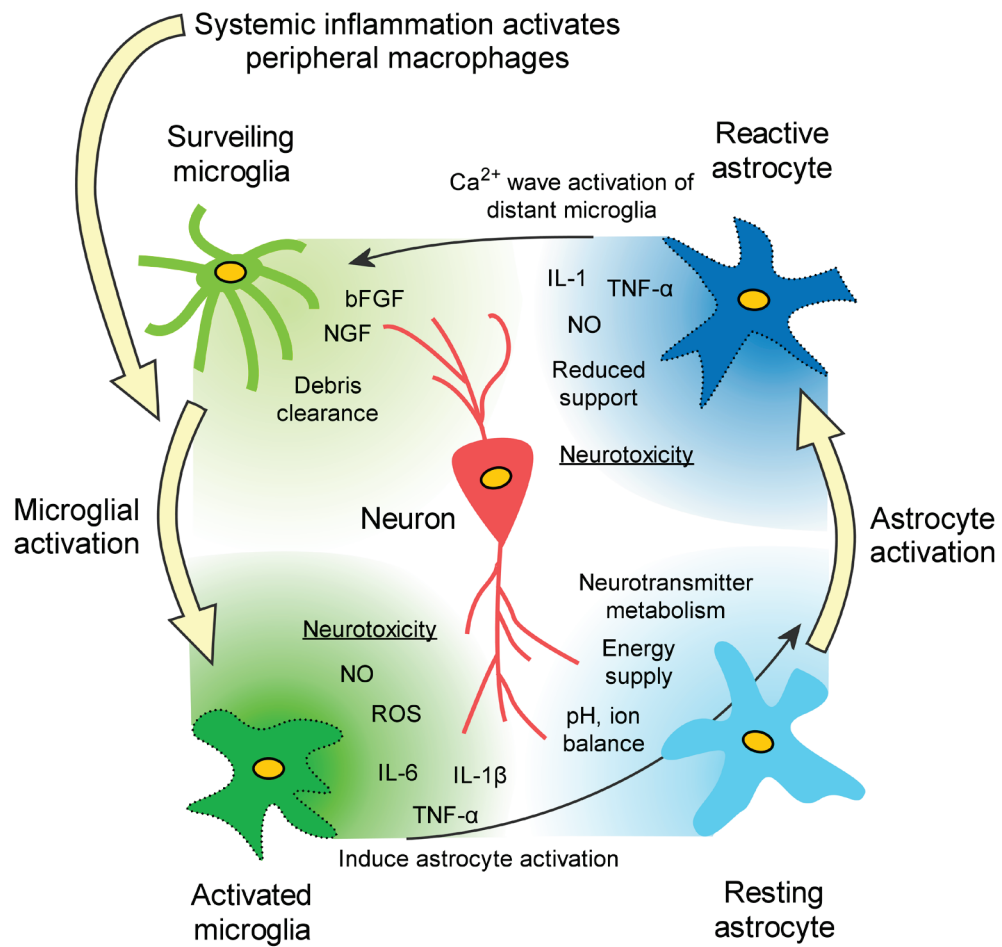
Figure 1.4 Surveilling and activated microglia.

Immunolabeling for Iba1 (green) in mouse hippocampus CA1 shows microglia in a resting (A) and activated (B) state following a single intraperitoneal injection of LPS. Scale bar is 20μm.
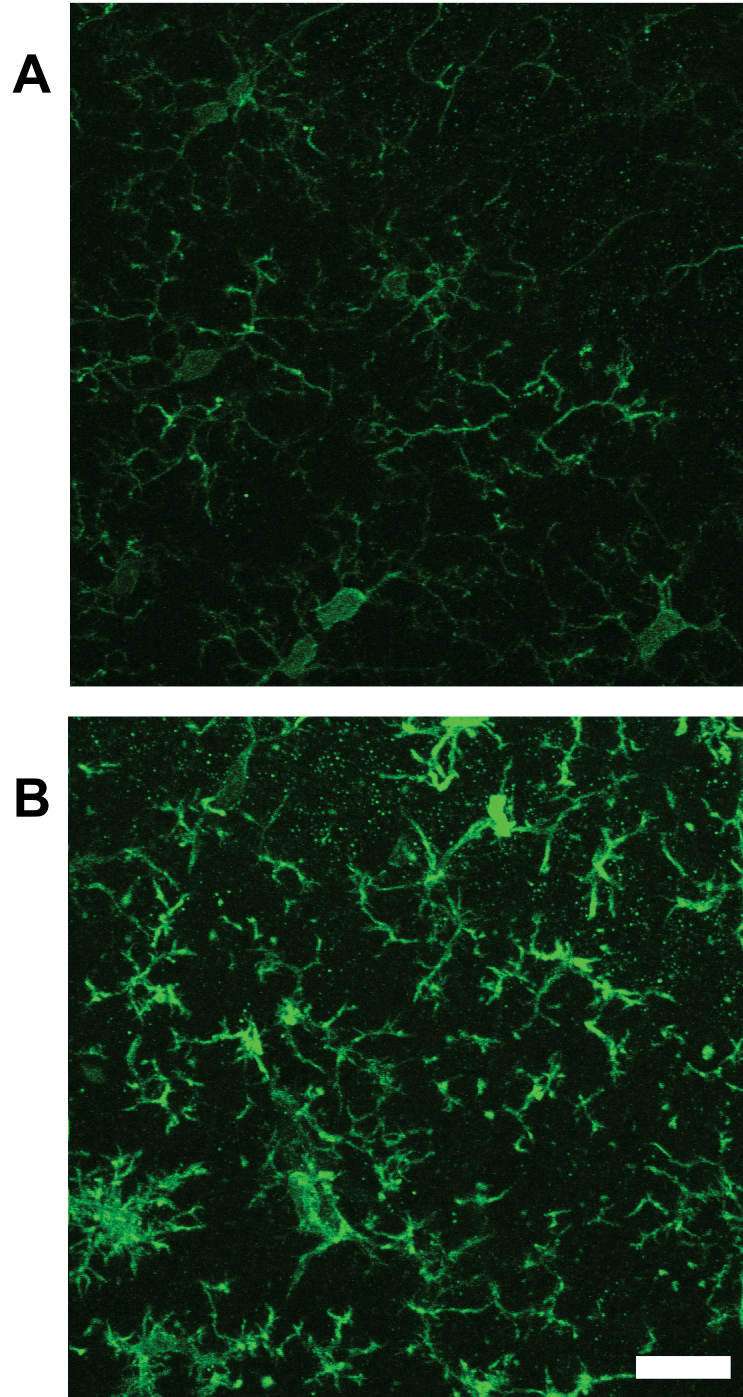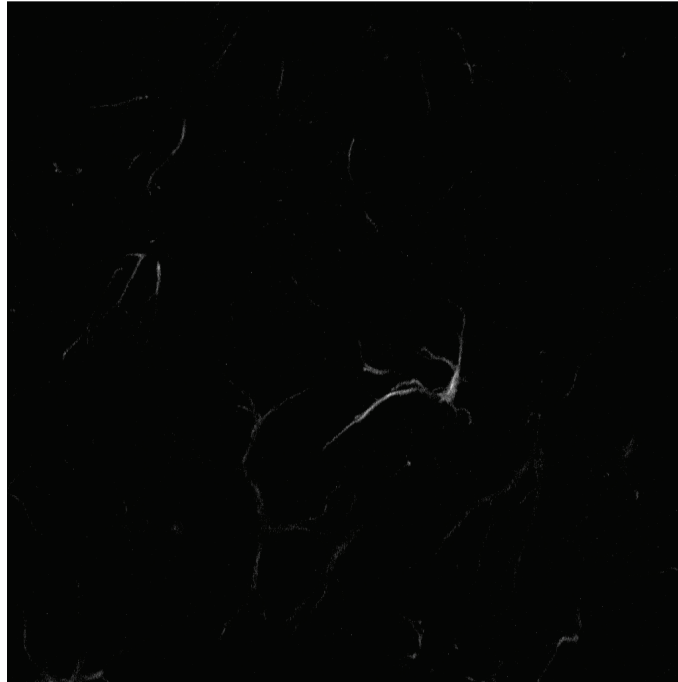
Figure 1.4

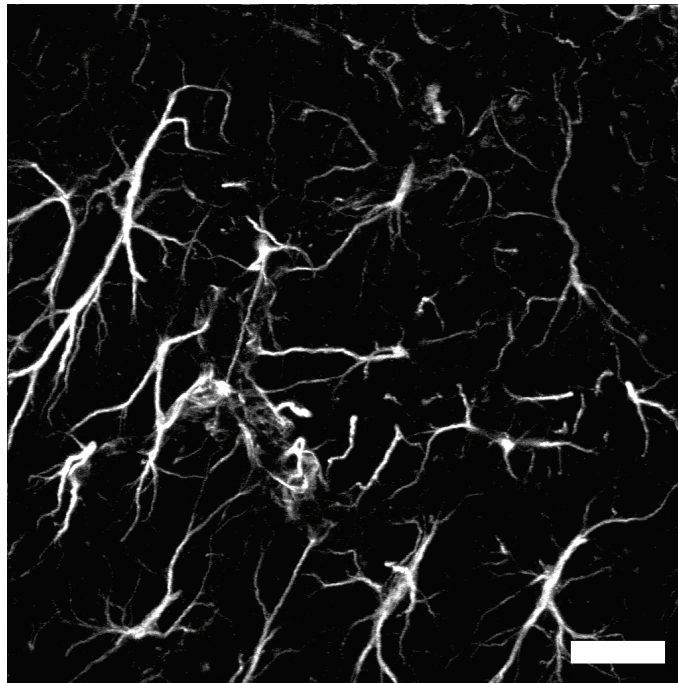**Figure 1.5 Resting and reactive astrocytes.**

Immunolabeling for GFAP (white) in mouse hippocampus CA1 shows astrocytes in a resting (A) and activated (B) state following a single intraperitoneal injection of LPS. Scale bar is 20μm.

Figure 1.5

Chapter 2 - Dendritic Spine and Synaptic Development in Healthy,

Unmanipulated Wild Type Mice.

Abstract

Synapses are points of contact between neurons which possess the membrane specializations and the cellular machinery necessary for mediating communication of electrical signals from one neuron to another. Two primary types of synapses can be distinguished, excitatory and inhibitory. The majority of excitatory presynaptic terminals make contact with small protrusions in the dendrite membrane termed dendritic spines. Dendritic spines vary in shape and size, which correlate with the strength of the synapse they form and possibly the level of electrical coupling with the dendritic shaft. Excitatory and inhibitory terminals can be differentiated with the use of protein expressed specifically at each synapse type. Synapses as well as dendritic spines form by the processes of synaptogenesis and spinogenesis, which are most exuberant during postnatal development, but remain active throughout adulthood. In this part of the investigation, synapto- and spinogenesis were studied in the CA1 region of mouse hippocampi. Using a combination of DiOlistic labeling, excitatory, inhibitory and general presynaptic terminal markers the progress of synaptogenesis from early postnatal period to adulthood was characterized. Synapse and spine numbers were shown to increase throughout the period of postnatal development into maturity.

Introduction

Hippocampus, with its highly structured laminar organization, easily identifiable cell types and a relatively simple set of circuits, lends itself to research of neural physiology, making it one of the most studied structures in the vertebrate and in particular, mammalian brain (Andersen et al., 2007). Being a brain region important to memory formation with easily manipulated circuits it has been widely used as a model of several forms of synaptic plasticity (Macdonald et al., 2006; Morris, 2006; Shapiro, 2001). These and other features make hippocampus a prime target for neuroanatomical studies, including morphometric investigation of dendritic spines. Dendritic spines are minute protrusions of dendritic membrane, reaching about 4μm in length. They are the postsynaptic sites for most of excitatory synaptic inputs in the mammalian brain (Grey, 1959) whose elongated shapes and bulbous heads compartmentalize postsynaptic signaling events, isolating them to individual synapses (Nimchinksy et al., 2002). Dendritic spines can assume a range of shapes and sizes, where longer spines with smaller heads are typically newer and less stable, while shorter spines with larger heads, more mature and stable (Ethell and Pasquale, 2005). Newly formed spines are very motile and changes in their shape can be observed on the order of minutes. On the contrary, mature spines can persist for months with only small changes to their geometry. Dendritic spine morphology and its dynamic nature have functional correlates in synaptic plasticity. It is believed that transient and persistent changes in synaptic strength that are associated with processes of learning of memory are in part accomplished by alterations to spine morphology (Bourne and Harris, 2007).

Dendritic spine development appears to occur by several mechanisms. The primary mode of spine formation is thought to take place via filopodial precursors, which

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much like spines are elongated protrusions, although much longer and more motile (Ziv and Smith, 1996). Filopodium rises out of a dendritic shaft to make contact with an axon and establish a functional synapse. Spine synapses may also be initiated by axonal terminals, with a shaft synapse preceding spine morphogenesis, or by pre- and postsynaptic structures appearing independently (Yuste and Bonhoeffer, 2004). Limited studies of rat and mouse brain development show that distinct brain regions may exhibit different combinations of mechanisms and different timelines of spinogenesis. As a whole, synaptogenesis is a complex process of which most of our knowledge has been obtained from neuromuscular junction and cell culture studies. In the vertebrate CNS axon growth cones extend to their targets with the help of guidance cues and through bidirectional communication with the target cell and activity dependent and independent mechanisms, synaptic specializations are established. Initially the connectivity is excessive and for some synapses, incorrect (Cohen-Cory, 2002). Through the process of synaptic pruning neuronal connectivity is refined and subsequently stabilized, while retaining a level of plasticity. Furthermore, only recently have we begun to appreciate that synaptogenesis is not a neuron-exclusive affair and that it relies on a contribution of glia (Bolton and Eroglu, 2009; Paolicelli et al., 2011).

In the hippocampal field CA1 stratum radiatum, dendritic spines of pyramidal neurons accept excitatory input from CA3 pyramidal neurons via Schaffer collaterals. Inhibitory input is received from local inhibitory interneurons synapsing onto dendritic shafts. Excitatory and inhibitory presynaptic terminals can be differentiated by immunodetection of proteins associated with specific terminals and thus with inhibitory or excitatory transmission. Synaptic development of CA1 pyramidal neurons was characterized at four timepoints, including postnatal day 8, 15, 22 and 3 months.

Dendritic spine density and morphology as well as densities of excitatory and inhibitory presynaptic terminals were used as indicators of synaptic development.

Materials and methods

<u>Mice</u>

C57Bl/6J mice were housed in an AAALAC accredited facility under a 12 hour light/dark cycle. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

<u>Intracardial perfusion</u>

Mice were anesthetized with Isoflurane (Phoenix, St. Joseph, MO) as 2%-5% vapor in pure oxygen, depending on mouse age (P8 – 5%, P15 – 3.5%, P22 – 3%, 3mo – 2%). Isoflurane vapor was produced by VSS vaporizer 32F (VSS, Rockmart, GA) at a constant 3.5 l/min. Mice were perfused first with ice cold PBS for 45 min., followed by ice cold 4% paraformaldehyde in PBS for 45 min. Mouse brains were extracted and post-fixed before further processing.

<u>Dendritic spine labeling, image acquisition and analysis</u>

Brains from perfused animals were post-fixed for 3h and sectioned into 250µm thick coronal slices using a Leica VT1000P vibrating blade microtome (Vibratome, Bannockburn, IL). Labeling of hippocampal neurons in brain slices was achieved using

DiOlistic approach (Wu et al., 2004). Tungsten particles coated with fluorescent lipophilic dye DiI (Invitrogen, Carlsbad, CA) were delivered by helium-powered ejection using the Helios gene gun system (Biorad, Hercules, CA) into brain slices. Labeled neurons were imaged using a confocal laser scanning microscope model LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY). 20-30 DiI-labeled CA1 hippocampal neurons were randomly selected for each group and dendrites in stratum radiatum (CA1) were imaged using a 63x water immersion objective (1.2 NA), 1x zoom at 0.3μm step intervals (z-stack). Series of 50-100 high-resolution optical sections (1024x1024 pixel format) were taken at 0.5-μm interval. Three-dimensional fluorescent images were created by maximum intensity projection of optical serial sections and encoded for blind analysis. The dendritic spine lengths and head sizes were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Quantification of the number and lengths of dendritic spines, as well as size of dendritic spine heads were performed as previously described (Henkemeyer et al., 2003; Shi and Ethell, 2006; Bilousova et al., 2009). Briefly, length of dendritic spines was measured from the point of attachment to the dendritic shaft to the tip of the spine. Spine head area was measured by selecting an outline of the spine head and measuring the area contained by the outline. To obtain dendritic spine density, lengths of dendrites containing the spines was measured. Statistical differences between age groups were compared by Student's *t*-test.

Immunolabeling of presynaptic terminals and image acqusition

Brains from perfused animals were post-fixed for overnight and sectioned into 100μm thick coronal slices using a Leica VT1000P vibrating blade microtome (Vibratome, Bannockburn, IL). Brain slices were permeabilized/blocked in PBS containing 10%

normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 0.3% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) for 3h at room temperature. Next, slices were incubated with primary antibodies, in PBS containing 10% donkey serum, and 0.1% Triton X-100 overnight at 4°C. The slices were then washed 3 times in a PBS solution containing 0.5% Tween 20 (Fisher Scientific, Fair Lawn, NJ) for 20min at room temperature, followed by incubation with secondary antibodies in PBS solution with 0.5% Tween 20 for 3h at room temperature. The slices were washed again 3 times in PBS solution with 0.5% Tween 20 for 20min at room temperature followed by a single 20min wash in PBS. The slices were mounted in Vectashield with DAPI, to visualize nuclei. Images were acquired using a confocal laser scanning microscope model LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY) and a 100x oil immersion objective (1.3 NA) as series of 10-30 high-resolution optical sections (1024x1024 pixel format) taken at 1μm interval. All images were acquired at the stratum radiatum layer of the hippocampal area CA1.

Primary antibodies: rabbit anti-vGlut1 (48-2400, Invitrogen, Carlsbad, CA), mouse anti-GAD65 (559931, BD Pharmingen, San Diego, CA), rabbit anti-Synapsin 1 (106 103, Synaptic Systems, Goettingen, Germany). Secondary antibodies: Alexa Fluor 488 donkey anti-mouse IgG H+L (A21202, Invitrogen, Carlsbad, CA), Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Invitrogen).

Analysis of images of immunolabeled presynaptic terminals

Presynaptic terminals were immunolabeled for vGlut1, GAD65 or Synapsin 1 and images were acquired as image series of optical sections as described above. Each

series began at the slice surface and single optical sections were extracted at the constant depth of 9μm for vGlut1 and Synapsin 1 and 6μm for GAD65. Quantification of immunopositive puncta density was performed with ImageJ software (http://rsb.info.nih.gov/ij/). A threshold appropriate for each staining was applied to eliminate background and minimize noise. Low thresholds were 80, 64 and 120 for vGlut1, GAD65 and Synapsin 1, respectively. Next, particles were counted by the "analyze particles" function. In order to avoid including noise and non-specific staining in the count, a range limit of 4 to 200 pixels was applied to vGlut1 and Synapsin 1 puncta. Due to the high quality of GAD65 labeling, a range limit was not necessary. Puncta density was calculated per 100μm^2.

Results

<u>Dendritic spines increase in density and mature morphologically during postnatal development.</u> C57Bl/6J mice were examined for dendritic spine density and morphology at postnatal days 8 (P8), 15 (P15), 22 (P22) and in adult animals at 3 months of age (3mo). DiOlistic labeling in fixed coronal brain slices of perfused mice was revealed by confocal microscopy and detailed images of dendritic arbors and their spines were obtained. The area of interest where dendritic spines were quantitatively analyzed was the stratum radiatum layer in CA1 region of the hippocampus. Density, length and head area of dendritic spines were measured. At P8 (Fig. 2.1A), dendritic spines were sparse, irregularly shaped, elongated and thin. At the next time point, P15 (Fig. 2.1B), spine numbers were increased, fewer short spines were present, yet they remained elongated and thin. At P22 (Fig. 2.1C), spines were more numerous than at

P15 and prominent spine heads were apparent, although many remained long and thin. Finally, in 3 month old mice (Fig. 2.1D) spines were mostly short with large heads and most abundant of all the time points examined in the study. Quantitative analysis of spine density (Fig. 2.2A) showed a progressive density increase over time, with largest increases occurring early, most dramatically between P8 to P15, where a two-fold surge in density was observed. Dendritic spine density peaked in 3 month old animals. Dendritic spines were at their longest at P8 (Fig. 2.2B) and incurred the largest decrease in length between P8 and P15. Spine length remained steady from P15 and P22 and was decreased between P22 and 3mo, where it reached the lowest value. Similarly to dendritic spine density, spine head area gradually increased over time, with the smallest heads observed at P8 and the largest at 3mo. Spine enlargement was most prominent between P15 and P22.

Cumulative distribution graphs of dendritic spine length (Fig. 2.3A) and spine head area (Fig. 2.3B) confirm the results presented as mean values (Fig. 2.2), showing a large increase in the proportion of shorter spines from P8 to P22, unchanged distribution from P15 to P22 and another increase in the number of shorter spines from P22 to 3mo. Spine head size progressively increased over time, with the largest spike in spine head area between P15 and P22.

Density of excitatory and inhibitory synaptic terminals increases during postnatal development. Presynaptic terminal density was assessed by immunolabeling of proteins associated with presynaptic specializations. Excitatory synapses were identified by presence of the vesicular glutamate transporter 1 (vGlut1), while inhibitory synapses

were detected by their expression of glutamic acid decarboxylase 65 (GAD65). Synapsin 1 was used as a general presynaptic marker, expressed at synapses irrespective of their neurotransmitters.

Expression of vGlut1 was punctate (Fig. 2.4) and its density increased until P22. The largest upsurge occurred between P8 and P15. Puncta density remained unchanged from P22 to 3mo. GAD65 expression was also punctate, however the shapes were larger and less regular (Fig. 2.5). It also increased over time, peaking at 3mo, but followed a different pattern. The largest change was observed between P8 and P15, where it remained steady until P22 and again increased slightly by 3 months. Synapsin 1 expression resembled that of vGlut1 (Fig 2.6) and steadily and regularly increased from P8 to 3mo as determined by puncta density.

Discussion

The investigation of the synaptic development of hippocampal pyramidal neurons of CA1 stratum radiatum shows a steady addition of new dendritic spines over the period of the first three postnatal weeks and into early adulthood. Similarly to data obtained in rat CA1, the density of dendritic spines roughly doubled between P15 and adulthood (von Bohlen und Halbach, 2009). At the first examined time point (P8), the spines were already present, which is in agreement with prior observations in rat neocortex, where spines first began to form in the middle of the first postnatal week. It is possible that the maximum spine density was attained between P22 and 3 months of age, given that in the rat neocortex spine numbers progressively increase over the first month of life and

are subsequently reduced (Miller, 1988). Establishing equivalencies in brain developmental stages between species is an area of extensive study and while there are several models for embryonic and fetal development (Clancy et al., 2007), drawing postnatal comparisons is more difficult. Nevertheless, similarly to mouse hippocampal pyramidal neurons, dendritic spine density on pyramidal neurons in human neocortex steadily increases from birth until puberty, where it peaks and begins to decline (Petanjek et al., 2011). It is important to consider that mice, rats and humans are among the species whose spinogenesis occurs after birth. This is in contrast to the guinea pig, which is born with highly developed brain and mature spines (Schüz, 1981). Therefore, while neuronal activity is important to spine development and maintenance (Yuste and Bonhoeffer, 2004), spine formation also follows a developmental program. This is further supported by the observation that during the course of spine formation the offset between individuals of the same species is less than one day (Jacobson, 1991)

The observed changes in spine morphology followed classical indicators of spine maturation (Ethell and Pasquale, 2005), as the spines progressively reduced their length and increased their head sizes, here measured as head areas. Interestingly, while the spine heads grew until P22 where they achieved near adult size, spine length, which was reduced from birth, was stable from P15 to P22 and further decreased between P22 and adulthood. This may indicate that while these two parameters are closely associated, they are not fully coupled. One observation that did not render itself to quantitative study was the irregularity of shapes of spines in young animals that became more regular and organized in adults. Additionally, dendritic shafts of P8 mice were had highly variable widths, in contrast to older animals. It is possible that some of the thicker areas of dendrites were in fact sites of shaft synapses, about to give rise to spines. In

terms of mechanisms of spine formation regarding the contributions of pre- and postsynaptic sites to spine formation, the study did not differentiate between the various models of spinogenesis.

Synaptic development was also investigated presynaptically, using general, excitatory and inhibitory markers of presynaptic terminals. Density of immunopositive puncta was obtained by unbiased software analysis. Given the magnification and resolution of immunolabeling images, each punctum corresponded to a presynaptic terminal. Excitatory synapses were quantified by vGlut1 density and exhibited an increase from birth to P22, at which point they attained adult levels. Since most excitatory synapses terminate on dendritic spines, one would expect the progression of the development of excitatory presynaptic terminals and dendritic spines to be correlated. Indeed, developmental vGlut1 puncta density increase closely follows spine density increase, but not exactly. This is most likely due to the fact that vGlut1 does not account for the entirety of excitatory synapses in CA1 stratum radiatum and neither do dendritic spines. Studies investigating vGlut1 expression and specifically postnatal changes in the mouse hippocampus have been qualitative and without the focus on development of presynaptic terminals (Nakamura et al., 2005).

GAD65 was used to quantify formation of inhibitory presynaptic terminals and similarly to excitatory synapses, a large increase in GABAergic synapses was observed during the first two weeks of life. The level of synapse density was maintained in the third postnatal week and experienced another increase between P22 and adulthood. It must be noted that until P10 GABA has depolarizing effects, when chloride reversal potential decreases below membrane potential (Sipila et al., 2005). Therefore at P7, GAD65 positive terminals are still excitatory. Prior studies of GABAergic synaptogenesis

in the rodent hippocampus made similar observations in regard to increase of synapse density (Rozenberg et al., 1989; Seress and Ribak, 1988). Direct comparisons with other investigations of GAD65 expression in development cannot be made, as they have not been performed at the level of a synapse for developing mouse hippocampus. However, in other model systems, postnatal GAD65 expression has been shown to be tightly coordinated to synapse formation and similarly to results of this study, gradual increases of GAD65 positive puncta have been observed (Greif et al., 1991; Guo et al., 1997).

Synapsin 1 is a membrane protein associated with synaptic vesicles, expressed at over 90% of synapses in the brain and as such it has been used in this and other studies as a general synaptic marker (De Camili et al., 1983). In the current study, synapsin 1 density increased in mice from P8 to 3 months of age, between every examined time point. This observation is in agreement with studies of synapsin in developing rodent cerebrum, which showed increasing synapsin expression to coincide with synaptogenesis (De Camili et al., 1983; Lohmann et al., 1978).

The differential progress of addition of excitatory and inhibitory synapses and synapses in general reflects distinct developmental programs for different modes of synaptic transmission. Furthermore, while glutamate and GABA are the major neurotransmitters in the mammalian CNS, not all synapses use them. In addition, some modulatory synapses utilize neuropeptides instead of classical neurotransmitters. The close parallels between rodent and human synaptogenesis validate the mouse as an excellent model system.

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Figure 2.1 Development of dendritic spines of hippocampal pyramidal neurons in the mouse.

Representative images of dendritic spines of hippocampal pyramidal neurons in CA1 stratum radiatum in mice at P8 (A), P15 (B), P22 (C) and 3 months of age (D). Dendritic spines were labeled DiOlistically with DiI. Images are maximum intensity projections of optical sections taken at 0.3µm intervals. Scale bar is 5µm.
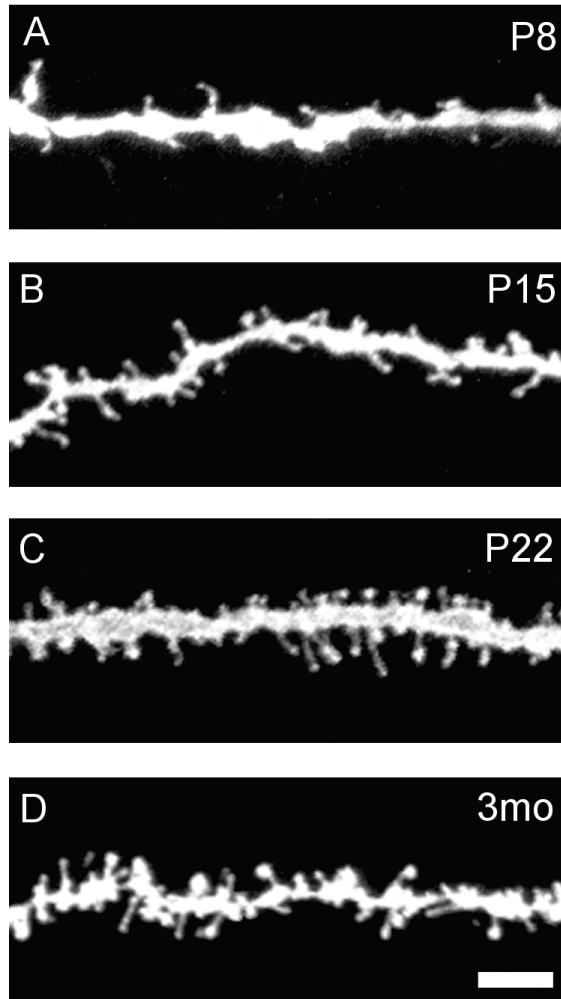
Figure 2.1

Figure 2.2 Quantitative morphometric analysis of dendritic spine development. Quantitative analysis of dendritic spine density (A), length (B) and head area (C) in P8, P15, P22 and adult 3 month old animals (3mo). P8: 3 mice, 943 spines (A,B), 768 spines (C). P15: 4 mice per condition, 6099 spines (A,B), 4630 spines (C). P22: 3 mice per condition, 5596 spines (A,B), 3894 spines (C). 3mo: 3 mice per condition, 5637 spines (A,B), 3793 spines (C). Statistical comparisons were performed using Student's *t*-test; * p<0.05; ** p<0.01; *** p<0.001. Error bars indicate S.E.M.
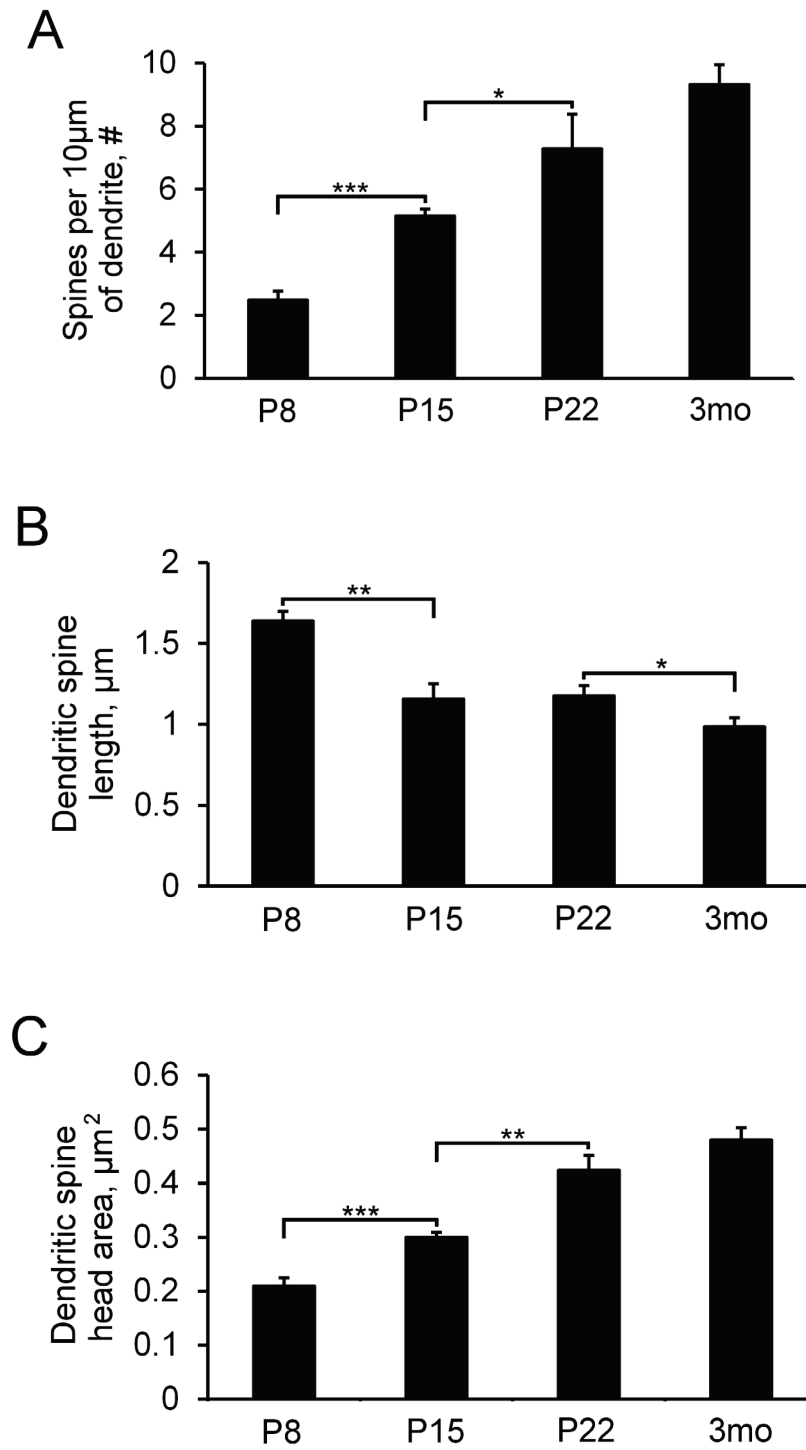
Figure 2.2

Figure 2.3 Quantitative morphometric analysis of dendritic spine development – distributions of spine measures.

Quantitative analysis of dendritic spine length (A) and dendritic spine head area (B) of P8, P15, P22 and 3 month old adult mice (3mo) represented as cumulative distributions. P8: 3 mice, 943 spines (A,B), 768 spines (C). P15: 4 mice per condition, 6099 spines (A,B), 4630 spines (C). P22: 3 mice per condition, 5596 spines (A,B), 3894 spines (C). 3mo: 3 mice per condition, 5637 spines (A,B), 3793 spines (C). Error bars are omitted for clarity.

Figure 2.3

Figure 2.4 Immunodetection of presynaptic excitatory terminals.

A subset of presynaptic excitatory terminals was immunolabeled for the vesicular glutamate transporter 1 (vGlut1) at P8 (A), P15 (B), P22 (C) and in adult 3 month old animals (3mo, D). Representative images are shown, scale bar is 20µm.
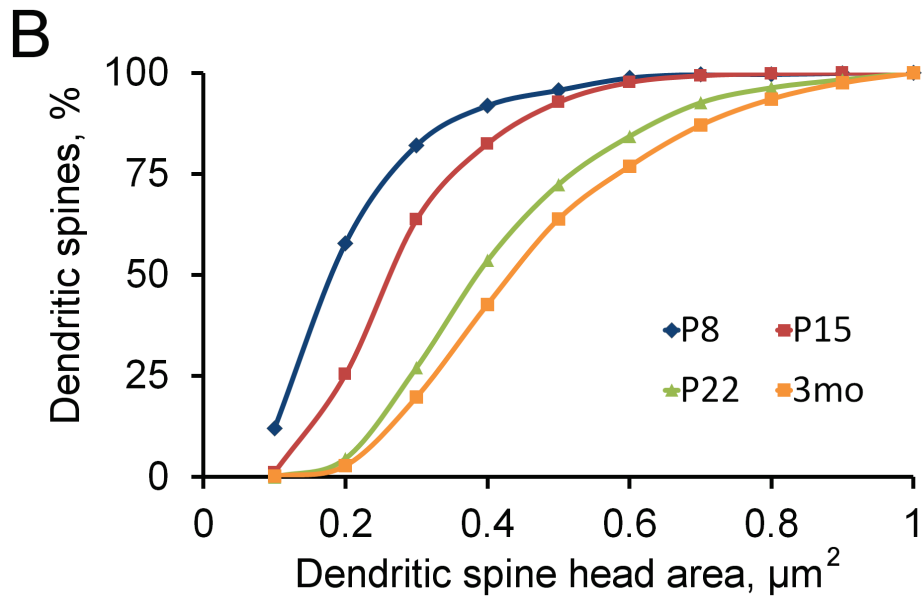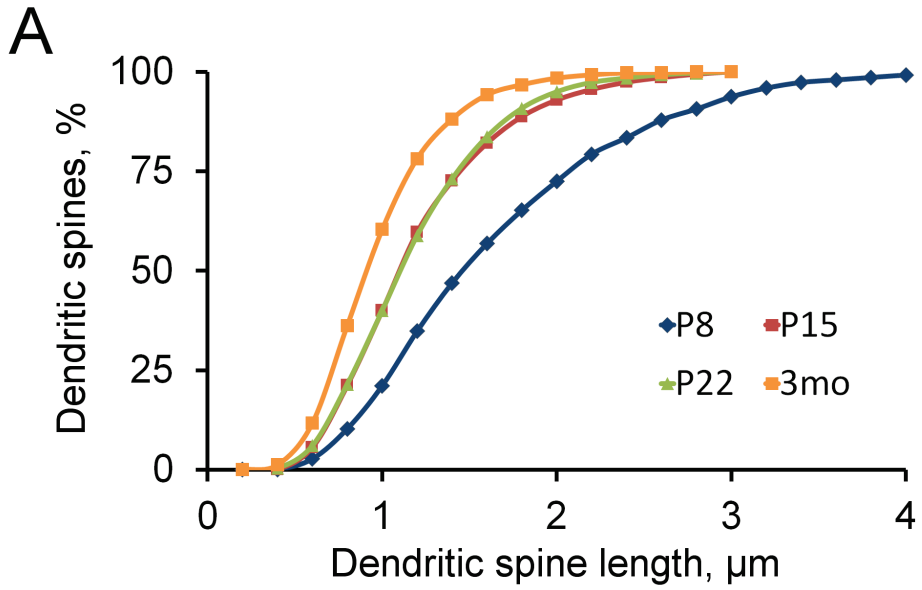
Figure 2.4

Figure 2.5 Immunodetection of presynaptic inhibitory terminals.

A subset of presynaptic inhibitory terminals was immunolabeled for the glutamic acid decarboxylase 65 (GAD65) at P8 (A), P15 (B), P22 (C) and in adult 3 month old animals (3mo, D). Representative images are shown, scale bar is 20μm.
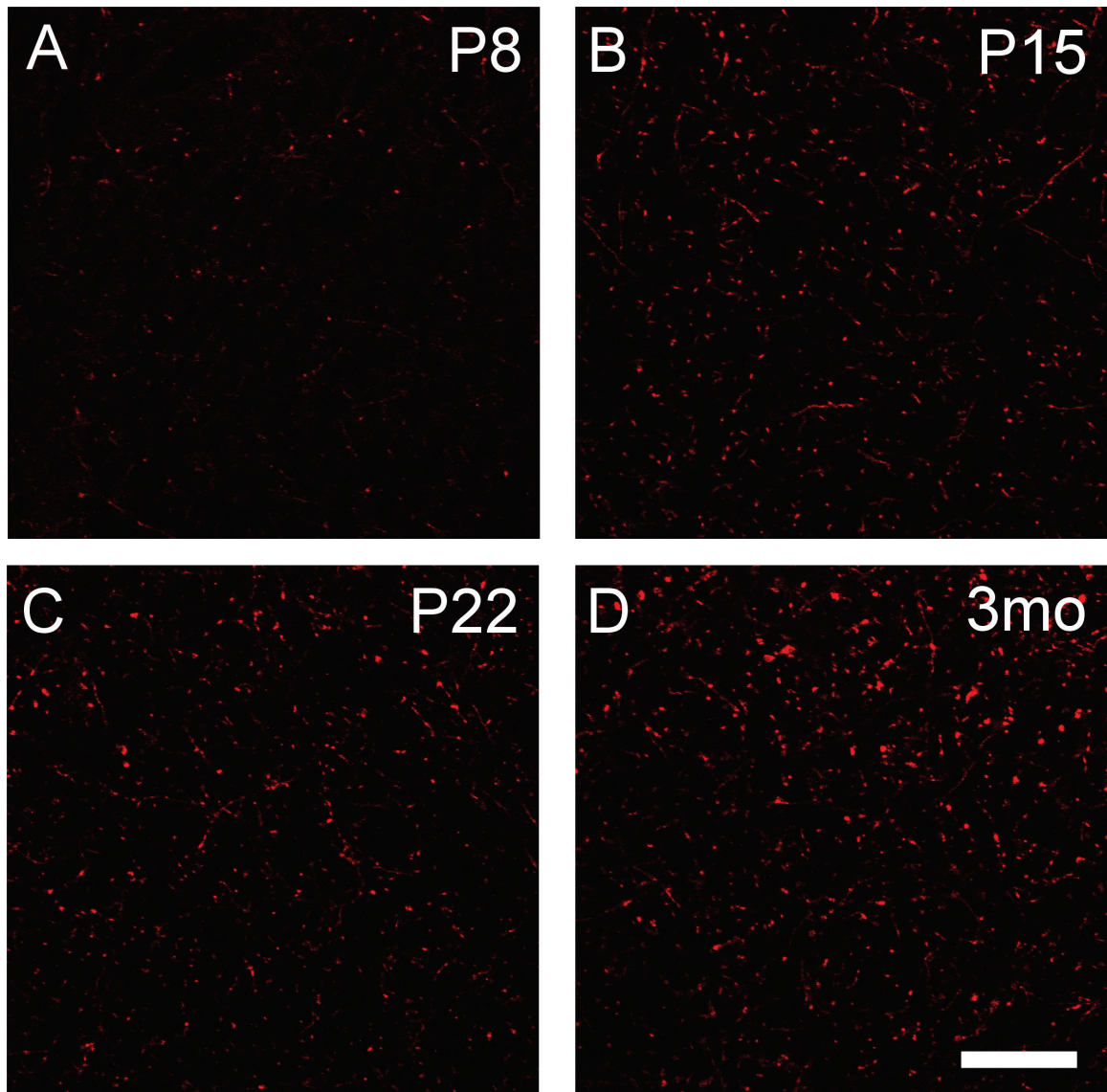
Figure 2.5

Figure 2.6 Immunodetection of presynaptic terminals.

A subset of excitatory and inhibitory presynaptic terminals was immunolabeled for Synapsin 1 at P8 (A), P15 (B), P22 (C) and in adult 3 month old animals (3mo, D). Representative images are shown, scale bar is 20µm.
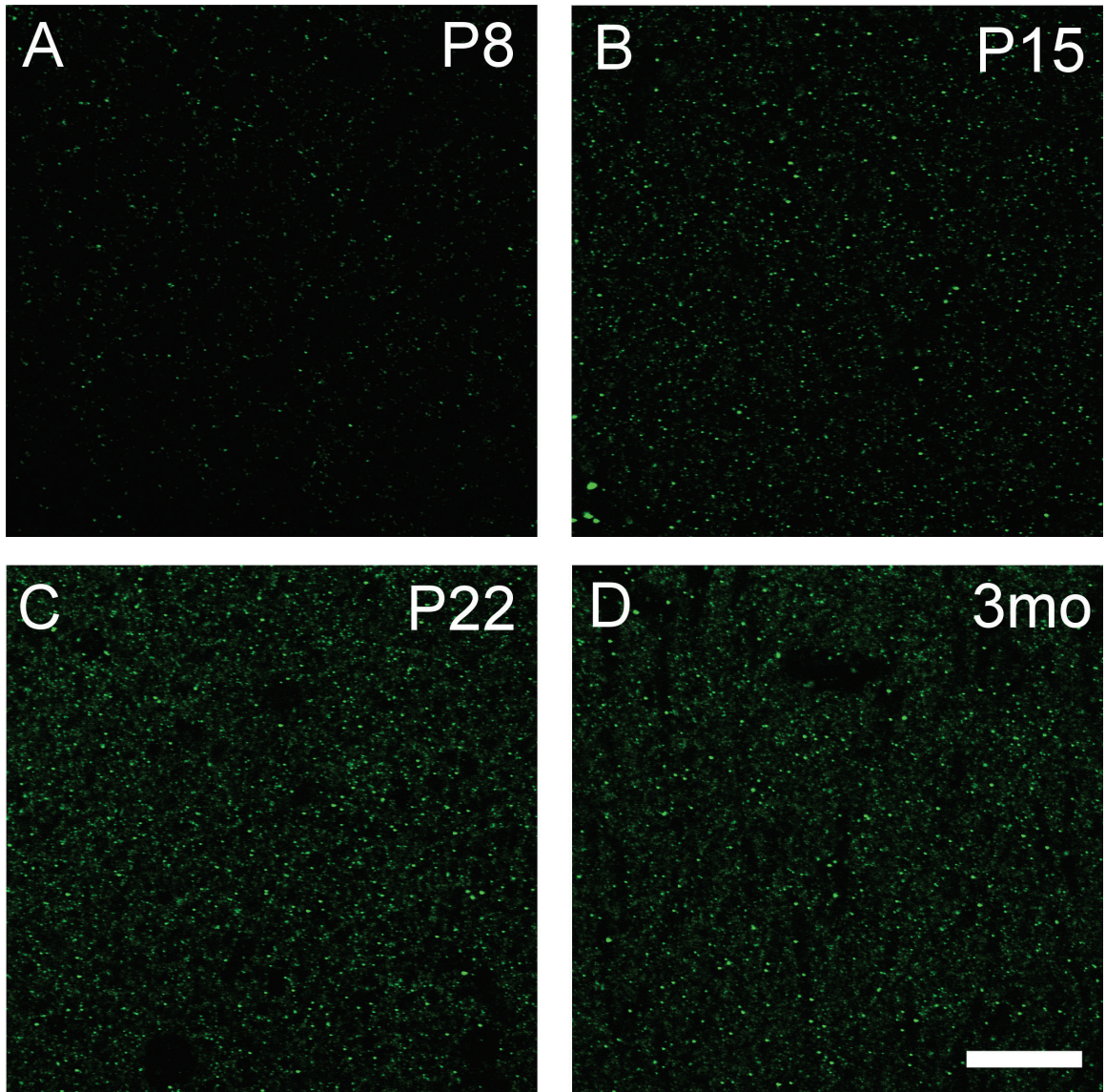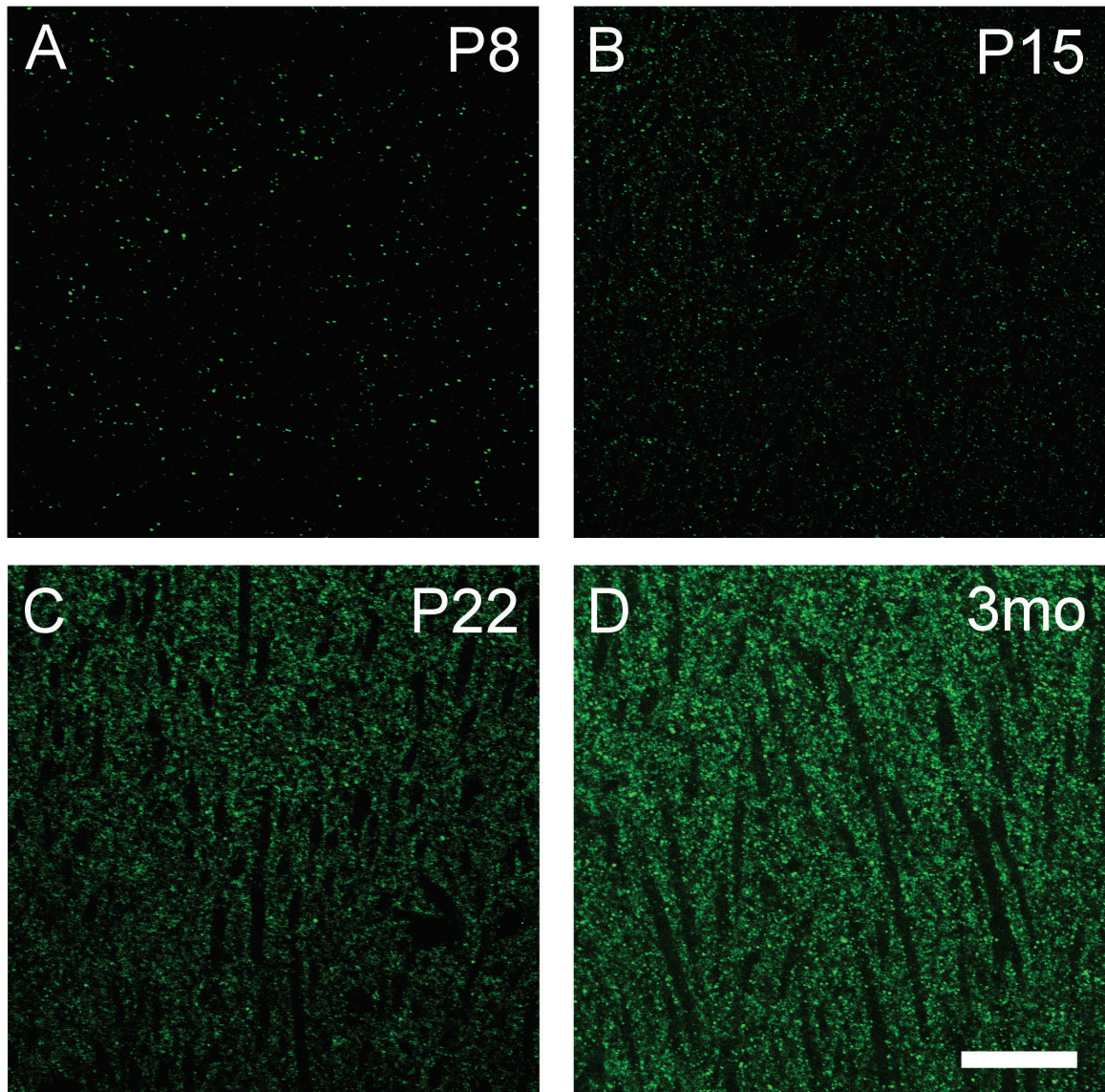
Figure 2.6

Figure 2.7 Quantification of presynaptic terminal immunolabeling.

Immunolabeling of presynaptic terminals for vGlut1 (A), GAD65 (B) and Synapsin 1 (C) was quantified by assessing the density of immunoreactive puncta per 100 μm^2. Analysis was performed in healthy, unmanipulated mice at P8, P15, P22 and in adult 3 month old animals (3mo). vGlut1: P8: 3 mice, 60 images; P15: 3 mice, 40 images; P22: 3 mice, 60 images, 3mo: 3 mice, 60 images. GAD65: P8: 3 mice, 60 images; P15: 3 mice, 60 images; P22: 2 mice, 40 images, 3mo: 3 mice, 60 images. Synapsin 1: P8: 3 mice, 60 images; P15: 3 mice, 60 images; P22: 2 mice, 40 images, 3mo: 3 mice, 60 images. Statistical comparisons were performed using Student's *t*-test; * p<0.05; ** p<0.01; *** p<0.001. Error bars indicate S.E.M.

Figure 2.7
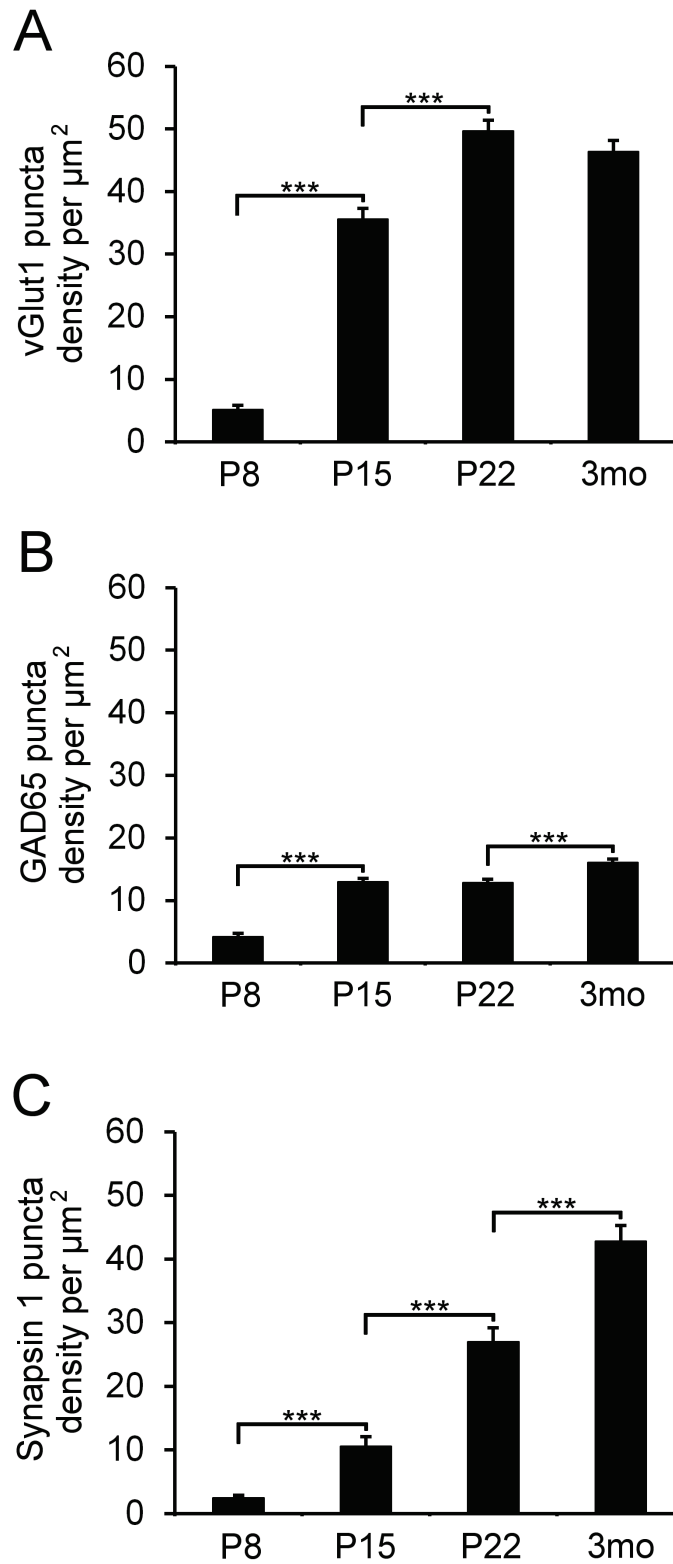
Chapter 3 - Effects of Systemic Inflammation on Synaptic Development In Mouse Hippocampus.

Abstract

Neurons comprise only one tenth of the entire cell population in the CNS. The rest of the cells are glial cells which serve to create a suitable environment for the neurons and ensure their proper function. Neurons in comparison to other cell types are very fragile; their extensive arbors of processes and high energy requirements make them vulnerable to even small changes in the condition of their milieu. Moreover, neurons have a very limited ability to regenerate, making their loss essentially permanent. Glial cells have capability to defend the CNS from invading pathogens, though by their very nature these defensive mechanisms are cytotoxic, therefore also harmful to neurons. Microglia, the only resident immune cells within the brain parenchyma, are capable of powerful inflammatory responses and can recruit astrocytes to contribute to inflammation. This can be detrimental to neurons, despite being aimed at their protection. In this study, systemic inflammation was induced peripherally in order to elicit glial activation. Then, the effects on synapse and spine formation in the developing postnatal mouse brain were investigated. We found that there exists a period of vulnerability of the process of synaptogenesis to the influences of inflammation.

Introduction

CNS is a so called "immune privileged site", due to its inflammatory responses significantly differing from those in the periphery and being generally subdued. Brain has only a minimal capacity for regeneration since neurons are post-mitotic and cannot proliferate. Although inflammation is a defensive mechanism, in the CNS it can produce irreparable damage and exacerbate the initial insult (Lucas et al., 2006). This is not always the case however and furthermore, the resident immune cells of the CNS – the microglia – serve important roles in the healthy brain that have only recently been coming to light (Polazzi and Monti, 2010, Tremblay and Majewska, 2011).

Systemic inflammation can activate microglia by diffusion or active transport of pro-inflammatory cytokines across the BBB, in addition to other mechanisms (Banks et al., 2002; Perry, 2004). Once activated, microglia produce and secrete inflammatory molecules, many of which are neurotoxic. A model of such a scenario, which is used to investigate effects of microglial activation, utilizes a major lipid component of the outer membrane of gram-negative bacteria, LPS (Bevenridge, 2001). To mimic systemic inflammation, LPS is administered intraperitoneally (IP), or less commonly, intravenously. Although microglia are competent to recognize and respond to LPS directly, BBB permeability to LPS is very poor and it is believed that its effect is indirect and mediated by peripheral mechanisms (Nadeau and Rivest, 1999). Pathogens exhibit evolutionarily conserved motifs in their DNA, dsRNA, ssRNA, lipopeptides and proteins, known as pathogen-associated molecular patterns (PAMPs) which are recognized by the innate immune system (Janeway and Medzhitov, 2002). LPS is one of the PAMPs and it is recognized by a member of Toll-like receptors, TLR4 which is an essential receptor for LPS signaling (Hoshino et al., 1999). The signaling cascade is initiated by

LPS binding to the LPS-binding protein (LBP) and followed by the formation of a signaling complex with TLR4, cluster of differentiation 14 (CD14) and myeloid differentiation 2 receptor (MD-2), which binds the extracellular domain of TLR4 (Nagai et al., 2002; O'Neill and Bowie, 2007). Several adaptor proteins are recruited to the complex and set off a number of signaling cascades, leading to release of various inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Dinarello, 1999; Leon et al., 2008; Luheshi, 1998). In turn, these cytokines cross the BBB, activate perivascular macrophages and stimulate vagal afferents, all resulting in microglial activation and thus an amplification of inflammatory response, by additional production of cytokines, prostaglandins and reactive oxygen species. These inflammatory molecules produce toxic effects on neurons while also inducing astrocyte reactivity, reducing their normal support activities (Liu and Hong 2002; Steele and Robinson, 2011).

Peripherally administered LPS has been widely used to investigate the effects of systemic inflammation on the CNS, from molecular mechanisms to behavior. It has been observed that the consequences of LPS injections are widespread and affect multiple brain regions to varying degrees. A detrimental effect on neurons by microglia activated with peripheral LPS are well described for dopaminergic neurons of the substantia nigra. LPS activation of microglia causes persistent degeneration of dopamine neurons, possibly by a NO mediated mechanism (Czapski et al., 2007; Gibbons and Dragunow, 2006). A single IP injection of LPS can result in a chronic activation of microglia and a prolonged, progressive loss of substantia nigra neurons in wild type adult mice, demonstrating a potential significance for Parkinson's disease (Qin et al., 2007). At high doses of LPS (10mg/kg) administered to rats, simulating sepsis, apoptosis and necrosis of neurons and glia is observed throughout the brain. Furthermore, distinct regions

exhibit different degrees of cell death which correlate with the levels of glial activation with the hippocampus being most vulnerable (Semmler et al., 2005). Significantly lower doses of LPS (1mg/kg) cause ultrastructural and morphological changes in hippocampal neurons characteristic of apoptosis and necrosis (Czapski et al., 2010). A separate study demonstrated that hippocampal neurogenesis is inhibited by a systemic administration of LPS (Monje et al., 2003). This effect could potentially be explained by the finding that the levels of neurotrophins, including BDNF, NGF and neurotrophin-3 are significantly reduced following an IP LPS injection (Zhiwei and Fang, 2006). Histological studies at the level of synapses and dendritic spines are only beginning and show the potential long term effects of LPS induced systemic inflammation to dendritic spines (Kondo et al., 2011).

Given that peripherally delivered LPS can produce inflammatory response powerful enough to result in cell death, presence of functional alterations is not surprising. Rats injected intraperitoneally with LPS have been tested for changes in synaptic transmission in the hippocampus using in vivo recordings. LPS affected several forms of synaptic plasticity at the CA1-subiculum projections – blocking LTP under high-frequency stimulation, but inducing potentiation following low-frequency stimulation. It also blocked paired-pulse facilitation at short instead of long stimulus intervals (Commins et al., 2001). Similarly, LPS inhibited LTP in the perforant path – DG granule cell synapses while increasing activation of stress related protein kinase p38 and the transcription factor nuclear factor κB (NFκB).

Systemic inflammation produces a set of behavioral changes which together constitute "sickness behavior" and include anorexia, decreased movement and social interaction, increased sleep, fever, and other symptoms (Dantzer, 2001). Using the IP

LPS model, amygdala has been shown to be involved in regulation of these behaviors (Engler et al., 2001). Furthermore, LPS-induced defects in synaptic transmission and plasticity suggest that the sickness behavior may include cognitive deficits, particularly memory impairments. Several studies have confirmed this hypothesis. LPS treated adult and juvenile rats showed disrupted contextual but not auditory-cue fear conditioning, indicating a disruption in the memory consolidation processes (Pugh et al., 1998). A study in mice using a two-way active avoidance conditioning paradigm showed LPS induced both learning and performance deficits, as animals exhibited reduced association between conditioned and unconditioned stimuli (Sparkman et al., 2005). Mice also show impairments in memory consolidation and reconsolidation after LPS injections in olfaction-based fear conditioning experiments (Kranjac et al., 2012).

Although studies have been performed on effects of LPS exposure on young and developing animals, they have been largely limited to intracerebral and intravenous injections of neonatal rats (Wang et al., 2006). A small number of inquiries that did investigate the effects of LPS-induced systemic inflammation on neonates, revealed lasting changes that altered adult neuroimmune and behavioral responses (Boissé et al., 2004; Spencer et al., 2005). One study aimed at addressing synaptic and cognitive changes in LPS treated rats. IP Injections were performed at P5, P14, P30 and P77 followed by NMDA receptor subunit mRNA level measurements in adulthood. Water maze and fear conditioning tests were also performed. The rats showed age dependent susceptibility to LPS in behavioral tests at P5 and P30 concomitantly with hippocampal reduction in NR1 mRNA. Adult rats injected at P14 experienced an increase in NR2A, B, C and D subunit mRNA. Cortical NR2B, C and D mRNAs were increased in adulthood in all groups (Harré et al., 2008). A study conducted in mice LPS injected at P4 and P5

demonstrated impaired avoidance learning in adult males but not females. Furthermore, an additional LPS challenge in adulthood resulted in a reduced IL-1β transcription in males (Kohman et al., 2008).

Materials and Methods

Mice

C57Bl/6J mice were housed in an AAALAC accredited facility under a 12 hour light/dark cycle. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

Lipopolysaccharide injections

Lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) injections were performed intraperitoneally at 5mg per kg in a total volume of 100ul using a 25 gauge needle.

Intracardial perfusion

Mice were anesthetized with Isoflurane (Phoenix, St. Joseph, MO) as 2%-5% vapor in pure oxygen, depending on mouse age (P8 – 5%, P15 – 3.5%, P22 – 3%, 3mo – 2%). Isoflurane vapor was produced by VSS vaporizer 32F (VSS, Rockmart, GA) at a constant 3.5 l/min. Mice were perfused first with ice cold PBS for 45 min., followed by ice

cold 4% paraformaldehyde in PBS for 45 min. Mouse brains were extracted and post-fixed before further processing.

Immunolabeling of presynaptic terminals and image acqusition

Brains from perfused animals were post-fixed for overnight and sectioned into 100µm thick coronal slices using a Leica VT1000P vibrating blade microtome (Vibratome, Bannockburn, IL). Brain slices were permeabilized/blocked in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 0.3% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) for 3h at room temperature. Next, slices were incubated with primary antibodies in PBS containing 10% donkey serum, and 0.1% Triton X-100 overnight at 4°C for vGlut1and GAD65 antibodies. In case of Iba1, GFAP, MAP2 antibodies, PBS containing 10% goat serum (Sigma-Aldrich), 0.5% Tween 20 (Fisher Scientific) was used. The slices were then washed 3 times in a PBS solution containing 0.5% Tween 20 for 20min at room temperature, followed by incubation with secondary antibodies in PBS solution with 0.5% Tween 20 for 3h at room temperature. The slices were washed again 3 times in PBS solution with 0.5% Tween 20 for 20min at room temperature followed by a single 20min wash in PBS. The slices were mounted in Vectashield with DAPI, to visualize nuclei. vGlut1 and GAD65 Images were acquired using a confocal laser scanning microscope model LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY) and a 100x oil immersion objective (1.3 NA) as series of 10-30 high-resolution optical sections (1024x1024 pixel format) taken at 1µm interval. Iba1, GFAP and MAP2 images were acquired using a 25x water immersion objective

(0.8 NA) as series of 30 optical sections taken at 1µm interval. All images were acquired at the stratum radiatum layer of the hippocampal area CA1.

Primary antibodies: rabbit anti-VGlut1 (48-2400, Invitrogen, Carlsbad, CA), mouse anti-GAD65 (559931, BD Pharmingen, San Diego, CA), rabbit anti-Iba1 (019-19741, Wako Chemicals USA, Inc., Richmond, VA), momouse anti-GFAP-Cy3 (C9205, Sigma-Aldrich), mouse anti-MAP2 (M4403, Sigma-Aldrich). Secondary antibodies: Alexa Fluor 488 donkey anti-mouse IgG H+L (A21202, Invitrogen, Carlsbad, CA), Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Invitrogen), Alexa Fluor 660 goat anti-rabbit IgG (A-21074, Invitrogen).

Analysis of images of immunolabeled presynaptic terminals

Presynaptic terminals were immunolabeled for vGlut1, GAD65 and images were acquired as image series of optical sections as described above. Each series began at the slice surface and single optical sections were extracted at the constant depth of 9µm for vGlut1 and Synapsin 1 and 6µm for GAD65. Quantification of immunopositive puncta density was performed with ImageJ software (http://rsb.info.nih.gov/ij/). A threshold appropriate for each staining was applied to eliminate background and minimize noise. Low thresholds were 80 and 64 for vGlut1 and GAD65, respectively. Next, particles were counted by the "analyze particles" function. In order to avoid including noise and non-specific staining in the count, a range limit of 4 to 200 pixels was applied to vGlut1. Due to the high quality of GAD65 labeling, a range limit was not necessary. Puncta density was calculated per 100µm^2.

Results

<u>Microglia exhibit ameboid morphology in young mice and ramified morphology in</u>

<u>adult mice.</u> Immunolabeling for Iba1, a microglia/macrophage specific protein has been

used to visualize microglial cells within the CA1 stratum radiatum of the hippocampus

(Fig. 3.1, 3.2) Microglia of healthy, unmanipulated, 8 days old C57Bl/6J wild type mice

display ameboid morphology, indicating an activated, inflammatory state (Fig. 3.1A, I;

3.2A, I) as their processes withdraw and thicken. This initial activation resolves within 2

weeks and microglia at postnatal day 22 (Fig. 3.1C, M; 3.2C, M) resemble those of adult,

3 month old animals (Fig. 3.1D, O; 3.2D, O). Microglia of healthy adult mice are highly

ramified, their processes are wispy and extend away from cell body. At P15, the shape

of microglia shows a residual activation, with processes slightly shorter and thicker than

in basal, non-activated state (Fig. 3.1B, K; 3.2B, K).

<u>Lipopolysaccharide induced inflammation results in age-dependent microglial and</u>

<u>astrocytic activation.</u> A single intraperitoneal injection of LPS produces observable

changes 24h after the injection in the morphology of microglia and astrocytes, specific to

the age of the animal. At P8, microglia, which are already displaying activated

morphology, become even more bulky and few thin processes remain (Fig. 3.1A, E, I, J;

3.2A, E, I, J). Higher amounts of apparent Iba1 immunoreactivity can also be observed.

Changes resulting from LPS injections are most dramatic at P15, where the nearly basal

microglial morphology shifts to an activated state, characterized by large, thick

processes (Fig. 3.1B, F, K, L; 3.2B, F, K, L). At P22, LPS induced inflammation also induces an activated morphology, although to a much lesser extent than at P15 (Fig. 3.1C,G,M,N; 3.2C,G,M,N). Microglial response in 3 month old adult animals was subdued compared to other time points with very small changes in cell shape (Fig. 3.1D,H,O,P; 3.2D,H,O,P). Brain sections were also stained for GFAP, which is a protein that is associated with intermediate filaments, primarily expressed in astrocytes and in the stratum radiatum layer of CA1 it can be used to identify astrocytes specifically. GFAP expression was upregulated under inflammatory conditions brought about LPS treatment, at all the examined time points (Fig. 3.1A-H). Distribution of astrocytes was observed to be non-uniform along the longitudinal axis and astrocytes tended to cluster around vasculature, with large blood vessels being associated with higher numbers of astrocytes. LPS injections also resulted in GFAP cytoskeleton becoming biased towards largest blood vessels, seemingly enwrapping them (data not shown).

Dendritic spine density in P15 mice is reduced acutely by a single LPS injection.
Coronal mouse brain sections prepared from wild type animals 24h after an intraperitoneal LPS injection were labeled DiOlistically and morphometric analysis of dendritic spines was performed. Dendritic spine density, length and head area were examined at P8 (P7+1d), P15 (P14+1d), P22 (P21+1d) and in 3 month old adults (3mo+1d) (Fig. 3.3A-H). Out of all time points that were investigated only P15 mice reacted to LPS induced systemic inflammation and a significant reduction in their dendritic spine density was observed (Fig .3.4A). Average spine length (Fig. 3.4B) and spine head area (Fig. 3.4C) were unaffected by LPS. Cumulative distributions of these

measures showed no differences across the ranges of spine length (Fig 3.5) and head size (Fig. 3.6).

LPS treatment causes changes in excitatory and inhibitory inputs. Presynaptic terminals in stratum radiatum CA1 of excitatory and inhibitory synapses were immunolabeled for vGlut1 and GAD65, respectively. Wild type mice were injected at P14 and sacrificed 24h (P14+1d) or 8 days (P14+8d) after the injection. Immunostaining revealed a punctuate pattern (Fig. 3.7) which was quantified to obtain puncta density corresponding to presynaptic structures of excitatory and inhibitory inputs. In P15 animals (P14+1d) vGlut1 puncta density was significantly lower in LPS injected mice as compared to control animals (Fig. 3.8A). Mice which were examined 8 days after the LPS injection showed an even larger difference between injected and non-injected animals. Injected P22 mice (P14+8d) had vGlut1 puncta density comparable to those of non-injected P15 mice, while the vGlut1 puncta density in P22 unmanipulated mice was significantly higher. In contrast to vGlut1, GAD65 puncta density was higher in LPS treated animals 24h after the injection. P22 mice injected 8 days earlier did not show a difference in GAD65 puncta density in comparison of LPS injected and non-injected conditions (Fig 3.8B).

Discussion

Systemic inflammation induced in mice by peripheral injections of LPS has been demonstrated in multiple studies to exert detrimental effects varying from cell death to impaired synaptic plasticity and cognitive deficits, depending on LPS dosage (Commins et al., 2001; Semmler et al., 2005; Sparkman et al., 2005). These effects are presumably due to microglial and astrocytic neurotoxicity (Garden and Möller, 2006) and reduction of support to neurons by reactive astrocytes (Steele and Robinson, 2011). In the present study, the effects of LPS-induced systemic inflammation on postnatal neuronal development in mice were investigated.

Glial immunodetection was performed by targeting Iba1 and GFAP to visualize microglia and astrocytes respectively. Microglia in P8 mice exhibited intermediate morphology between amoeboid and ramified. At later time points they became increasingly more ramified. This conversion in morphology is a normal developmental process, as microglial precursors that colonize the brain have amoeboid morphology (Davis et al., 1994). It is possible that it is a reflection a functional state other than simply migration. LPS injections had different effects 24h after injection at different ages of mice on cell shape and possibly Iba1 expression. At P8 microglia in the control animals have activated morphology and systemic inflammation further reduces their processes. The effect of LPS is most dramatic at P15, when microglia are becoming ramified and are reverted into amoeboid shape as a result of the LPS treatment. P22 mice also respond to the injections, although not to the same degree. In adult animals microglial response was least pronounced. It appears that inflammatory responses are developmentally regulated and depend on the basal microglial state at the particular age. In terms of Iba1 expression, it has been shown that in activated microglia Iba1 may become upregulated

(Imai and Kohsaka, 2002). This is also possibly occurring in the injected mice in the present study, however due to alterations in cell shape, Iba1 distribution also changes, producing a different signal pattern, which affects its apparent strength. Astrocyte reactivity in response to inflammation was also observed as increases in GFAP expression at all ages. Morphological changes were more subtle and most visible around large blood vessels, where astrocytic processes appeared to reorient themselves towards the vasculature. Astrocyte activation likely occurred in response to microglial activation, as multiple studies have shown that microglia become activated first and promote astrocytic activation (Liu et al., 2011).

Dendritic spines were affected by systemic inflammation 24h after injection of LPS only in P15 mice, where a slight reduction in spine density was observed. Spine morphology, on the other hand, did not change, similarly to mice at other ages. P15 animals also exhibited a decrease in excitatory presynaptic terminals compared to controls, as measured by vGlut1 puncta density. Furthermore, P22 mice, injected 8 days earlier at P14, had vGlut1 puncta density at levels of P15 control mice, while the control P22 mice gained synapses over this developmental period. This suggests that mice experiencing a systemic inflammation were unable to form new presynaptic terminals and therefore functional synapses. These effects are most likely due to neurotoxic effects of activated microglia and astrocytes, although the mechanism and the events that take place are not clear. One possibility is that microglial and astrocytic activation exerts cytotoxic effects on neurons (Bruce-Keller, 1999; Garden and Möller 2006; Liu et al., 2011) with detrimental influences on synapse formation that could be compounded by astrocytes neglecting their supportive duties (Steele and Robinson, 2011). New spines are inherently less stable (Bhatt et al., 2009), hence newly formed synapses

could be more sensitive to inflammatory conditions in their environment. Alternatively, these synaptic effects of inflammation could be explained by anomalies in synaptic pruning, in which microglia are known to participate (Paolicelli et al., 2011; Stevens et al., 2007). A less likely possibility, although one that ought to be mentioned, is a pathological elimination of new synapses by a process of synaptic apoptosis (Mattson MP et al., 1998). The effects of LPS-induced inflammation on GAD65-positive inhibitory synapses are intriguing. 24h after LPS injection there was a small increase in GAD65-positive inhibitory terminals in P15 mice. However, in P22 mice, 8 days after the injection administered at P14, this change was absent. This result indicates a possible sensitivity of excitatory terminals compared to inhibitory ones, a microglial selectivity for excitatory terminals, or both. Since in the period from P15 to P22 no new inhibitory terminals were added as a part of normal development, it is not known whether a systemic inflammation could affect the development of inhibitory synapses. The transient increase in inhibitory synapses in P15 mice injected at P14 could indicate an attempt to drive up overall inhibition which is in agreement with the decrease in the number of excitatory terminals. This would indicate a wider influence of inflammatory responses, going beyond simple pathological neurotoxicity of microglia.

The observed effects of LPS-induced systemic inflammation in P22 mice injected at P14 would predict an overall lower amount of excitatory drive in LPS injected mice, given their much lower vGlut1-positive excitatory terminal density. In turn, the mice should be more resistant to seizures than not injected controls. An experiment on seizure susceptibility of LPS treated mice was performed by a collaborating laboratory (Hsu and Binder, 2011 - unpublished observations), using the pentylenetetrazole (PTZ) model of epilepsy (da Silva et al., 1998) and 40mg/kg dose of PTZ. Although the number

of seizures and seizure duration were the same for the experimental and the control groups, the survivability of LPS-injected mice was at 80%, as compared to 40% survivability of the control mice. This result indicates that the systemic inflammation induced by LPS leads to a reduction in overall excitatory drive brought about by lower numbers of excitatory synaptic terminals.

The results of this study show differential susceptibility to systemic inflammation of developing, postnatal mice depending on age, with P14 animals being vulnerable to the immune response of microglia and possibly astrocytes. Relative to P14, younger and older mice, including adults did not react in a similar manner, as their dendritic spines and presynaptic terminals appeared unaffected. Presumably, the immunological profile of P14 mice renders their immune systems more prone to induce neuronal damage, either through higher sensitivity or stronger responses. Systemic inflammation influences neuronal function and is likely to have wide-ranging and long-lasting effects. To date, there is only one published study investigating effects of LPS-induced inflammation on dendritic spines, which was performed in adult animals. The authors have observed a decrease in spine density 56 days after LPS injection, but not after 2, 7, or 28 days (Kondo et al., 2011). It is therefore probable that mice which undergo a systemic inflammation during postnatal development will be affected by it as adults. On the contrary, the more plastic brains of younger animals may be able to cope better with such challenges.

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Figure 3.1 Glial and macrophage morphology in CA1 stratum radiatum of control and LPS injected wild type mice.

Confocal images of immunofluorescent labeling of microglia and macrophages (Iba1), astrocytes (GFAP) and neurons (MAP2) in unmanipulated (A-D) and LPS treated (E-H) wild type mice. Images show labeling at P8 (P7+1d, A,E,I,J), P15(P14+1d, B,F,K,L), P22(P21+1d, C,G,M,N) and in 3 month old animals (3mo+1d, D,H,O.P), 24h after LPS injection.

High magnification images of Iba1-positive cells in P8 (I,J), P15 (K,L), P22 (M,N) and adult (O,P) hippocampi of control (I,K,M,O) and LPS-treated (J,L,N,P) mice. Scale bars are 50μm for panels A-H and 10μm for panels I-J.

Figure 3.1

Iba1 GFAP MAP2

3mo+1d P21+1d P14+1d P7+1d
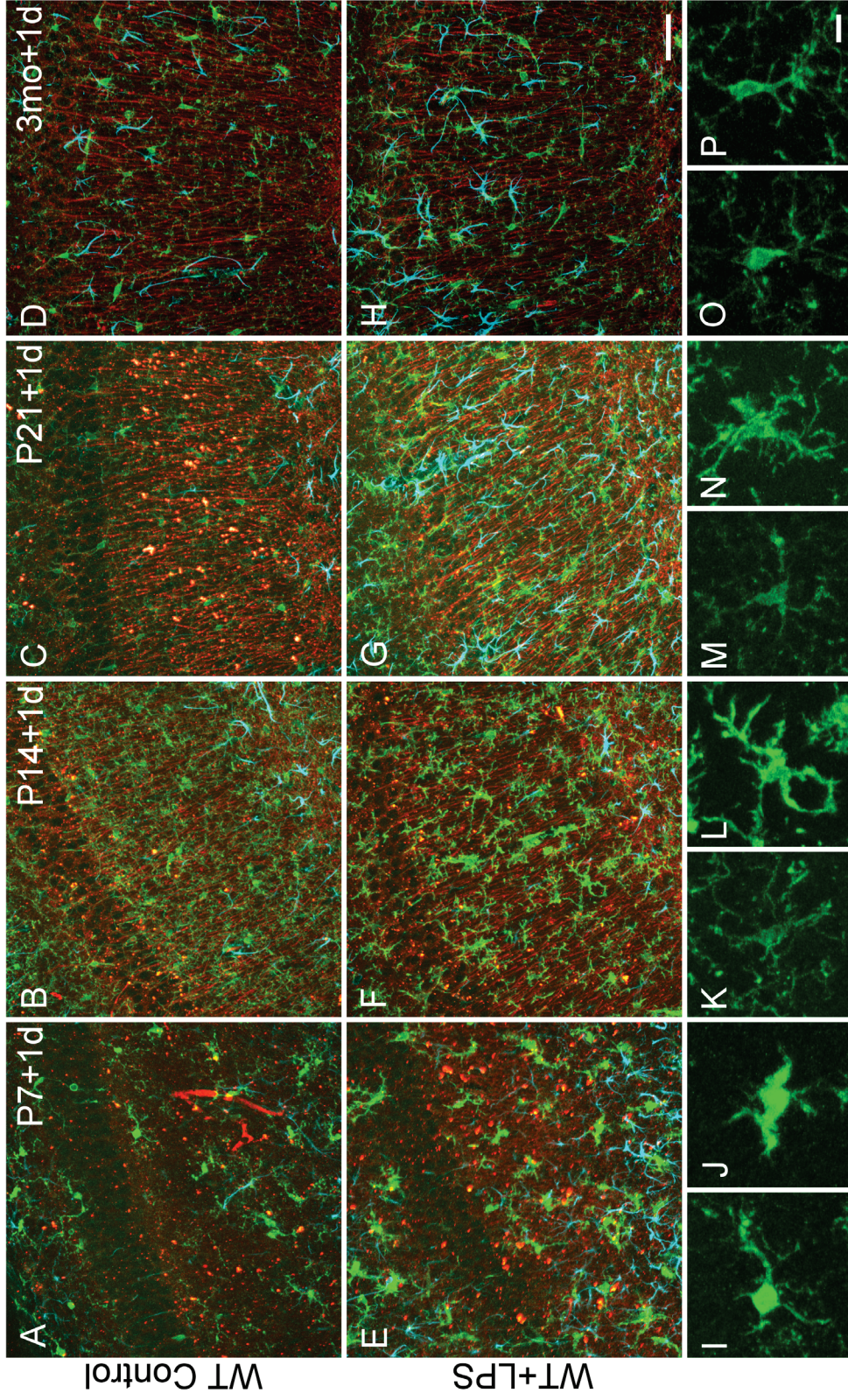
WT Control

WT+LPS

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Figure 3.2 Microglial and macrophage morphology in CA1 stratum radiatum of control and LPS injected wild type mice.

Confocal images of immunofluorescent labeling of microglia and macrophages (Iba1) in unmanipulated (A-D) and LPS treated (E-H) wild type mice. Images show labeling at P8 (P7+1d, A,E,I,J), P15(P14+1d, B,F,K,L), P22(P21+1d, C,G,M,N) and in 3 month old animals (3mo+1d, D,H,O.P), 24h after LPS injection.

High magnification images of Iba1-positive cells in P8 (I,J), P15 (K,L), P22 (M,N) and adult (O,P) hippocampi of control (I,K,M,O) and LPS-treated (J,L,N,P) mice. Scale bars are 50μm for panels A-H and 10μm for panels I-J.

Figure 3.2

Iba1

P7+1d   P14+1d   P21+1d   3mo+1d

WT Control

WT+LPS

A | B | C | D
E | F | G | H
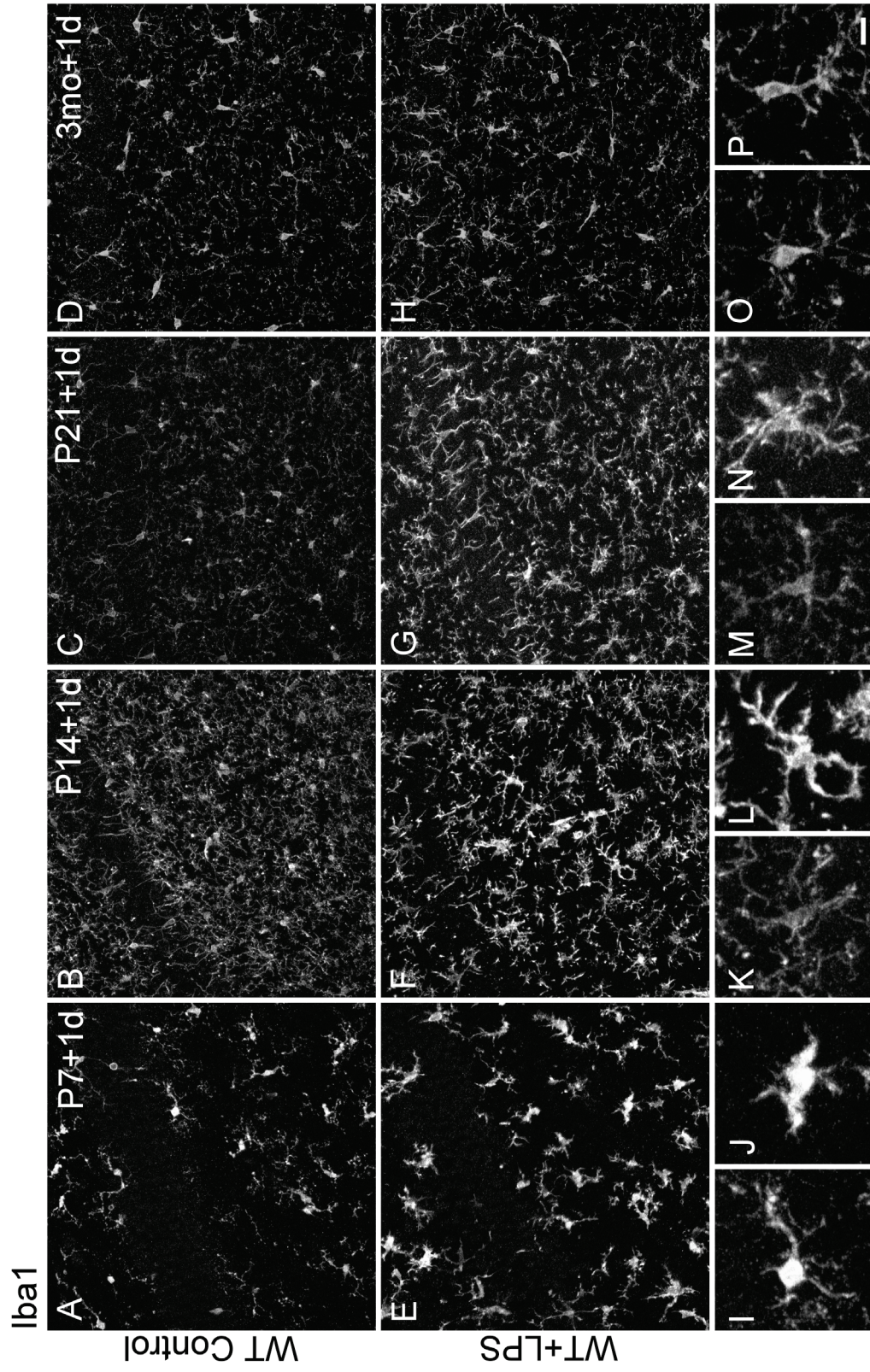I | J | K | L | M | N | O | P

105

Figure 3.3 Dendritic spines of hippocampal pyramidal neurons in unmanipulated and LPS treated mice.

Representative images of dendritic spines of hippocampal pyramidal neurons in CA1 stratum radiatum in mice at P8 (P7+1d, A,B), P15 (P14+1d, C,D), P22 (P21+1d, E,F) and 3 months of age (3mo+1d, G,H), 24h after LPS injection. Dendritic spines were labeled DiOlistically with DiI. Images are maximum intensity projections of optical sections taken at 0.3µm intervals. Scale bar is 5µm.

Figure 3.3

WT Control   WT+LPS

A   P7+1d   B

C   P14+1d   D
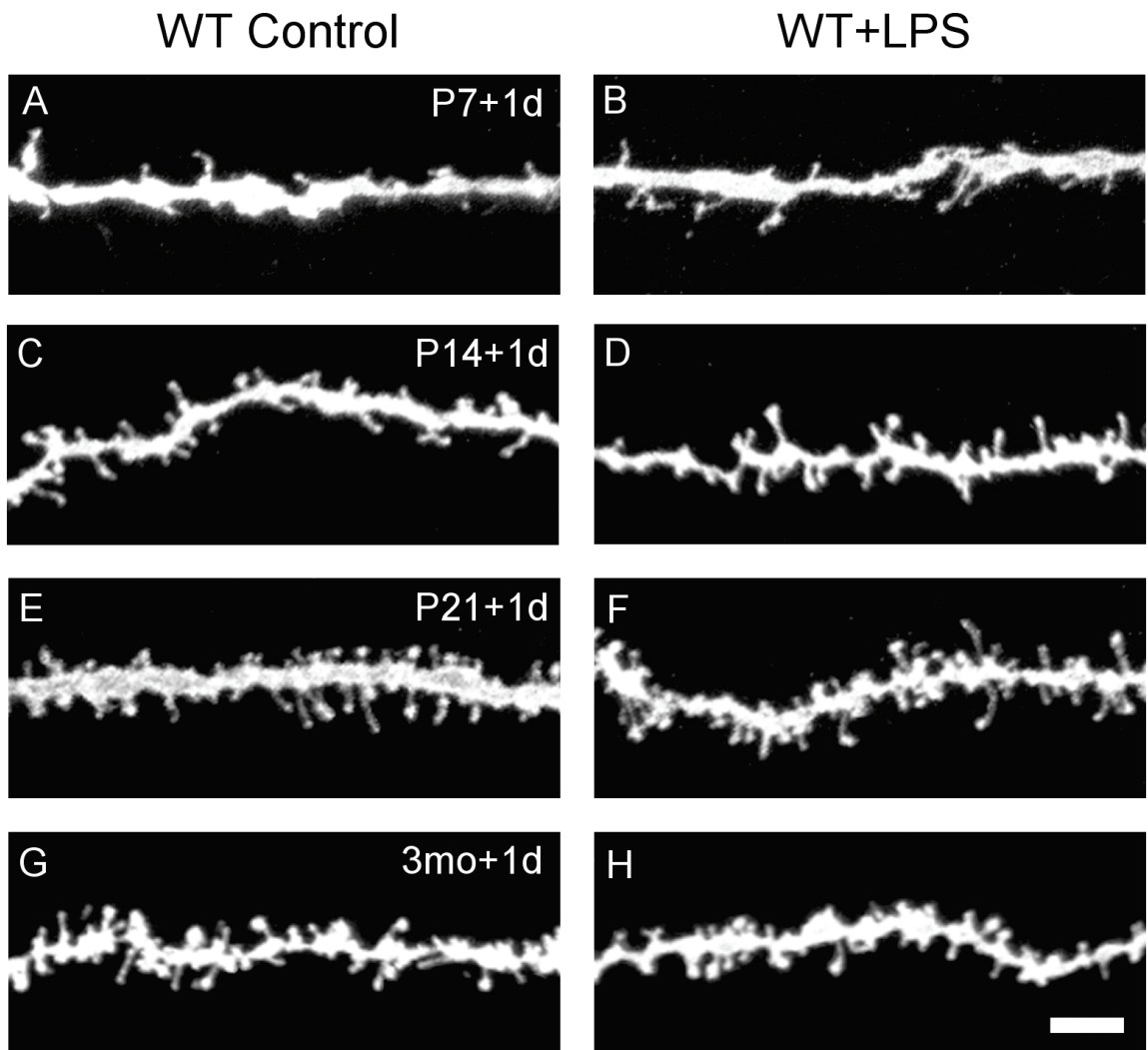
E   P21+1d   F

G   3mo+1d   H

Figure 3.4 Quantitative morphometric analysis of LPS-induced inflammation on dendritic spine density and morphology.

Quantitative analysis of dendritic spine density (A), length (B) and head area (C) in P8 (P7+1d), P15 (P14+1d), P22 (P21+1d) and adult 3 month old animals (3mo+1d) 24h after LPS injection. P8: 3 control, 4 LPS mice, 943 control, 1683 LPS spines (A,B), 768 control, 1353 LPS spines (C). P15: 4 mice per condition, 6099 control, 4690 LPS spines (A,B), 4630 control, 3524 LPS spines (C). P22: 3 mice per condition, 5596 control, 5243 LPS spines (A,B), 3894 control, 3578 LPS spines (C). Adult: 3 mice per condition, 5637 control, 2595 LPS spines (A,B), 3793 control, 1771 LPS spines (C).Statistical comparisons were performed using Student's t-test; * $p<0.05$. Error bars indicate S.E.M.
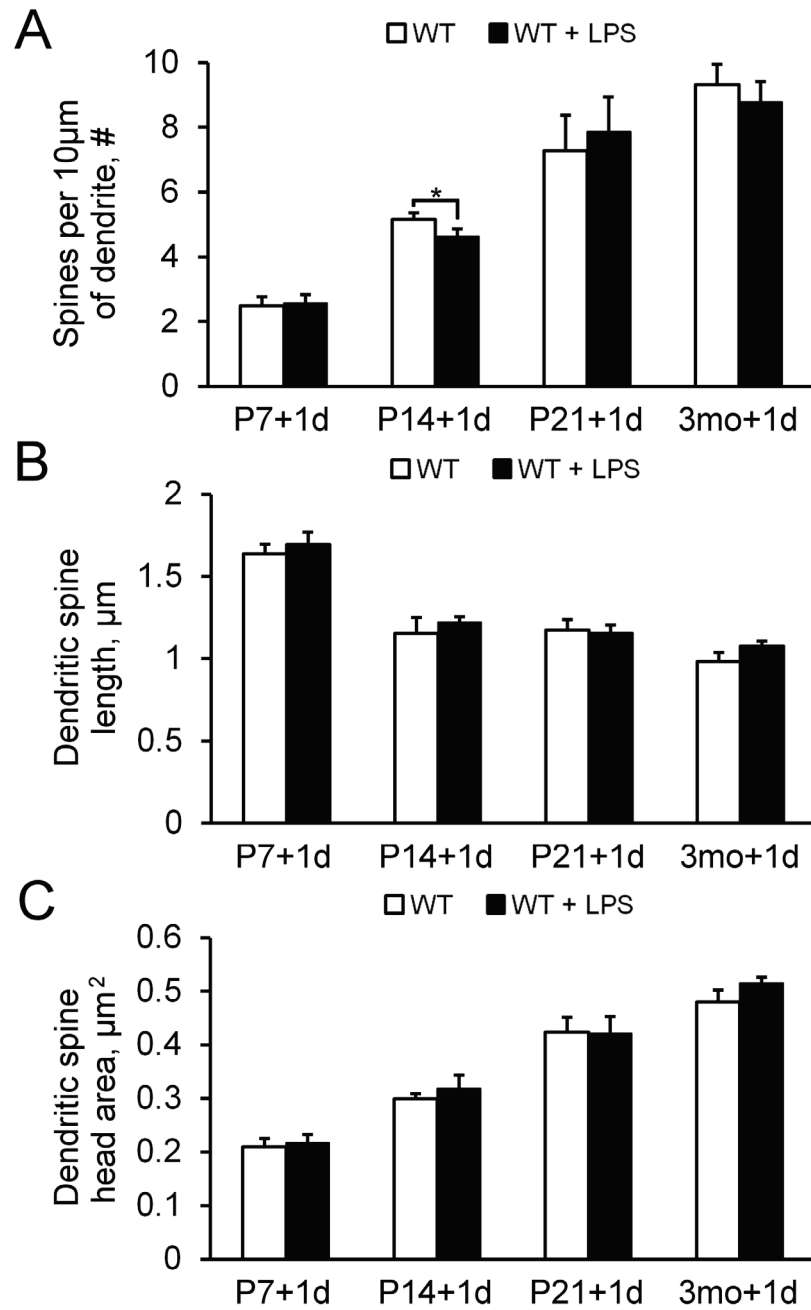
Figure 3.4

Figure 3.5 Dendritic spine length cumulative distributions in unmanipulated and LPS treated wild type mice.

Quantitative analysis of dendritic spine density length in P8 (P7+1d, A), P15 (P14+1d, B), P22 (P21+1d, C) and adult 3 month old animals (3mo+1d, D) 24h after LPS injection represented as cumulative distribution graphs. P8: 3 control, 4 LPS mice, 943 control, 1683 LPS spines. P15: 4 mice per condition, 6099 control, 4690 LPS spines. P22: 3 mice per condition, 5596 control, 5243 LPS spines. Adult: 3 mice per condition, 5637 control, 2595 LPS spines. Error bars indicate S.D.

Figure 3.5

Figure 3.6 Dendritic spine head area cumulative distributions in unmanipulated and LPS treated wild type mice.

Quantitative analysis of dendritic spine head areas in P8 (P7+1d, A), P15 (P14+1d, B), P22 (P21+1d, C) and adult 3 month old animals (3mo+1d, D) 24h after LPS injection represented as cumulative distribution graphs. P8: 3 control, 4 LPS mice, 768 control, 1353 LPS spines. P15: 4 mice per condition, 4630 control, 3524 LPS spines. P22: 3 mice per condition, 3894 control, 3578 LPS spines. Adult: 3 mice per condition, 3793 control, 1771 LPS spines. Error bars indicate S.D.
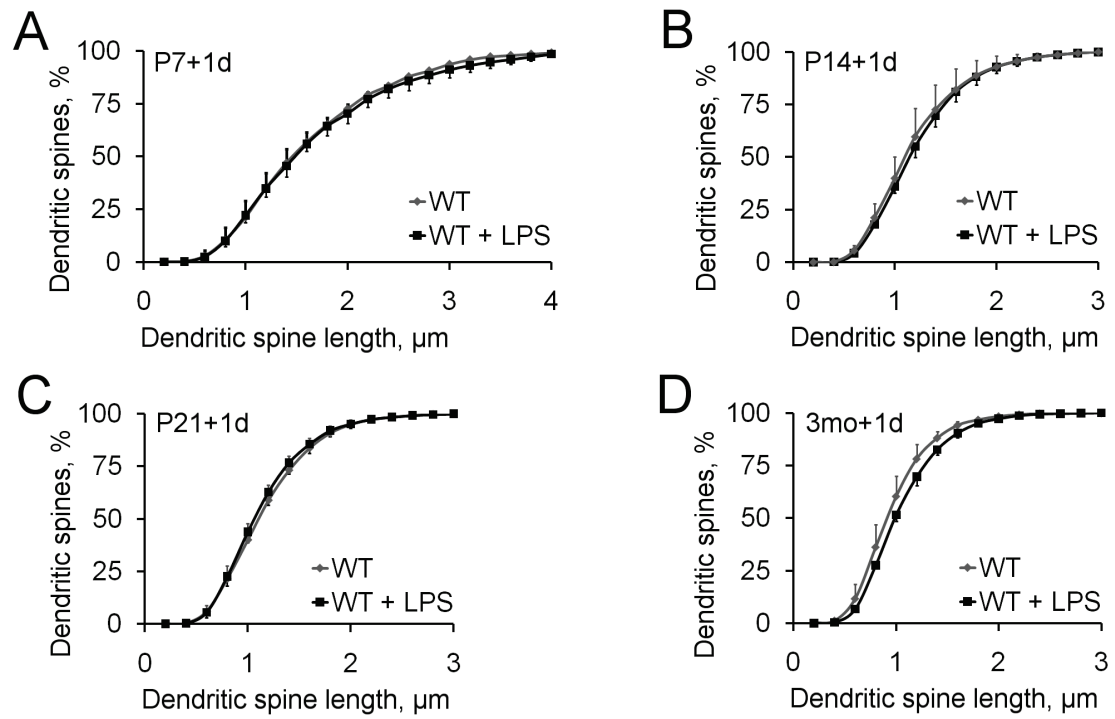
Figure 3.6
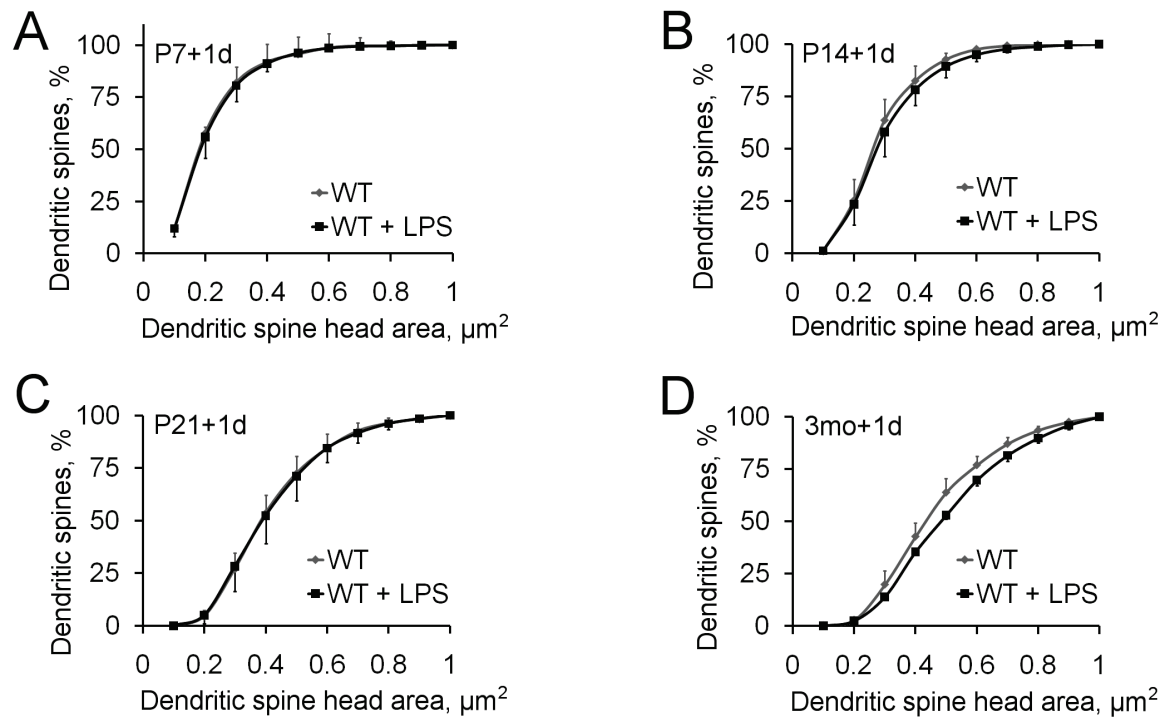
Figure 3.7 Immunolabeling of excitatory and inhibitory presynaptic terminals in unmanipulated and LPS treated mice.

Confocal images of presynaptic terminal immunolabeling in P15 (P14+1d) unmanipulated (A) and LPS treated (B) wild type mice. Excitatory terminals are labeled for vGlut1 (green), inhibitory terminals are labeled for GAD65 (red). Scale bar is 10µm.

Figure 3.7

A P14+1d GAD65 vGlut1 WT

B P14+1d GAD65 vGlut1 WT+LPS
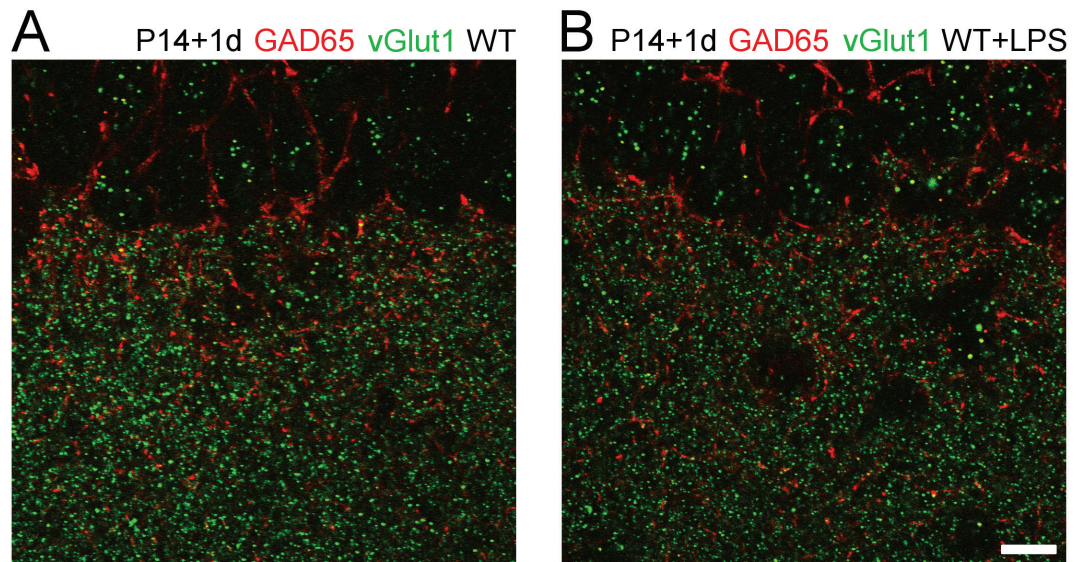
Figure 3.8 Quantification of presynaptic terminal immunolabeling in unmanipulated and LPS treated wild type mice.

Immunolabeling of presynaptic excitatory terminals by vGlut1(A) and inhibitory terminals by GAD65 (B) in unmanipulated and LPS treated mice was quantified in P15 animals 24h after LPS injection (P14+1d) and in P22 animals 8 days after LPS injection (P14+8d). vGlut1: P14+1d: 3 mice per condition, 40 control images, 50 LPS images; P14+8d: 2 mice per condition, 60 images per condition. GAD65: P14+1d: 3 mice per condition, 60 images per condition; P14+8d: 2 mice per condition, 40 images per condition. Statistical comparisons were performed using Student's *t*-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate S.E.M.

Figure 3.8
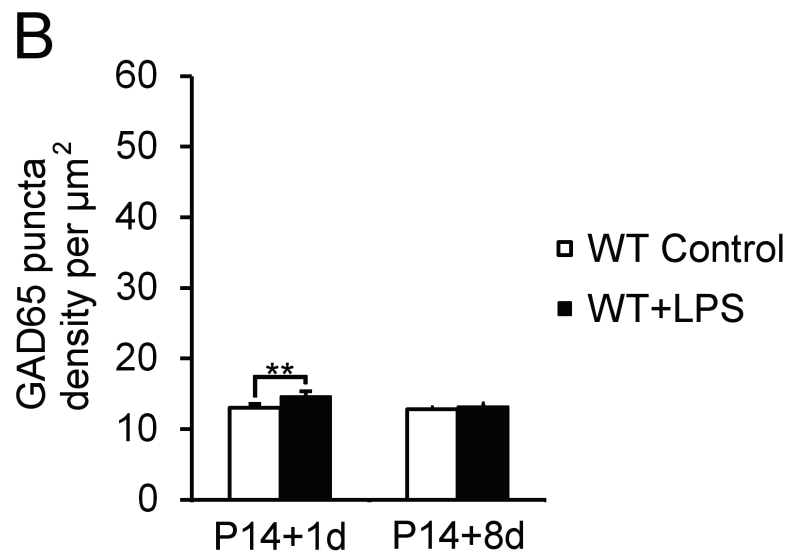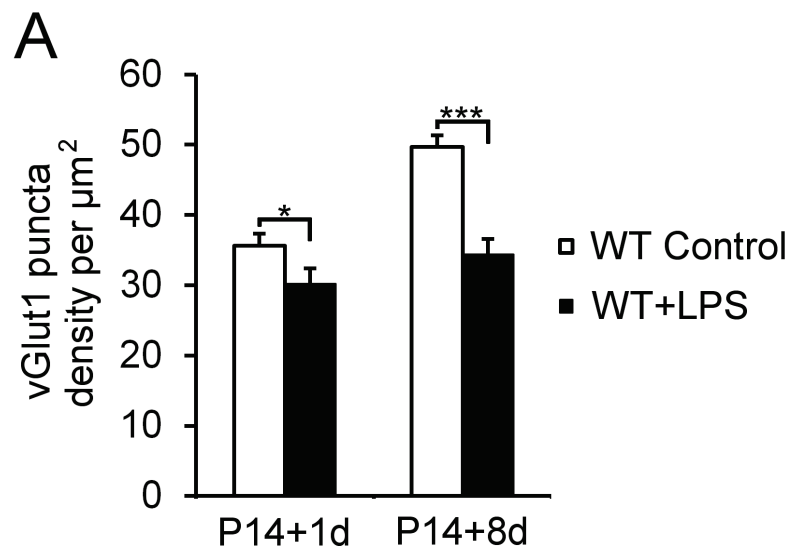
Chapter 4 - Effects of inflammation on synaptic development of TREM2 KO mice

Abstract

Nasu-Hakola disease is a recessive genetic neurodegenerative disorder characterized by cyst-like lesions in the bones and severe dementia in its late stages. The neurological symptoms begin in the 3rd decade of life with slight behavioral changes and progress into crippling dementia that incapacitates its victims who become bed-ridden and die usually in their 50's. Post-mortem analysis of their brains revealed neuronal degeneration and microglial activation suggesting involvement of inflammatory processes. We used a mouse model of the disease to study its effects on synapse development and showed defective synapse formation in the mouse hippocampi.

Introduction

Triggering receptor expressed on myeloid cells-2 (TREM2) is a member of a family of at least three receptors characterized by an immunoglobulin superfamily domain in their extracellular portion (Colonna, 2003). TREM2 is expressed on cell membranes of activated macrophages, immature dendritic cells, osteoclasts and microglia. Precise expression of TREM2 in the brain is still under debate, as some studies have detected its presence on microglia and oligodendrocytes (Kiilainen et al., 2005; Thrash et al., 2009), another study on microglia and a subpopulation of neurons (Sessa et al., 2004), yet another on microglia only (Takahashi et al., 2005). Interestingly, not all microglia express TREM2 even those found in close proximity to each other,

indicating a heterogeneity in microglial population, possibly resulting from differences in each cell's microenvironment (Schmid et al., 2009). Expression of TREM2 is also regulated developmentally (Thrash et al., 2009).

TREM2 lacks a cytoplasmic signaling tail and associates with DNAX adaptor protein-12 (DAP12), a member of a transmembrane adaptor protein family containing immunoreceptor tyrosine-based activation motifs (ITAMs). Upon TREM2 activation, ITAM of DAP12 becomes phosphorylated, initiating a signaling cascade and inducing phosphorylation of the extracellular signal-regulated kinase (ERK; Takahashi et al., 2005). The signaling pathway of TREM2 upregulates the chemokine receptor CCR7 and promotes microglial migration towards its ligands, CCL19 and CCL21 and is distinct from TLR pathway, as it does not enhance NFκB activity (Neumann and Takahashi, 2007; Takahashi et al., 2005). Although TREM2 has been considered an orphan receptor, recently a ligand has been proposed. The heat shock protein 60 (Hsp60) is a mitochondrial chaperone that can be expressed on cell surface. It was shown to be a low affinity agonist for TREM2 and to increase phagocytic activity of a microglial cell line (Stefano et al., 2009).

The primary known function of TREM2 signaling is clearance of neural tissue debris. DAP12 knockout mice exhibit thalamic hypomyelinosis, synaptic degeneration, accumulation of synaptic vesicles in axons and developmentally arrested oligodendrocytes. Furthermore, electrophysiological recordings showed aberrant GABAergic mini inhibitory post-synaptic currents (mIPSCs), which related to impaired startle response in these mice (Kaifu et al., 2003). These defects may result from inadequate clearance of apoptotic cells and accumulation of cellular debris. An investigation into TREM2 function revealed that its signaling through DAP12 plays a role

in phagocytosis. TREM2 knockdown in microglia inhibited phagocytosis of apoptotic neurons, while its overexpression promoted phagocytosis and decreased pro-inflammatory responses of microglia (Takahashi et al., 2005). A separate study supported and expanded on these results, demonstrating expression of TREM2 ligands on neurons in primary and cell line neuronal cultures. Inducing apoptosis in the neurons increased the expression of the ligand and stimulated TREM2 signaling, whereas an anti-TREM2 antibody blocked the stimulation and reduced phagocytosis of apoptotic neurons by microglia. Additionally, Chinese hamster ovary cells transfected with TREM2 gained phagocytic ability and engulfed apoptotic neurons (Hsieh et al., 2009).

The lack of TREM2 signaling results in Nasu-Hakola disease, also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL). It's a rare and fatal recessively inherited disorder, arising from loss-of-function mutations in either TREM2 or DAP12 (Paloneva et al., 2002). It was first described independently in Finland and Japan as characterized by bone cysts, neurodegeneration and dementia (Hakola, 1972; Nasu et al., 1973). Patients suffering from PLOSL experience four stages of the disease. The initial, latent stage, up to about 20 years of age is asymptomatic. In the next, osseous stage, the patients report pain and mild swelling in the ankles and feet and pathological fractures in the extremities occur several years later. By their mid-thirties, early neurological symptoms set in, involving personality changes, aphasia, memory deficits and gait disturbances among others. The patients enter the last, late neurological stage in their forties, characterized by profound dementia and die by their sixth decade of life, typically of respiratory or urinary infections (Bianchin et al., 2004). Brains of PLOSL victims exhibit demyelination, atrophy and extensive activation of microglia (Paloneva et al., 2002). It is hypothesized that microglial

deficiencies resulting from loss of TREM2/DAP12 signaling are the underlying reason for PLOSL CNS phenotype (Takahashi et al., 2005).

Materials and Methods

Mice

C57Bl/6J mice were housed in an AAALAC accredited facility under a 12 hour light/dark cycle. All mouse studies were done within NIH and Institutional Animal Care and Use Committee guidelines.

Lipopolysaccharide injections

Lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) injections were performed intraperitoneally at 5mg per kg in a total volume of 100ul using a 25 gauge needle.

Intracardial perfusion

Mice were anesthetized with Isoflurane (Phoenix, St. Joseph, MO) as 2%-5% vapor in pure oxygen, depending on mouse age (P8 – 5%, P15 – 3.5%, P22 – 3%, 3mo – 2%). Isoflurane vapor was produced by VSS vaporizer 32F (VSS, Rockmart, GA) at a constant 3.5 l/min. Mice were perfused first with ice cold PBS for 45 min., followed by ice cold 4% paraformaldehyde in PBS for 45 min. Mouse brains were extracted and post-fixed before further processing.

<u>Immunolabeling of presynaptic terminals and image acqusition</u>

Brains from perfused animals were post-fixed for overnight and sectioned into 100µm thick coronal slices using a Leica VT1000P vibrating blade microtome (Vibratome, Bannockburn, IL). Brain slices were permeabilized/blocked in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 0.3% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) for 3h at room temperature. Next, slices were incubated with primary antibodies in PBS containing 10% donkey serum, and 0.1% Triton X-100 overnight at 4°C for vGlut1and GAD65 antibodies. In case of Iba1, GFAP, MAP2 antibodies, PBS containing 10% goat serum (Sigma-Aldrich), 0.5% Tween 20 (Fisher Scientific) was used. The slices were then washed 3 times in a PBS solution containing 0.5% Tween 20 for 20min at room temperature, followed by incubation with secondary antibodies in PBS solution with 0.5% Tween 20 for 3h at room temperature. The slices were washed again 3 times in PBS solution with 0.5% Tween 20 for 20min at room temperature followed by a single 20min wash in PBS. The slices were mounted in Vectashield with DAPI, to visualize nuclei. vGlut1 and GAD65 Images were acquired using a confocal laser scanning microscope model LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY) and a 100x oil immersion objective (1.3 NA) as series of 10-30 high-resolution optical sections (1024x1024 pixel format) taken at 1µm interval. Iba1, GFAP and MAP2 images were acquired using a 25x water immersion objective (0.8 NA) as series of 30 optical sections taken at 1µm interval. All images were acquired at the stratum radiatum layer of the hippocampal area CA1.

Primary antibodies: rabbit anti-VGlut1 (48-2400, Invitrogen, Carlsbad, CA), mouse anti-GAD65 (559931, BD Pharmingen, San Diego, CA), rabbit anti-Iba1 (019-19741, Wako Chemicals USA, Inc., Richmond, VA), momouse anti-GFAP-Cy3 (C9205, Sigma-

Aldrich), mouse anti-MAP2 (M4403, Sigma-Aldrich). Secondary antibodies: Alexa Fluor 488 donkey anti-mouse IgG H+L (A21202, Invitrogen, Carlsbad, CA), Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Invitrogen), Alexa Fluor 660 goat anti-rabbit IgG (A-21074, Invitrogen).

Analysis of images of immunolabeled presynaptic terminals

Presynaptic terminals were immunolabeled for vGlut1, GAD65 and images were acquired as image series of optical sections as described above. Each series began at the slice surface and single optical sections were extracted at the constant depth of 9µm for vGlut1 and Synapsin 1 and 6µm for GAD65. Quantification of immunopositive puncta density was performed with ImageJ software (http://rsb.info.nih.gov/ij/). A threshold appropriate for each staining was applied to eliminate background and minimize noise. Low thresholds were 80 and 64 for vGlut1 and GAD65, respectively. Next, particles were counted by the "analyze particles" function. In order to avoid including noise and non-specific staining in the count, a range limit of 4 to 200 pixels was applied to vGlut1. Due to the high quality of GAD65 labeling, a range limit was not necessary. Puncta density was calculated per 100µm^2.

Results

<u>TREM2 KO mice have lower levels of excitatory input than wild type mice.</u>

Presynaptic terminals of stratum radiatum CA1 synapses in brain sections of TREM2 KO mice were immunolabeled for vGlut1 and GAD65 and compared to those of wild type

mice. The number of excitatory presynaptic terminals as measured by vGlut1 puncta density is significantly lower in TREM2 KO mice than wild type mice at P15 and P22 (Fig. 4.1A). While in wild type animals vGlut1 puncta density increases from P15 to P22, it decreases in TREM2 KO mice within the same timeframe. Inhibitory input assessed by measuring GAD65 puncta density does not differ between wild type and TREM2 KO animals at P15, however at P22 TREM2 KO strain has a higher level of GAD65 puncta density (Fig. 4.1B).

LPS injections have a differential effect on excitatory and inhibitory inputs in wild type and TREM2 KO mice. A single injection of LPS results in lower vGlut1 puncta density 24h post-injection at P15 (P14+1d) in both wild type and TREM2 KO mice (Fig. 4.2A). However, in P22 mice injected at P14 (P14+8d) vGlut1 levels are the same in LPS and control TREM2 KO mice, while in wild type mice they are significantly reduced in LPS injected animals as compared to controls (Fig. 4.2B). GAD65 puncta density increases 24h after LPS injection in wild type animals at P15 and remains the same in TREM2 KO mice (Fig. 4.3A). In P22 mice injected 8 days earlier (P14+8d) GAD65 puncta density is the same in LPS injected wild types as controls, but is significantly lower in LPS injected TREM2 KO mice in comparison to their controls (Fig 4.3B).

Discussion

TREM2 is expressed on microglia, where its signaling mediates phagocytosis of apoptotic neurons (Stefano et al., 2009; Takahashi et al., 2005). Deficiency of TREM2 signaling results in a lethal neurodegenerative disorder, Nasu-Hakola disease, or

PLOSL. It is thought that the dysfunction of the ability of microglia to phagocytose apoptotic cells leads to accumulation of cell remains, which activates microglia, inducing inflammation and causing neuronal damage. Dying cells contribute more tissue debris which is not cleared, driving the cycle of inflammation (Neumann and Takahashi, 2007). TREM2 KO mice are a model of this disorder and exhibit interesting features compared to wild type mice under normal and inflammatory conditions. In comparison to wild type mice, the density of presynaptic excitatory terminals in their hippocampus is lower at P15 and P22. While there is a developmental increase in the density of the terminals in wild type mice from P15 to P22, TREM2 deficient mice experience a significant reduction over the same period. This is in contrast to inhibitory, GAD65-positive terminals, which slightly, but significantly increase in numbers during this time, while they stay the same in wild type animals. It appears that the state of inflammation inherent to TREM2 deficiency has a similar effect on the development of TREM2 KO mice as LPS induced inflammation has on wild type mice. The increase in inhibitory terminals could be related to the similar, but transient increase in LPS treated wild type mice.

TREM2 KO mice also react differently to systemic inflammation induced by peripheral LPS injections. While they do show an acute reduction in excitatory terminals in P15 animals 24h after injection, similarly to wild types, 8 days after injection administered at P14, excitatory terminal densities are the same in controls and LPS injected mice. Presumably, LPS prevented the developmental formation of new synapses in wild type mice, yet since TREM2 KO's did not form new synapses over the same developmental period, their levels remained the same. However, TREM2 deficient mice added new inhibitory terminals from P15 to P22 and this increase was prevented by LPS injections. Wild type animals, which did not form new inhibitory terminals over

this period, did not experience a change. These results suggest that synaptic development in TREM2 KO mice is compromised, that LPS induced inflammation exerts both acute (24h) effects and hinders development over the long term.

Similarly to P22 wild type mice injected at P14, TREM2 KO mice were tested for their seizure susceptibility (Hsu and Binder, 2011 - unpublished observations). Since TREM2 KO animals have a significantly lower density of excitatory terminals, and higher density of inhibitory terminals, their seizure threshold is expected to be higher. A dose of PTZ (40mg/kg) which induces seizures in wild type mice with 40% survival rate, failed to produce seizures in TREM2 KO mice. Seizures could still be induced at higher doses. LPS injected mice have reduced numbers of inhibitory terminals and their overall levels of inhibition should be lower than those of unmanipulated controls. This manifests itself by PTZ inducing seizures in LPS treated TREM2 KO mice, although with 100% survivability. Potentially, their reduced excitatory drive resulting from lower excitatory terminal density protects them from seizure mortality (Fig. 4.4).

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Figure 4.1 Excitatory and inhibitory presynaptic terminal density in wild type and TREM2 KO mice.

Immunolabeling of excitatory presynaptic terminals for vGlut1 (A) and inhibitory terminals for GAD65 (B) was quantified by assessing the density of immunoreactive puncta per 100 µm^2. Analysis was performed in unmanipulated wild type (WT) and TREM2 KO mice at P15 and P22. vGlut1: WT: P15: 3 mice, 40 images; P22: 3 mice, 60 images; TREM2 KO: P15: 2 mice, 40 images; P22: 2 mice, 40 images. GAD65: WT: P15: 3 mice, 60 images; P22: 2 mice, 40 images; TREM2 KO: P15: 4 mice, 80 images; P22: 3 mice, 60 images. Statistical comparisons were performed using Student's *t*-test; * p<0.05; ** p<0.01; *** p<0.001. Error bars indicate S.E.M.

Figure 4.1

Figure 4.2 Excitatory presynaptic terminal density in wild type and TREM2 KO P15 LPS injected mice 24h after injection and P22 LPS injected mice 8 days after injection.

Immunolabeling of excitatory presynaptic terminals for vGlut1 in P15 (P14+1d, A) 24h after injection and P22 (P14+8d, B) 8 days after injection in wild type (WT) and TREM2 KO mice was quantified by assessing the density of immunoreactive puncta per 100 μm^2 and compared to controls. WT: P15: 3 control mice, 40 images, 4 LPS mice, 50 images; P22: 3 mice, 60 images per condition; TREM2 KO: P15: 2 control mice, 40 images, 2 LPS mice, 30 images; P22: 2 control mice, 40 images; 3 LPS mice, 60 images. Statistical comparisons were performed using Student's t-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Error bars indicate S.E.M.
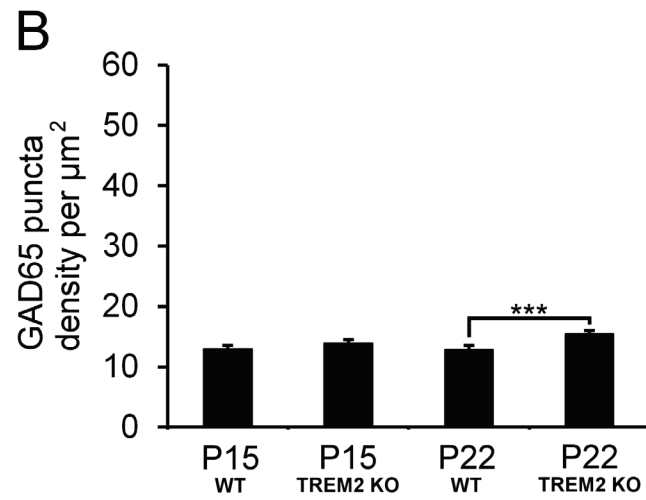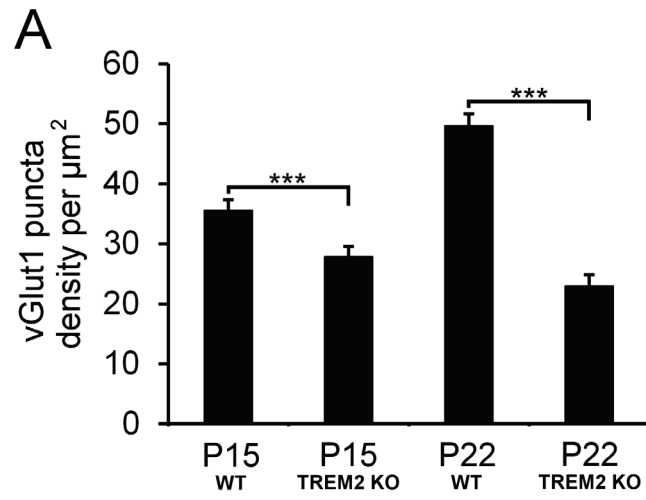
Figure 4.2

Figure 4.3 Inhibitory presynaptic terminal density in wild type and TREM2 KO P15 LPS injected mice 24h after injection and P22 LPS injected mice 8 days after injection.

Immunolabeling of inhibitory presynaptic terminals for GAD65 in P15 (P14+1d, A) 24h after injection and P22 (P14+8d, B) 8 days after injection in wild type (WT) and TREM2 KO mice was quantified by assessing the density of immunoreactive puncta per 100 μm^2 and compared to controls. WT: P15: 3 mice, 60 images per condition; P22: 2 mice, 40 images per condition; TREM2 KO: P15: 4 control mice, 80 images, 5 LPS mice, 100 images; P22: 3 control mice, 60 images; 5 LPS mice, 100 images. Statistical comparisons were performed using Student's t-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Error bars indicate S.E.M.
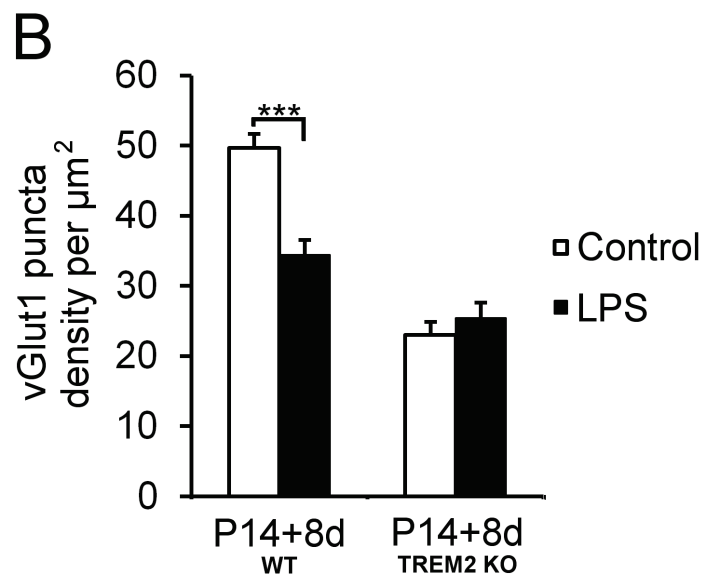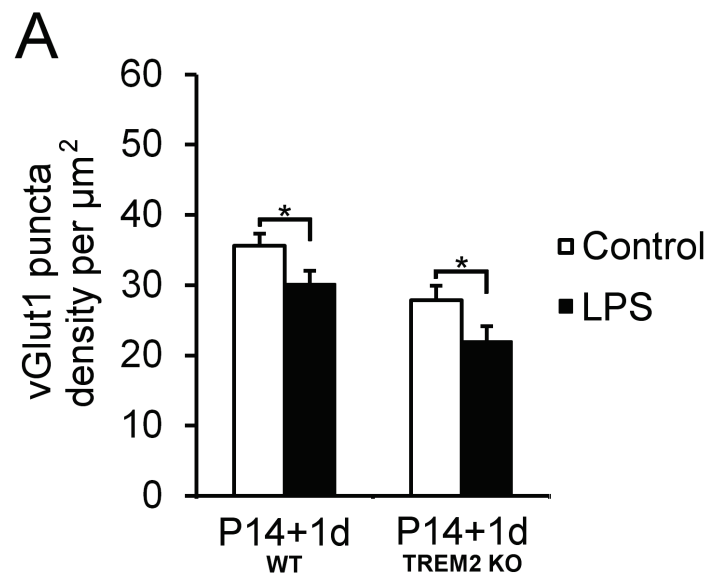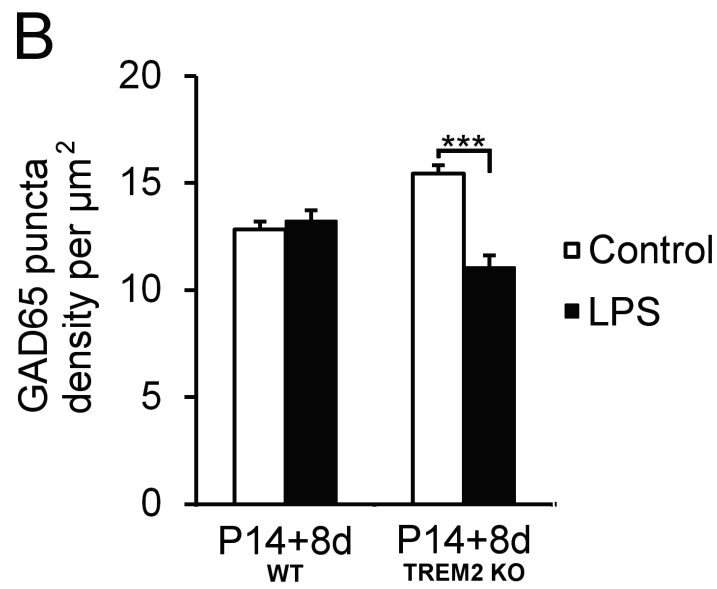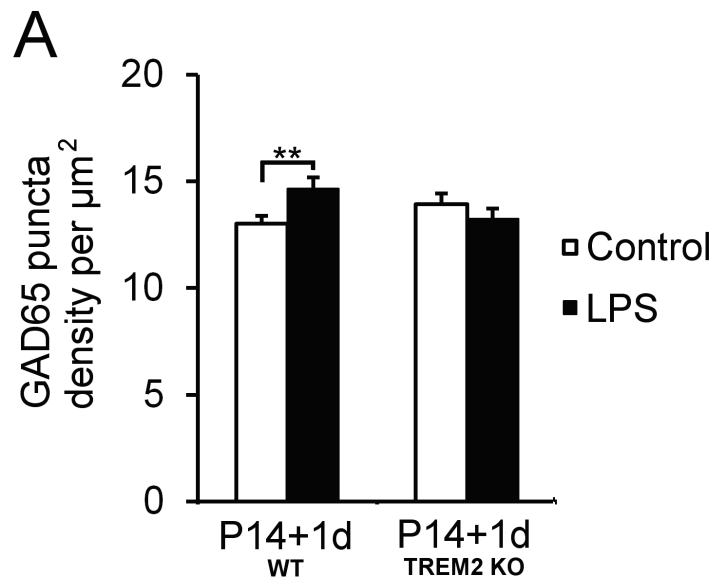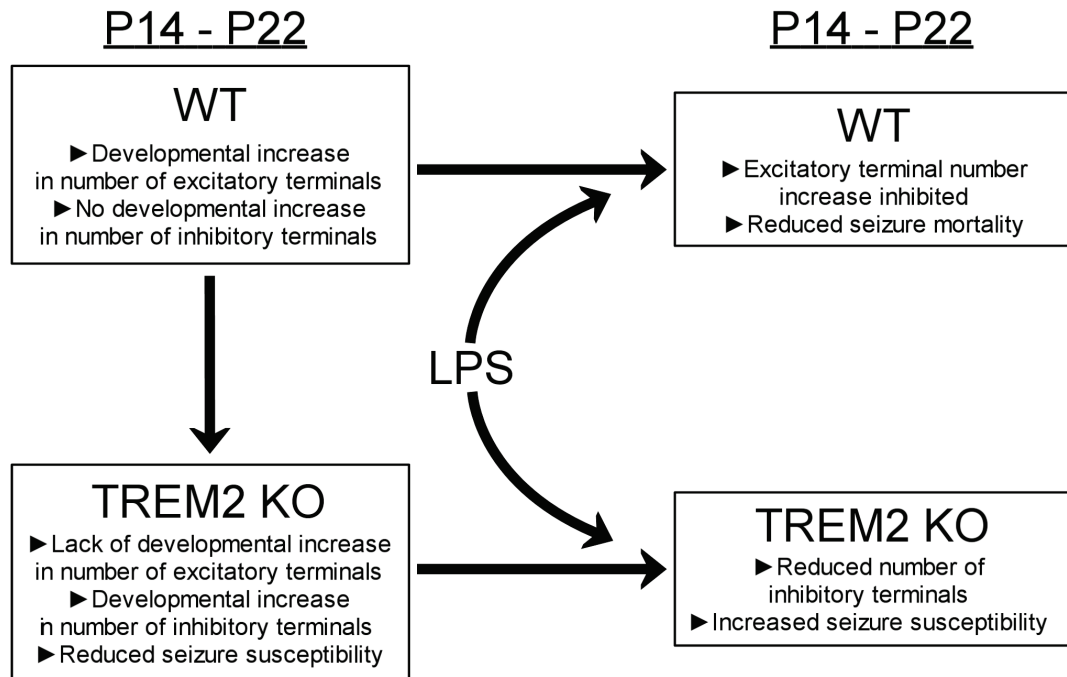
Figure 4.3

Figure 4.4 Flowchart of the effects of LPS induced inflammation on wild type and TREM2 KO mice.

Comparison of effects of systemic inflammation between wild type and TREM2 KO P22 mice injected at P14.

Figure 4.4

Chapter 5 – Conclusion

Conclusion

The human CNS is composed of hundreds of billions of neurons, ten times as many glia with 164x1012 synaptic connections making it a functional unit (Colón-Ramos, 2009; Tang et al., 2001). Even the humble mouse can boast 75 million neurons (Williams, 2000). These staggering numbers reflect the complexity of the process that is brain development. Given the sensitivity of neurons and the potential for damage that can be inflicted by the very cells that support and protect them, it is remarkable major defects are relatively rare. After all, it is the very purpose of glia to ensure correct CNS functioning throughout all the developmental stages, including aging. However, under certain conditions, even small insults can have long-lasting detrimental effects as a result of glial activation (Kondo et al., 2011). Postnatal period is especially critical for synaptic development, as it is the stage when the vast majority of synapses are formed, both in humans (Petanjek et al., 2011) and rodents (Miller, 1988). Challenges to the immune system during this time resulting in glial activation can have deleterious consequences in the adulthood (Boissé et al., 2004; Harré et al., 2008; Spencer et al., 2005). The present study focused on influence of systemic inflammation on synaptic development in the mouse hippocampus.

Synapses are points of contact between neurons with extensive membrane specializations where neuronal communication occurs. In the mammalian brain, synapses are mostly chemical, as opposed to electrical, meaning that the electrical signal is converted into a chemical one, in form of a neurotransmitter puff, which is

detected by the postsynaptic neuron and converted into a change of electrical potential (López-Muñoz and Alamo, 2009). Synapses are a crucial component in neuronal information processing and changes to synaptic efficacy as well as their formation and elimination are thought to represent memory and learning processes (Martin et al., 2000). Synaptic dysfunction underlies a wide range of neurological, psychiatric and mental disorders, thus it is important to understand what factors can lead to abnormal synapse development (Melom and Littleton, 2011). Chemical synapses are primarily excitatory and inhibitory and can be unambiguously differentiated by electrophysiological means in live tissue and by histological methods in post-mortem tissue. They express unique sets of proteins, which can be visualized by immunolabeling. Postsynaptic effects were investigated in this study by analyzing the morphologies of structural specializations of excitatory synapses – dendritic spines.

Dendritic spines are minute protrusions in dendrite membrane that accept the majority of excitatory inputs in the vertebrate CNS (Gray 1959). Although their roles are still enigmatic, it is believed that they act mainly as biochemical compartments isolating signaling events to individual synapses (Nimchinsky et al., 2002). Spine morphology is closely related to synapse strength and its alterations correspond to functional plasticity of the synapse, experiencing enlargement or shrinkage, as the synapse is potentiated or depressed (Matsuzaki et al., 2004; Zhou et al., 2004). Spines can change their shape within minutes, yet they are not uniformly motile, since large spines with large heads are more mature and stable, whereas thin, elongated spines are more motile. Additionally, newly formed spines are more likely to be transient and eliminated than older spines, which are more persistent (Holtmaat et al., 2005; Zuo et al., 2005). Pathology of dendritic spine morphology and density also associated with many neurological

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disorders, including several forms of mental retardation, Alzheimer's disease and schizophrenia. However, the relationship of spine abnormalities to symptomology of these disorders is not yet clear (Fiala et al., 2002).

Glial cells have long been neglected by researchers in favor of neurons since they are not electrically excitable and hence less conducive to study, but also thought to play only vaguely defined "supportive" roles. It has since became clear just how involved glial cells are in CNS physiology, including information processing. Astrocytes comprise the largest population of cells in the CNS and serve functions essential to brain function, including energy metabolism, extracellular ion and pH balance and neurotransmitter metabolism (Volterra and Meldolesi, 2005). Furthermore, astrocytes participate in synaptogenesis, regulation of synaptic transmission and plasticity. With their processes wrapping synapses or closely apposed to them, these findings gave rise to the "tripartite synapse" model, indicating that astrocytes participate in interneuronal synaptic communication (Haydon, 2001).

Microglia are the immune cells of the CNS, constantly surveying their environment in search of potential threats to CNS functioning, including invading pathogens, injury and hypoxic/ischemic damage (Hanisch and Kettenmann, 2007; Wake et al., 2009). Our knowledge of microglia in non-pathological states has been limited and their activity has been thought to contribute to rather than to prevent neuronal damage (Giaume et al., 2007; Kettenmann et al., 2011). It is now known that microglial activation can be beneficial to the threat resolution and furthermore, that their roles extend into synapse development (Paolicelli et al., 2011) and possibly synaptic plasticity (Stellwagen and Malenca, 2006).

Nevertheless, microglia do have the potential to induce damage to nervous tissue due to the cytotoxic nature of their responses targeting invading pathogens. Neurons can become innocent bystanders in microglial fight against invaders (Bruce-Keller, 1999; Garden GA, Möller, 2006). Microglial inflammatory response also leads to astrocyte activation, causing further damage, in large part due to decrease in support provided to neurons (Liu and Feng, 2011; Steele and Robinson, 2011).

The current study was aimed at elucidating the effects of a systemic immune response on postnatal synaptic development in mouse hippocampus. The LPS model of systemic inflammation was used, with the mice injected intraperitoneally. In this model, it is thought that microglial activation is not a result of LPS acting directly on these cells, but rather of a release of pro-inflammatory molecules by peripheral immune cells (Nadeau and Rivest, 1999) which diffuse or are actively transported across the BBB or a combination of both. Each mouse in the experimental condition received a single injection of LPS at P7, P14, P22 or at 3 months of age. Mice were sacrificed 24h post injection, or in case of P14 mice, either 24h or 8 days after injection. Morphometric analysis of dendritic spines revealed a lower spine density in P15 LPS treated mice compared to controls and no changes to spine morphology at any of the examined time points. This result suggested that spine development in P14 mice was vulnerable to systemic inflammation. Over the course of postnatal development investigated in this study, spine density rose steadily from P8 through P14, P22 to adulthood. Therefore it is possible that the lower spine density observed in P15 LPS treated mice reflected a lack of growth of new spines between P14, the time of injection and P15, when the mice were sacrificed. Alternatively, the spines could have been eliminated as they were being formed. It is also possible that there was no selectivity in spine elimination and that

existing spines and newly forming spines were being eliminated together. It could be suggested that new spines, being less stable could be more vulnerable than established spines.

The effects of systemic inflammation on presynaptic terminals were also investigated. VGlut1, a vesicular glutamate transporter was used to identify excitatory synapses (Fremeau et al., 2004), while GAD65, one of the GABA-producing enzymes was used to detect inhibitory synapses (Soghomonian and Martin, 1998). Both of these proteins are expressed by presynaptic terminals and specifically mark the presynaptic portion of the synapse. The density of immunopositive vGlut1 and GAD65 puncta was used as the indicator of the number of the terminals. Similarly to dendritic spine density, vGlut1 and GAD65 puncta densities increased during postnatal development and into adulthood. Following the results of spine analysis, effects of LPS induced inflammation on P14 mice were investigated. The density of excitatory terminals was also lower in LPS injected mice compared to the controls, however the number of inhibitory terminals was higher in the experimental group. The reasons for this are not clear but could potentially reflect an overall reduction of excitation as a form of pathology. When the post-injection period was extended to 8 days and P14 injected mice were sacrificed at P22, the difference in excitatory terminal density between the two conditions was much wider. While the control animals formed new synapses from P14 to P22, the LPS injected animals did not. Inhibitory terminal density was unaffected. This result supports the hypothesis of systemic inflammation halting excitatory synaptogenesis.

TREM2 KO animals are a model of a neurodegenerative Nasu-Hakola disease, or PLOSL. They experience a state of persistent inflammation stemming from the dysfunction of a signaling pathway mediating cellular debris clearance by microglia

(Neumann and Takahashi, 2007). Similarly to LPS treated wild type mice, TREM2 deficient animals did not experience excitatory synaptic terminal formation. This made them less susceptible to seizures than normally developing wild type mice at P22. Interestingly, TREM2 KO mice treated with LPS at P14, also did not form new excitatory synapses and furthermore, experienced a loss of inhibitory terminals. At the same time they became more susceptible to seizures, presumably due to the loss of inhibitory synapses.

The results obtained by this study show that at different stages of postnatal development the CNS can be differentially affected by systemic inflammation. There appears to be a period vulnerability, where glial activation has deleterious effects on synaptogenesis. Perhaps this is due to a lowered threshold of resistance exhibited by neurons and with a stronger stimulus comparable outcomes could be generated at other ages. Alternatively, the developmental state of the immune system itself at this particular time point may be prone to an exaggerated inflammatory response. Given the dramatic consequences of inflammation 8 days after induction by LPS in P14 mice, long term inflammation could conceivably also have an effect in younger and/or older mice, albeit in a delayed manner. Synaptic development of TREM2 KO underscores the potential of damage that can result from an inflammatory response. Further research is needed to elucidate the mechanism behind the observed effects and the long term consequences of systemic inflammation on synaptic development.

In conjunction with already available data, the present study raises questions regarding the possible impact inflammation can have on developing human brain. Throughout life we experience many non-life threatening instances of inflammation resulting typically from common infectious diseases which resolve without apparent

harm. What kind of effect do they have on CNS development? It seems that in most cases, if present, such effects are undetectable. Human brains appear to be well suited to cope with minor insults. This however, is not always the case. Inflammation has been shown to be involved in etiology of several neurodevelopmental disorders, including autism, schizophrenia and cerebral palsy, which also exhibit synaptic abnormalities. Given the relative prevalence of these diseases and small bouts of inflammation, it is possible that a combination of a predisposition and inflammation experienced at a critical time point could trigger the disorder. Future inquiries into these issues will provide the much needed answers.

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