Interactions of Aging, Inflammation, and Neuronal Function: Synaptic Plasticity in Older Rodents is Altered Following a Peripheral Immune Challenge

by

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Abstract

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Thesis directed by Associate Professor Adjunct Susan L. Patterson

It is well accepted that early stages of cognitive decline and memory loss involve altered synaptic plasticity in the hippocampus and related structures. It has been proposed that calcium (Ca^{2+}) homeostasis in principal neurons of the hippocampus becomes 'dysregulated' with age. Specifically, a decline in *N*-methyl-D-aspartate receptors (NMDA receptors) and an increase in L-type voltage-dependent Ca^{2+} channels may reduce overall neuronal excitability and synaptic plasticity. This is believed to shift synaptic plasticity in the aged brain away from long-term potentiation (LTP) and toward long-term depression (LTD). Another major change that occurs with age is increased brain inflammation. It has been demonstrated in aged rodents that a peripheral immune challenge with *Escherichia coli* triggers a prolonged inflammatory response in the hippocampus, which impairs long-term memory consolidation in the days following. Because this process is dependent upon synaptic plasticity in region CA1 of the hippocampus, we explored LTP and LTD there as well changes in NMDA receptors and L- type Ca^{2+} channels. We found that a peripheral infection in aged rats decreases LTP following theta-burst stimulation but increases LTD following low frequency stimulations, both of which are dependent upon NMDA receptors. However, infection decreases a more robust LTP

following high frequency stimulation in young rats, and reduces the contribution of L-type Ca^{2+} channels in both young and aged rats. These effects are not associated with major changes in NMDA receptors or L-type Ca^{2+} channels in the synapse, and only the GluN2B subunit of NMDA receptor is significantly increased. Inflammation has been demonstrated to increase neural excitability, yet the overall effect of inflammation in our studies was reduced LTP and enhanced LTD. It is therefore possible that some of the changes in Ca^{2+} homeostasis and synaptic plasticity that occur with age are either caused by or activated as a neuroprotective mechanism against increased inflammation.

This work is dedicated to Jen, who has patiently supported me, and our two boys, Cole and Sam. They are the best part of life and will hopefully never grow up.

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Chapter 1

Introduction

Aging and Cognitive Decline

Improvements in social, economic, and medical standards in wealthy industrialized nations have caused an unprecedented increase in the average life expectancy in just four generations (Burger, Baudisch et al. 2012). Unfortunately, the reduction of mortality rates has led to a proliferation of age-related diseases for which there are few effective treatments. A major concern for the global healthcare system will be the management of an enormous population, an estimated 81 million by 2040, suffering from dementia and severe memory loss (Ferri, Ames et al. 2004). These patients in particular present a stressful burden on both healthcare workers and family members because of their need for full time care and monitoring. Current recommended preventative strategies like diet and exercise simply offer a tenuous solution, as they would only further increase the age of the population and, therefore, the likelihood of dementia (Brayne 2007). Continuing basic research into the causes and mechanisms of cognitive decline provides the best means for developing an effective and long-term strategy for a growing elderly population.

Dementia is not a specific disease but rather a general syndrome that encompasses a number of separate neuropsychological pathologies, including Alzheimer's disease and vascular dementia (Chapman, Williams et al. 2006). A diagnosis usually requires symptoms like memory loss and language difficulty that are severe enough as to interfere with daily, social, and occupational activities (Kaplan et al. 1994) More recently, a transitional stage between normal cognitive ageing and dementia has been loosely defined as mild cognitive impairment (MCI) (Stephan, Matthews et al. 2011). During this period, individuals experience a decline in memory performance that is noticeable to friends, family, and themselves, but not significant enough to meet the criteria for dementia (Petersen 2011). Whether MCI is a prodrome of dementia or a separate pathology entirely, it is nevertheless predictive of dementia (Stephan, Matthews et al. 2011).

The best current predictor of cognitive decline is simply age. The prevalence of dementia is 10% for those 65 and older, 30% for those 85 and older, and more than 50% for those over the age of 93 (Chapman, Williams et al. 2006). Additionally, the prevalence of MCI is estimated as high as 20% for those 65 and older and 29% for those 85 and older (Chapman, Williams et al. 2006). Unfortunately, measurable cognitive decline, independent of any known pathology, begins much earlier than 65. Cross-sectional and longitudinal studies demonstrate that working memory, reasoning, spatial visualization, and information processing speed begin to decline in healthy educated adults between their 20s and 30, and accelerates after the age of 60 (Hedden and Gabrieli 2004; Finch 2009; Salthouse 2009). Some of these

cognitive changes correlate with physiological changes in brain as it ages. These include volumetric decreases in frontal and temporal lobes, decreased dopamine and serotonin activity in the prefrontal cortex and striatum, and decreased or more diffuse activation of the prefrontal cortex. However, a major symptom of dementia and MCI is a steep decline in episodic memory, and this is almost certainly caused by changes in the aging hippocampus and its related structures.

An outstanding question in field of neuroscience is why the hippocampus is so vulnerable to aging. Although it was once believed that this vulnerability was due neurodegeneration caused by the progressive spread of Alzheimer's disease into the hippocampus (Small, Schobel et al. 2011), there is now an enormous amount of data, which will be discussed below, that attributes memory loss with earlier alterations in synaptic plasticity. If this is true, and it seems likely that it is, then the question becomes, "Why is synaptic plasticity in the hippocampus so vulnerable to age?" There have been numerous hypotheses and theories proposed that relate to more general issues of aging, including oxidative stress, mitochondrial dysfunction, and protein homeostasis (Bishop, Lu et al. 2010). Another theory that has gained prominence is inflammation.

Aging and Inflammation

It was once believed that aging led to a down-regulation of the innate immune response (Franceschi, Capri et al. 2007). However, an initial observation that peripheral blood mononuclear cells from healthy older adults were more

inflammatory than cells from younger adults launched the eventual reversal of this theory (Fagiolo, Cossarizza et al. 1993). It is now widely accepted that inflammation increases with age; and the term 'inflammaging' has been coined to describe this process (Franceschi, Capri et al. 2007; Perry, Cunningham et al. 2007; Freund, Orjalo et al. 2010). During acute inflammation in the periphery, tissue damage or the presence of foreign organisms induces an innate pro-inflammatory response that includes the release of cytokines and chemokines. This process usually ceases within hours to days. But as human gets older, this inflammatory response can become chronic, lasting for years. An important feature of chronic inflammation is that pro-inflammatory mediators are increased above baseline, but well below levels observed during acute inflammation. For example, two important pro-inflammatory cytokines, interleukin 6 (Il-6) and tumor necrosis factor alpha (TNFα) are 2- to 4-fold higher in serum of older individuals compared to younger individuals. This low-grade chronic inflammation has been associated with pathological diseases that are usually acquired with age, including diabetes, atherosclerosis, and cancer. (Freund, Orjalo et al. 2010).

As in the periphery, inflammation in the brain, frequently referred to as neuroinflammation, also increases with age. Neuroinflammation can be induced directly by neurodegeneration, or indirectly by sickness, injury, surgery, etc in the periphery (Glass, Saijo et al. 2010). During such an insult, macrophages in the peripher y release pro-inflammatory cytokines, which communicate with the brain via neural and circulatory routes to activate microglia, the major resident immune

cell of the brain (Perry, Cunningham et al. 2007). In contrast to macrophages in other tissues, microglia normally maintain a down-regulated, resting phenotype in the absence of insult, but upon stimulation, their morphology transforms and they secrete more pro-inflammatory cytokines like interleukin-1 beta (IL-1B), Il-6, and TNF (Ransohoff and Perry 2009). Older individuals are more likely to have an exaggerated neuroinflammatory response following a peripheral insult; and there is some evidence in humans and extensive evidence rodent that increased neuroinflammation is the result of microglial 'priming' (Sheng, Griffin et al. 1998; Wong 2013). Whether this is caused by increased signaling from the periphery, by another aspect of brain aging (neurodegeneration, oxidation, vascular changes, etc) or by an intrinsic change like cellular senescence, microglia are nevertheless sensitized to stimulation, releasing larger amounts of cytokines for a longer duration following a systemic inflammatory challenge (Barrientos, Frank et al. 2010; Cunningham 2011).

It has been hypothesized that as inflammation in periphery becomes less regulated with age, a sickness or injury could trigger or accelerate cognitive decline through sustained neuroinflammation (Zipp and Aktas 2006; Giunta, Fernandez et al. 2008). In agreement with this, clinical evidence demonstrates increased levels of inflammatory markers in serum before the diagnosis of dementia and Alzheimer's disease; and peripheral inflammation alone can induce symptoms akin to MCI (Stephan, Matthews et al. 2011). Furthermore, dementia patients with elevated pro-inflammatory cytokines in serum demonstrate an expedited loss of cognitive

function; and treatments that reduce systemic inflammation can slow the progression (Rosenberg 2005; Cunningham 2011). As mentioned before, dementia usually involves a loss of hippocampal function. It is probably not coincidental that the hippocampus is more vulnerable to the deleterious effects of microglia, just as it is more vulnerable to age. Some studies have demonstrated that the density of microglia is higher in the hippocampus than other brain region (Lawson, Perry et al. 1990; Mouton, Long et al. 2002; Wong 2013); and it has been widely observed in rodents, monkeys, and humans that more microglia transition to an activated phenotype in the aged hippocampus (Choi and Won 2011). Multiple studies, including those directly related to this dissertation, have demonstrated that aged rodents have an exaggerated inflammatory response in the hippocampus following an insult to the periphery. This in turn causes deficits in spatial and contextual memories that depend on the hippocampus (Barrientos, Higgins et al. 2006; Barrientos, Watkins et al. 2009). Therefore, if age-related inflammation can deteriorate hippocampal-dependent memories in rodents, it is certainly plausible that the age-related loss of episodic memory in humans could also be related to inflammation.

Aging and the Hippocampus

Based on numerous lesion studies in humans and animals, it is well accepted that the hippocampus and related structures are critical for recent episodic and spatial memories, but less so for older memories (Bird and Burgess 2008). Several

different theories have tried to explain this. For example, the Declarative Theory states that acquisition and conscious recollection of a new episodic memory depends on the concerted activity of the hippocampus and adjacent regions of the medial temporal lobe. But as the memory ages, it becomes "consolidated" to the neocortex and no longer depends on the hippocampus (Squire, Cohen et al. 1984). Alternatively, the Multiple Trace Theory proposes that all episodic memories, no matter how old, are always dependent on the hippocampus (Nadel and Moscovitch 1997). According to this theory, the hippocampus connects and stores a "trace" of neocortical information regarding the contextual information of a place and experience. Each time a memory is retrieved, a new trace is created. So as the memory ages and/or is repeatedly rehearsed, it become less vulnerable to disruption because it is represented by multiple traces. Regardless of how it might work, most theories identify the reciprocal connections between the hippocampus and neocortex as an important component of memory (Bird and Burgess 2008).

The hippocampus is defined by three structures, the dentate gyrus, CA3, and CA1. It receives multiple inputs from the neocortex and subcortical nuclei, but for simplicity, only the neocortical inputs will be described. The junction between hippocampus and neocortex is the entorhinal cortex. The hippocampus is commonly represented as a single, tri-synaptic circuit in which information flows from the entorhinal cortex to the hippocampus and back to the entorhinal cortex in a sequential manner (EC→DG→CA3→ CA1→EC). The superficial layers of the entorhinal cortex receives highly processed sensory information from the neocortex

via the perirhinal and parahippocampal cortex and projects to dentate gyrus via the perforant pathway. The dentate gyrus projects to CA3 pyramidal cells through the mossy fiber pathway. CA3 pyramidal cells then project to CA1 pyramidal cells through Schaffer collaterals. Finally, CA1 pyramidal cells, along with the subiculum, provide the major output of the hippocampus to the deep layer V of the entorhinal cortex. In turn, the entorhinal cortex re-connects via the parahippocampal gyrus to neocortical sites that produced the original input to the hippocampus (Bird and Burgess 2008).

The hippocampus has important anatomical features that are critical for memory. The dentate gyrus is theorized to have a role in 'pattern separation', in which contextual (temporal and spatial) information of similar episodes is made more dissimilar to create unique memories (Teyler and Rudy 2007). There are roughly ten times more granules cells in dentate gyrus than pyramidal cells in the entorhinal cortex, causing an expansion of the perforant pathway as it targets the hippocampus. This expansion could be critical for pattern separation. The CA3 (and CA1) is theorized that have a role in 'pattern completion', In this process, the hippocampus can retrieve the entire memory trace when presented with only a fragment of the original pattern (Bird and Burgess 2008). This is probably due to the fact that major output of CA3 neurons is not to CA1, but rather to other CA3 neurons through auto-association fibers along the longitudinal axis of the hippocampus. The dentate gyrus and CA3 working in conjunction as a 'pattern separator' and 'pattern completer' has been shown to be important for rapid

acquisition and later recall of spatial memories. In, addition, the entorhinal cortex can bypass the dentate gyrus and CA3 and connect directly to CA1 (Nakashiba, Young et al. 2008). And finally, the CA1 integrates converging input from CA3 and entorhinal cortex to create an allocentric spatial map of one's surroundings and is important for the consolidation of episodic memories (Bartsch, Schonfeld et al. 2010).

Structural and functional magnetic resonance imaging in humans has demonstrated that subregions of the hippocampus are differentially affected by age and age-related pathologies. The hippocampus as a whole decreases 2-3% in volume every decade in healthy adults, but this is not related to any significant cognitive decline (Hedden and Gabrieli 2004). Only later in life (>70 years), when the hippocampus begins to shrink at an accelerated rate (1% per year), does it become predictive of diminished memory performance. High-resolution imaging has shown that volume loss under normal aging is more profound in the dentate gyrus and CA3 (Small, Schobel et al. 2011). Volume loss in CA1 and also occurs with age, but tends to be associated with age-related vascular disease that have an inflammatory component, like hypertension, ischemia, and hypoxia, The entorhinal cortex usually maintains it volume until advanced age in healthy adults. Conversely, in a pathological condition like Alzheimer's, there is extensive volume loss in entorhinal cortex, CA1, and subiculum, without any additional loss of volume in DG and CA3 (Small, Schobel et al. 2011). The hippocampus and specific subregions exhibit a general decline in basal metabolism as measured by fMRI and blood oxygen level-

dependent (BOLD) respsonses. However, in the case of aging, a basal BOLD response can be confounded by underlying neurovascular factors and might not truly reflect neural metabolism or activity. For example, non-demented older adults with a reduced ability to discriminate similar objects actually displayed hyperactivity in the DG and CA3 during a task that assessed pattern separation (Yassa, Lacy et al. 2010). Furthermore, patients with MCI, a possible indicator of early AD, had similar hyperactivity in DG and CA3 but conversely had hypoactivity in the entorhinal cortex during the same pattern separation task (Yassa, Stark et al. 2011).

The cause of hippocampal volume loss in humans depends on the subregion and whether the nature of aging is normal or pathological. In Alzheimer's disease, which is clearly pathological, the erroneous regulation of the amyloid precursor protein and tau provokes a slow accumulation of extracellular plaques and intracellular neurofibrillary tangles (Small and Gandy 2006). This eventually causes cell death in the entorhinal cortex and CA1, and leads to devastating memory loss. However, under conditions of normal aging, most post-mortem histological studies in humans indicate that there is no substantial loss of principle neurons in the hippocampus, despite discernable volume loss and impairments in cognition (Hedden and Gabrieli 2004). Furthermore, aged rodents, like their human counterparts, also experience memory impairments, even though they do not develop Alzheimer's disease and do not exhibit significant neuronal loss (Morrison and Baxter 2012). In fact, studies of normally aging rodents have revealed that age-

related memory decline begins with early alterations in synaptic plasticity, long before there is gross neurodegeneration of the hippocampus (Finch 2009).

Synaptic Plasticity: Long-Term Potentiation and Long-Term Depression

Synaptic plasticity exists throughout the rodent brain and is believed to be the cellular basis for numerous behaviors, including learning and memory (Malenka and Bear 2004). It is usually evoked in the form of long-term potentiation (LTP), which, in its most basic form, represents an enhancement of excitatory transmission at glutamatergic synapses (Malenka and Bear 2004). LTP was initially discovered in the dentate gyrus (Bliss and Gardner-Medwin 1973), but is now, along with long-term depression (LTD), most commonly studied within the Schaffer collaterals of hippocampal slices, where axons of CA3 pyramidal neurons terminate on apical dendrites of CA1 pyramidal neurons (Bliss, Collingridge et al. 2007). Because hippocampal slices are easy to prepare and the Schaffer collaterals remain well preserved after dissection, an enormous amount of data regarding the mechanism of synaptic plasticity has been generated from CA3-CA1 glutamatergic synapses (Bliss, Collingridge et al. 2007). It should be noted that stimulations that reliably induce LTP and LTD in CA1 of hippocampal slices also cause similar persistent plasticity in CA1 of freely behaving rodents, although not always (Manahan-Vaughan and Schwegler 2011; Buschler, Goh et al. 2012).

LTP has certain properties that accord with various principles of long-term memory, including Donald Hebb's famous postulate. Hebb proposed that when a

presynaptic neuron reliably causes the firing of a postsynaptic neuron, the synaptic connection between them strengthens (Hebb, 1949). LTP faithfully fulfills this and others aspects of Hebb's theory, including input specificity (only active synapses are strengthened) and associativity (weak stimulation of two pathways strengthens both if done simultaneously, but not independently) (Markram, Gerstner et al. 2011). Finally, much like a memory trace, LTP is persistent, and can last anywhere from minutes to hours *in vitro*, and days to month *in vivo* (Kumar 2011).

LTD is another form of 'Hebbian' plasticity that has the opposite effect of LTP. Although Hebb did not propose an active way to weaken synapses, others proposed the possibility of LTD as the inverse to 'Hebbian' strengthening (Stent 1973; Markram, Gerstner et al. 2011). Years later, LTD was discovered. Whereas synaptic strengthening is induced by a brief, but intense stimulus, synaptic weakening is induced by a weak but sustained stimulus (Malenka and Bear 2004). As with LTP, LTD demonstrates input specificity, associativity, and persistence (Markram, Gerstner et al. 2011). While it is debated whether LTP and LTD are a related form or two distinct forms of synaptic plasticity, they do employ similar and distinct mechanisms (Braunewell and Manahan-Vaughan 2001).

Induction of Synaptic Plasticity

LTP is most commonly induced by brief bursts of high frequency electrical stimulation (typically 100 Hz). A single, one-second burst of 100 Hz typically produces a brief form of LTP that lasts 30 minutes to 2 hours, while multiple burst

of 100 Hz causes LTP that last longer than 3 hours (Kumar 2011). An even higher frequency stimulation protocol uses multiple bursts of 200 Hz to achieve LTP (Grover and Teyler 1990). Since 100 and 200 Hz is beyond the typical firing rate of a neuron, a more physiological pattern, theta burst stimulation (TBS) was developed. The theta burst is based off a theta rhythm (~5 Hz), which is produced by the coordinated firing of hippocampal pyramidal neurons while an animal explores and learns an environment (Bland 1986). By delivering multiple bursts of four 100 Hz pulses, five times a seconds, researchers are able to effectively induce LTP that lasts for hours in a hippocampal slice (Larson, Wong et al. 1986; Raymond 2007). LTP can also be chemically induced by tetraethylammonium (TEA), a voltage-gated potassium channel blocker that broadens the action potential and reduces the afterhyperpolarization phase, leading to increased neurotransmitter release general excitability (Aniksztejn and Ben-Ari 1991; Huber, Mauk et al. 1995; Mathie, Wooltorton et al. 1998).

As opposed to LTP, LTD is commonly induced by long, low frequency stimulations. Although the frequency (1-10 Hz) and the number of pulses vary across protocols, the most commonly used is 900 pulses at 1 Hz. Finally, LTD, like LTP, can be chemically induced by group I metabotropic glutamate receptor agonists (Malenka and Bear 2004; Massey and Bashir 2007).

The cellular mechanisms that mediate LTP and LTD in the Schaffer collaterals are vast, but are usually categorized by their involvement in either the induction or maintenance of synaptic plasticity. The induction of most forms of

LTP, especially by brief 100 Hz stimulation, theta-burst stimulation, and intermediate ranges of high frequency stimulation (20-50 Hz), rely mainly on *N*-methyl-D-aspartate (NMDA) receptors; and have been termed 'NMDA receptor-dependent LTP (Malenka and Bear 2004; Rebola, Srikumar et al. 2010). Importantly, the induction of LTD by low frequency paradigms is also dependent upon NMDA receptors. When CA3 axonal fibers are stimulated, glutamate is released from presynaptic terminals and binds to two ionotropic glutamate receptors at CA1 postsynaptic terminals: α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors and NMDA receptors. Upon glutamate binding, AMPA receptors rapidly open and the resulting influx of sodium ions (Na^+) causes synaptic depolarization. However, NMDA receptors have a magnesium ion (Mg^{2+}) blocking the pore of the channel at rest. When glutamate binds to NMDA receptors, there must be a sufficient level of depolarization via AMPA receptors (or another source) to expel the Mg^{2+} block. In this way, the NMDA receptor acts as a molecular coincidence detector because they are both ligand- and voltage-sensitive. Once activated, NMDA receptors influx calcium ions (Ca^{2+}), which initiate signaling cascades important for plasticity. While paradigms that induce LTP cause a brief and rapid rise of synaptic Ca^{2+}, those that induce LTD cause a prolonged but modest rise of intracellular Ca^{2+}. The exact dynamics of the intracellular Ca^{2+} rise seem to be critical for the direction and magnitude of plasticity (Massey and Bashir 2007; Yashiro and Philpot 2008).

There are other forms of LTP induction that recruit additional sources of Ca^{2+} in the postsynaptic terminal. While LTP produced by brief stimulations of 100 Hz and theta-burst are entirely dependent upon NMDA receptors, even stronger stimulations (200 Hz, multiple trains of theta bursts) activate L-type voltage-dependent Ca^{2+} channels (L-VDCC or L-type Ca^{2+} channels) (Grover and Teyler 1990). These forms of LTP have been termed 'VDCC-LTP' or 'compound LTP' (Blundon and Zakharenko 2008; Foster 2012). L-type Ca^{2+} channels are enriched in the hippocampus and are found in clusters on the soma, along dendrites, and on dendritic spines of pyramidal neurons (Greer and Greenberg 2008). They require either a strong depolarization or a back-propagating action potential to open, but once so, they flux in large amounts of Ca^{2+} (Regan, Sah et al. 1991). L-type channels and are closely linked to the endoplasmic reticulum (ER), which spans dendrites and act as an intracellular Ca^{2+} store. The influx of Ca^{2+} via L-type channels activates ryanodine receptors (RyR) on the ER, releasing additional Ca^{2+} into the dendrite and postsynaptic terminal (Foster 2012). In addition, tetraethylammonium (TEA) also induces a compound LTP that can be pharmacologically divided into an NMDA receptor and L-type Ca^{2+} channels component (Huber, Mauk et al. 1995).

Maintenance of Synaptic Plasticity

The maintenance of LTP and LTD requires a diverse array of signaling molecules, many of which are regulated by Ca^{2}+ (Malenka and Bear 2004). As noted before, the duration of LTP typically depends on the strength of stimulation. This is

because the magnitude of the intracellular Ca^{2+} rise that follows LTP induction determines the degree of signaling cascade. Early-phase LTP (E-LTP), which lasts ~30 minutes to ~2 hours, requires protein kinase activity and ion channel trafficking. Late-phase LTP (L-LTP), which lasts beyond 3 hours, requires the synthesis of additional ion channels, signaling molecules, and growth factors from transcription and translation (Kumar 2011). Two important signaling molecule for E-LTP are calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA), both of which indirectly activated by an intracellular rise in Ca^{2+} via calmodulin and adenyl cyclase, respectively. Once activated, CamKII and PKA directly phosphorylate the GluA1 subunit of AMPA receptors, increasing AMPA channel conductance rates, and also have an indirect role in the trafficking and insertion of additional AMPA receptors into the synapse. The net effect of these two processes is an enhancement of the EPSP following LTP induction (Shepherd and Huganir 2007; Kessels and Malinow 2009; Lisman, Yasuda et al. 2012). There are a number of other Ca^{2+}-dependent kinases involved in the maintenance of LTP, including protein kinase C (PKC), protein kinase M zeta (PKMξ), phosphatidylinositol 3-kinase (PI3 kinase), mitogen-activated protein kinases (MAPKs), and tyrosine kinases Src and Fyn (Malenka and Bear 2004).

In contrast to LTP, the prolonged but modest rise of intracellular Ca^{2+} that occurs during LTD preferentially activates phosphatases rather than kinases, which is correlated with the dephosphorylation of AMPA receptors. LTD also initiates the binding of clathrin adaptor protein AP-2 to the GluA2 subunit, leading to the rapid

internalization of AMPA receptors (Shepherd and Huganir 2007; Kessels and Malinow 2009). It is clear that the exo- and endocytosis of synaptic AMPA receptors is a major mechanism regulating synaptic plasticity.

LTP and LTD are also associated with the morphological restructuring of synapses. Excitatory synapses usually form on small protrusions on the dendrite called dendritic spines. Dendritic spines vary in shape and size, and range from the immature, thin and elongated shape to a more mature, mushroom-like shape, with a large head and thin neck. A disc-like structure termed the postsynaptic density (PSD) forms beneath the membrane at the head of the synapse and contains scaffolding proteins that organize glutamate receptors, ion channels, intracellular signaling molecules and cytoskeletal components. It generally believed that the induction of L-LTP increases spine head volume, PSD area, and the number of synaptic AMPA receptors (Malenka and Bear 2004; Bosch and Hayashi 2012). LTP can also cause the emergence of new spines. Conversely, LTD causes spine retraction and loss (Nagerl, Eberhorn et al. 2004). The expansion or contraction of spine heads appears to be mediated by the polymerization and depolymerizatoin rate of the actin filaments (F-actin); and just as NMDA receptor antagonists and Ca^{2+} blockers inhibit the induction of LTP and LTD, they also inhibit the structural modifications that accompany them (Bosch and Hayashi 2012). Intriguingly, dendritic spine expansion following LTP was demonstrated to be more persistent in smaller, thin spines, but only transient in mature, mushroom-like spines

(Matsuzaki, Honkura et al. 2004). It line with this, larger spines are more likely to undergo LTD than smaller spines (Yashiro and Philpot 2008).

Metaplasticity

Despite the fact that many stimulation protocols used to induce synaptic plasticity are likely biologically implausible (Markram, Gerstner et al. 2011), it is widely accepted that the hippocampus is probably using some form of LTP and LTD to acquire and store new information. Nevertheless, LTP and LTD cannot exist in the brain as simple forms of Hebbian-like plasticity because they would leave neural networks prone to destabilization and excitotoxicity (Cooper and Bear 2012). There seems to be other mechanisms in place that prevent network saturation or rundown by LTP or LTD. The theory of 'metaplasticity' states that prior neural activity at a synapse affects how it responds to later activity (Abraham 2008). For example, induction of LTP in CA1 reduces subsequent LTP by the same stimulus. Importantly, this is not simply the result of ceiling effect because it can be overcome by increasing the stimulus intensity. Rather it seems that LTP elevates the threshold for subsequent LTP. Conversely, LTP reduces the threshold for future LTD. Metaplasticity creates an important caveat to synaptic plasticity which is that one must consider the history of the synapse when trying to predict its response to a given stimulus.

The theory of metaplasticity can be best explained by the Bienenstock, Cooper, Munro computational model of synaptic plasticity (Cooper and Bear 2012). The BCM theory was developed in 1982 to explain the effects of monocular deprivation on plasticity in the visual cortex. However, it has proven to be much a broader principle of synaptic plasticity and predicted the existence of LTD in the hippocampus. The model has two features that explain both the selectivity and stability of synaptic plasticity. First, it describes bidirectional plasticity as a function of postsynaptic activity. According to the theory, synapses have threshold level of response to a given stimulus termed the modification threshold (θ). If postsynaptic activity is less than the modification threshold, the synapse will undergo depression, but if activity is greater than the modification threshold, the synapse will undergo potentiation. The second and probably most important feature of the BCM theory is that the modification threshold automatically adjusts with direction of synaptic plasticity. If a synapse undergoes potentiation, the modification threshold slides to the right. This means that the synapse now requires a greater level of postsynaptic activity to maintain itself, and an even greater level to undergo further potentiation. If the activity falls below the new modification threshold, the synapse undergoes depression, and the modification threshold slides back to the left. When one replaces the level of postsynaptic activity on the x-axis with the typical frequency ranges of hippocampal plasticity studies, the BCM model accurately predicts why previous LTP reduces future LTP, but enhances future LTD (Figure 1.1). More recent derivations of the BCM model have

theorized that bidirectional synaptic plasticity is regulated by postsynaptic intracellular Ca^{2+} dynamics, and place NMDA receptor composition in a critical role. (yeung cl 2004, philpot)

Figure 1.1

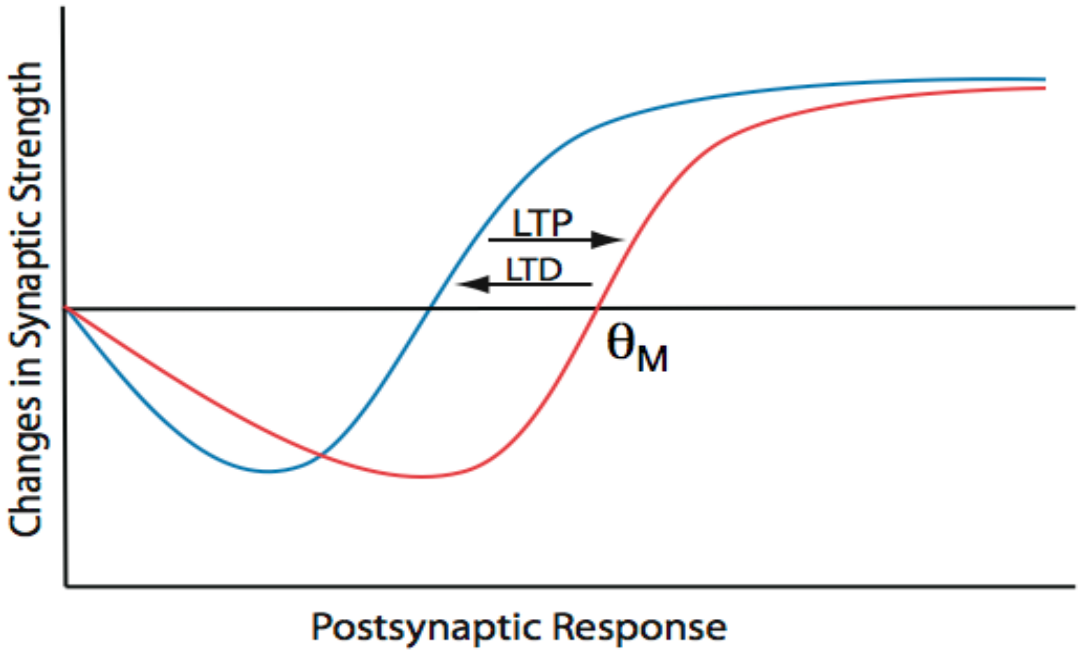

Figure 1.1 The Bienenstock, Cooper, Munro computational model of synaptic has two important features. (1) Bidirectional synaptic plasticity (LTP or LTD) is mediated as a function of postsynaptic activity. If activity is above the modification threshold (θ_M) the synapse will undergo potentiation. If activity is below the modification threshold, the synapse will undergo depression. (2) The modification

threshold is adjustable. If a synapse undergoes potentiation, the modification threshold shifts to right, making further potentiation harder, but depression easier. If a synapse undergoes depression, the modification threshold shifts to left, making further depression harder, but potentiation easier.

Aging and Synaptic Plasticity

It is no coincidence that the three sources of Ca^{2+} needed for the induction of synaptic plasticity (NMDA receptors, L-type Ca^{2+} channels, and intracellular Ca^{2+} stores) are also the major components of the 'Calcium Hypothesis of Brain Aging' (Thibault, Gant et al. 2007; Kumar, Bodhinathan et al. 2009). This hypothesis states that age-related changes of Ca^{2+} sources leads to altered Ca^{2+} homeostasis, thereby changing the intrinsic excitability and plasticity of aged principle neurons. Altered Ca^{2+} homeostasis has been detected in both aged humans and rodents, and shows greater expression in CA1 of the hippocampus in both species. The theory has been utilized to explain very specific deficits in LTP in aged rodents (Foster 2012).

Many rodent strains demonstrate alterations in LTP in Schaffer collaterals with advanced age; however, this decline is not observed with all stimulation protocols used. LTP elicited by weak paradigms (25-50 Hz, one theta burst, etc) appears to be vulnerable to age, but more robust LTP paradigm (100, 200 Hz, etc) produces equivalent LTP in young and old rodents alike (Burke and Barnes 2006; Burke and Barnes 2010). As note before, the induction of LTP by a weak protocol relies on Ca^{2+} from NMDA receptors while stronger protocols recruit additional Ca^{2+} from L-type channels and RyRs (Grover and Teyler 1990). Some aged rodents strains that have decreased NMDAR-LTP conversely display increased NMDAR-LTD (Norris, Korol et al. 1996). The selectivity of these plasticity deficits would indicate that aging preferentially reduces NMDA receptor function in the hippocampus. A number of studies suggest that this is true. Many aged rodent

strains have less NMDA receptor mRNA and protein in the hippocampus and demonstrate reduced receptor agonist/ antagonist binding (Magnusson, Brim et al. 2009). Furthermore, the NMDA receptor component of synaptic transmission is consistently reduced in CA1 of aged rodents; and NMDA receptors contribute less Ca^{2+} following robust LTP inductions (Norris, Halpain et al. 1998; Shankar, Teyler et al. 1998; Foster 2012). It is not exactly known why NMDA receptor function diminishes with age, but it has been linked to oxidative stress and a reduction in d-serine, an important glial-derived co-agonist (Magnusson, Brim et al. 2009). Alternatively, evidence suggests that NMDA receptor function is indirectly reduced by an age-related increase in L-type channel activity (Landfield and Pitler 1984; Foster 2012).

As opposed to NMDA receptors, aging causes increased membrane expression of L-type channels and enhanced L-type Ca^{2+} currents in CA1 pyramidal neurons; but how this affects learning and memory is debated (Thibault and Landfield 1996; Kumar 2011). An early study demonstrated that young and aged rats could achieve a similar magnitude of LTP in CA1 following a robust LTP protocol (200 Hz). However, a pharmacological dissection revealed that a reduction in the NMDA receptor component in aged compared to young rats was compensated for by a greater L-type channel component (VDCC-LTP) (Shankar, Teyler et al. 1998). Using the same LTP protocol, a more recent study demonstrated that aged rats with a large VDCC-LTP performed better on a spatial memory task than age-matched rats with a smaller VDCC-LTP, despite both groups having reduced NMDAR-LTP

(Boric, Munoz et al. 2008). The authors suggested an age-related compensatory mechanism that preserves Ca^{2+} homeostasis as NMDA receptor activity fades. However, it has been demonstrated that increased L-type channel activity indirectly reduces NMDA receptor function by altering the neuronal membrane potential. (Kumar, Bodhinathan et al. 2009) L-type channel activity stimulates additional Ca^{2+} release from intracellular stores via RyRs. In the dendrites, this large rise in intracellular Ca^{2+} activates SK channels, causing potassium (K^+) efflux and hyperpolarization. Following a burst of action potentials, this Ca^{2+}-dependent, K^+ efflux mediates a slow afterhyperpolarization (sAHP), which is a well-known biomarker of aging . Both the magnitude and duration of the sAHP is increased in aged rats, and blocking either L-type channels or RyRs reduces it (Disterhoft and Oh 2006). Because NMDA receptors are voltage-gated and require sufficient depolarization to open, dendritic hyperpolarization and a larger sAHP is thought to reduce NMDAR activity (Foster 2012). Treatments that reduce the sAHP in aged rodents, including blocking L-type channels, RyRs, or SK channels, increase weak forms of NDMAR-LTP (5-25 Hz) and enhance NMDAR-LTD in aged animals (Norris, Halpain et al. 1998). From this evidence it has been proposed that as the entry of Ca^{2+} shifts from NMDA receptors to L-type channels and intracellular Ca^{2+} stores in aged animals, the threshold of synaptic plasticity shifts toward LTD and away from LTP (i.e. metaplasticity) (Kumar 2011).

Inflammation and Synaptic Plasticity

As noted earlier, microglia activation and chronic inflammation has been well characterized in the aged brain, particularly in the hippocampus (Perry, Cunningham et al. 2007; Wong 2013). This has prompted many researchers to consider what proportion of age-related effects on synaptic plasticity is actually caused by increased brain inflammation (Lynch 2010). *In vivo* recordings in the dentate gyrus of aged rats have found that decreased LTP is consistently correlated with increased levels of pro-inflammatory cytokines (Il-1B, Il-6, TNFα, and interferon-γ), decreased levels of anti-inflammatory cytokines (Il-4), and increased expression of markers of microglial activation (Nolan, Maher et al. 2005; Griffin, Nally et al. 2006; Maher, Clarke et al. 2006). Furthermore, reversal of microglial activation by minocyline, inhibition of IFNγ and Il-1B by insulin-like growth factor (IGF-1), and manipulation of Il-4 levels all restored LTP in aged rats. Other treatments that lower inflammation also have similar restorative effects on *in vivo* LTP (Cowley, O'Sullivan et al. 2012; Murphy, Cowley et al. 2012). Reduced LTP (100 Hz) in CA1 of hippocampal slices also been demonstrated following peripheral inflammation (Di Filippo, Chiasserini et al. 2013); and chronic inflammation induced by continual intracerebroventricular infusion of lipopolysaccharides, a potent antigen of microglia, reduced NMDA receptor-dependent LTP and LTD and L-type Ca^{2+} channel dependent LTP (Min, Quan et al. 2009). Others have demonstrated that Il-1β reduces glutamate release (Murray, McGahon et al. 1997), inhibits voltage-gated Ca^{2+} channel currents (Plata-Salaman and ffrench-Mullen

1994), decreases surfaces expression of AMPA receptors (Lai, Swayze et al. 2006), and increases GABAergic inhibition (Hellstrom, Danik et al. 2005), all of which could participate in the down-regulation of LTP. Very little is known regarding the effects of chronic inflammation on LTD.

While many studies are fairly in agreement that chronic inflammation in the brain reduces LTP, there is less consensus of how acute inflammation affects LTP, with particular regards to Il-1β. It has been demonstrated that acute bath application (15-30 minutes) of Il-1β to hippocampal slices reduces LTP induction in the dentate and CA1 (Bellinger, Madamba et al. 1993; Coogan and O'Connor 1997). In CA1, Il-1β reduced LTP following a 100 Hz tetanus. In the dentate, Il-1β treatment reduced electrical LTP following a 100 Hz tetanus and chemical LTP following a brief application of tetraethylammonium (TEA), which indicates a possible effect on NMDA receptors and/or L-type Ca^{2+} channels (Coogan and O'Connor 1999). In fact, it was later shown in the dentate of hippocampal slices that Il-1β by itself depresses isolated NMDA receptor-dependent excitatory postsynaptic potential (NMDA-EPSP) amplitude without altering AMPA-EPSP amplitude (Coogan and O'Connor 1997). Possibly in agreement with this, bath application of Il-1β was also shown to induce LTD in CA1, without the need of low frequency stimulation (Ikegaya, Delcroix et al. 2003). It should be noted that at least some Il-1β is needed for the full expression of LTP because Il-RA reduces the maintenance phase of potentiation, and Il-1β, Il-1α, and Il-6 gene expression is robustly induced by LTP tetenization (Schneider, Pitossi et al. 1998; Ross, Allan et al. 2003). Even

the very act of preparing hippocampal slices increases cytokine expression (Jankowsky, Derrick et al. 2000).

However, there is also a substantial amount of data that suggests that pro-inflammatory cytokines facilitate neuronal excitability and excitotoxicity. Cytokines are commonly viewed as pro-convulsants in epilepsy as there is a strong association between cytokine levels and seizure occurrence and duration; and blockade of cytokine action by IL-1 receptor antagonist (IL-1Ra) reduces seizure severity. Interestingly, peripheral inflammation also enhances seizure generation and reduces the threshold needed to induce seizures (Galic, Riazi et al. 2012). Although Il-1β is important for the reduction of LTP, it counter intuitively may be important for increasing neural excitability, and NMDA receptors appear to be its target. It has been observed in culture that Il-1β enhances NMDA receptor Ca^{2+} currents (Yang, Liu et al. 2005)) and work by Vivianni *et al.* have demonstrated a specific mechanism via phosphorylation of NMDA receptors(Viviani, Bartesaghi et al. 2003; Viviani, Gardoni et al. 2006). This increases NMDA receptor binding to PSD-95, resulting in synaptic stabilization and greater Ca^{2+} influx (Sanz-Clemente, Nicoll et al. 2012). Furthermore, the Il-1 receptor (Il-1R) directly interacts with the NMDA receptor subunits, NR2B, and enriches there following Il-1β treatment (Gardoni, Boraso et al.).

L-type Ca^{2+} channels are also affected by cytokines. Separate treatments of cultured neurons with Il-1β, Il-6, and Il-8 all caused decreased L-type Ca^{2+} channel membrane expression (Zhou, Tai et al. 2006; Vereyken, Bajova et al. 2007). There

are other effects of cytokines in cultured cells that also suggest a role in excitability, including decreased glutamate uptake by astrocytes and an acute decrease in $GABA_A$ mediated potentials (Hu, Sheng et al. 2000; Wang, Cheng et al. 2000).

It is difficult to reconcile findings that cytokines reduce synaptic plasticity with findings that cytokines enhance neural excitability. A similar dichotomy seems to exist for cytokine effects on NMDA receptors and L-type Ca^{2+} channels. Some have proposed that the differences are related to concentration of cytokines, the length of exposure, and the preparation used (cultures, slices, or *in vivo*) (Viviani, Gardoni et al. 2007; Galic, Riazi et al. 2012). For example, acute application of Il-1β causes GABAergic attenuation, but longer exposure causes facilitation. If cytokines directly effect neurons, synapses, and ion channels, as has been shown, then it is possible that longer exposures could induce homeostatic changes in neurons to reduce overall levels of excitability, which would impact synaptic plasticity and metaplasticity.

Aging, Inflammation, and Synaptic Plasticity

Many of the age-related changes that alter Ca^{2+} homeostasis and change synaptic plasticity may be part of larger function to preserve neuronal viability. It has been proposed that as neurons age, a decrease in NMDA receptor activity and an increase in L-type Ca^{2+} channel activity and intracellular Ca^{2+} release might functionally reduce excitotoxicity, but as a consequence, alter synaptic plasticity (Kumar, Bodhinathan et al. 2009). However, it is not well understood how chronic

inflammation interacts with brain aging. Do changes in Ca^{2+} homeostasis and synaptic plasticity occur before the onset of low-grade inflammation and microglia sensitization? If so, then how does increased expression of cytokines interact with these changes? If cytokines acutely enhance NMDA receptor function while reducing L-type Ca^{2+} channel currents, as has been shown in culture, this could act counter to the neuroprotective effects of aging. Older neurons might become more excitable, which could lead to aberrant and unspecific potentiation of synapses in NMDA receptor-dependent manner. According to the rules of BCM-like metaplasticity, this would increase and decrease the threshold for NMDA receptor-dependent LTP and LTD, respectively. An effect like this could explain the contradiction of how acute inflammation increases neural excitability yet chronic inflammation reduces synaptic potentiation. Another possibility is that microglia sensitization and chronic inflammation precede the age-related alterations in Ca^{2+} homeostasis. In this case, chronic cytokine expression could be the cause of the reduced NMDA receptor currents and NMDA receptor-dependent LTP observed with age. This might then lead to a compensatory up-regulation of L-type Ca^{2+} channel activity and intracellular Ca^{2+} release to maintain Ca^{2+} levels and prevent neuronal death.

Experimental Synopsis

This following experiment in this dissertation used a rodent model developed and characterized by colleagues that mimics the disruption of memory that the

elderly can experience following a sickness or major injury (Barrientos, Higgins et al. 2006; Barrientos, Frank et al. 2009; Barrientos, Watkins et al. 2009). It demonstrates that a peripheral infection causes an enduring elevation of the Il-1β in the hippocampus of pre-senescent, aged F344xBN rats. The F1 hybrids of a cross between F344 and Brown Norway rats (F344xBN) are widely used for aging studies because they are genetically healthier and live longer (~34-39 months) than other inbred rat strains (~25-30 months) (Markowska and Savonenko 2002; Newton, Forbes et al. 2005). At 24 months, when other rat strains have spatial memory deficits, F344xBN rats have only minimal impairments (Foster 2012) However, following an intraperitonneal injection of *Escherichia coli* (*E. coli*), 24 month-old F344xBN rats exhibit a robust neuroinflammatory response in the hippocampus, and Il-1β levels remain elevated (> 2 fold over basal) in the hippocampus for at least 8 days. During this period, aged rats demonstrate specific deficits for contextual fear conditioning, a hippocampal-dependent memory task. Importantly, only long-term memory, and not short-term memory, is affected (Barrientos, Watkins et al. 2009). It is therefore possible that exaggerated Il-1β in aged rats following a peripheral immune challenge might alter synaptic plasticity in the hippocampus.

The initial experiments in Chapter 2 were designed by my advisor, Dr. Susan Patterson, to characterize the basic parameters of synaptic transmission, E-LTP, L-LTP in region CA1 of aged rats following peripheral inflammation. The experiments in Chapter 3 were meant to determine if a prolonged inflammation in the

hippocampus altered metaplasticity as well as the synaptic content of NMDA and AMPA receptors. Finally, experiments in Chapter 4 were meant to determine if age and/or a peripheral infection affected LTP that is dependent on L-type Ca^{2+} channels.

Chapter 2

Synaptic Correlates Of Increased Cognitive Vulnerability With Aging: Peripheral Immune Challenge and Aging Interact to Disrupt Theta-BurstL-LTP in Hippocampal Area CA1

Abstract

Variability in cognitive functioning increases markedly with age, as does cognitive vulnerability to physiological and psychological challenges. Exploring the basis of this vulnerability may provide important insights into the mechanisms underlying aging-associated cognitive decline. As we have previously reported, the cognitive abilities of aging (24-month-old) F344xBN rats are generally good, but are more vulnerable to the consequences of a peripheral immune challenge (an i.p. injection of live *E. coli*) than those of their younger (3-month-old) counterparts. Four days after the injection, the aging, but not the young rats show profound memory deficits, specific to the consolidation of hippocampus-dependent memory processes. Here, we have extended these observations, using hippocampal slices to examine for the first time the *combined* effects of aging and a recent infection on several forms of synaptic plasticity. We have found that the specific deficit in long-

lasting memory observed in the aged animals following infection is mirrored by a specific deficit in a form of long-lasting synaptic plasticity. The late-phase long-term potentiation (L-LTP) induced in area CA1 using theta burst stimulation is particularly compromised by the combined effects of aging and infection – a deficit that can be ameliorated by intra-cisterna magna administration of the naturally occurring anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra). These data support the idea that the *combination* of aging and a negative life event such as an infection might produce selective, early-stage failures of synaptic plasticity in the hippocampus, with corresponding selective deficits in memory.

Introduction

Although it is not clear that a decline in the ability to learn and remember is a normal feature of aging, it is clear that *variability* in cognitive functioning increases with aging in humans (Laursen 1997; Unverzagt, Gao et al. 2001) and animals (Gage, Dunnett et al. 1984; Barnes and McNaughton 1985; Deupree, Turner et al. 1991; Gallagher, Bizon et al. 2003). An intriguing clue about sources of this variability comes from the observation that aging increases cognitive *vulnerability* to challenging life events such as infection (Wofford, Loehr et al. 1996), surgery (Bekker and Weeks 2003), heart attack and psychological stress (VonDras, Powless et al. 2005).

Because little is known about the mechanisms that mediate aging-associated increases in cognitive vulnerability, we have developed a rodent model to study

them (Barrientos, Higgins et al. 2006). 24-month-old Fischer344/Brown Norway rats generally do not display significant physical or cognitive impairments prior to a brief infection produced by an intraperitonneal injection of *E. coli*. However, after recovering from the active infection, the aged animals show significant impairment in hippocampus-dependent memory tasks (e.g. contextual fear and place learning); the young animals generally do not (Barrientos, Higgins et al. 2006).

Data from conventional aging models examining variability in cognitive functioning with aging *per se* suggest that when age-related deficits in hippocampus-dependent learning occur, they do not arise from a loss of hippocampal neurons or synapses (Rapp and Gallagher, 1996; Geinisman et al., 2004), but rather from more subtle alterations in synaptic efficacy (Rapp, Stack et al. 1999; Smith, Adams et al. 2000). Not surprisingly, some age-related neurodegenerative disorders (e.g. Alzheimer's Disease) manifest themselves as disorders of synaptic plasticity prior to the onset of overt cellular pathology (Selkoe 2002). Thus, synaptic plasticity, particularly LTP, has been extensively studied in aging and disease models (Barnes and McNaughton 1985; Deupree, Turner et al. 1991; Diana, Domenici et al. 1995; Bach, Barad et al. 1999; Tombaugh, Rowe et al. 2002) (Martin, Grimwood et al. 2000; Bliss, Collingridge et al. 2003).

These earlier studies have reinforced the idea that synaptic plasticity has multiple forms: short-term forms (e.g., early-phase LTP or E-LTP) involving the covalent modification of existing proteins, and long-lasting forms (e.g. late-phase LTP or L-LTP) that require transcription and translation (Bliss, Collingridge et al.

2007). It has also become apparent that different stimulation paradigms can evoke similarly sized, and similarly enduring, manifestations of synaptic plasticity – for example L-LTP - that nonetheless arise from distinct biochemical processes, and may reflect different information storage processes with differential vulnerabilities to disruption (e.g. Woo et al., 2000; Patterson et al., 2001). Thus, examining the impact of aging and immune challenge on these processes should ultimately provide mechanistic insights into aging-associated cognitive vulnerability.

In the present study, we have examined several forms of synaptic plasticity in hippocampal slices from young and aged rats, with and without a recent history of *E. coli* infection as a first step in utilizing the slice system to examine the cellular and molecular mechanisms underlying the memory deficits evoked by immune challenge in aged animals.

Materials and Methods

Animals

The rats were 3- and 24-month old male Fischer344/Brown Norway F1 crosses from the NIA Aged Rodent Colony. Animals were pair housed, on a 12-hr light dark cycle, with *ad libitum* access to food and water. Upon arrival, rats were allowed to acclimate to the animal facility for two weeks before experiments were begun. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

***E. coli* Infection**

One day prior to the start of experimentation, stock *E. coli* cultures (ATCC 15746; American Type Culture Collection, Manassas, VA) were thawed and cultured overnight (15–20 h) in 40 mL of brain-heart infusion (BHI; DIFCO Laboratories, Detroit, MI) in an incubator (37 ^{O}C, 95% air + 5% CO_2). The number of bacteria in individual cultures was quantified by extrapolating from previously determined growth curves. Cultures were then centrifuged for 15 min at 3000 rpm, the supernatants were discarded, and the bacteria were resuspended in sterile phosphate buffered saline (PBS), to achieve a final dose of 2.5 X 10 ^{9} CFU in 250µl. All animals received an intraperitonneal (i.p.) injection of 250µl of either *E. coli* or the vehicle (sterile PBS).

Blocking CNS Consequences of the Peripheral Infection

Cisterna magna, rather than intra-hippocampal or ICV injections were utilized because this procedure does not require surgery - which can itself produce memory impairments in aging animals. Twenty-four month old rats were briefly anesthetized using halothane, and a 27-gauge needle connected to a 25 µl Hamilton syringe via PE50 tubing was inserted into the cisterna magna. Interleukin-1 specific receptor antagonist (IL-1Ra) or vehicle (endotoxin-free saline from Abbott Laboratories, North Chicago, IL, USA) was then injected into the

cisterna magna. The IL-1Ra (112 μg; Amgen, Thousand Oaks, CA) was administered in a total volume of 3 μl. Immediately after this procedure, the rats received an i.p. injection of either *E. coli* or vehicle.

Electrophysiology

Physiology experiments were performed 4-5 days after the initial infection. This time point was chosen based on several observations: (1) all of the animals have completely recovered from the acute infection after four days (symptoms such as fever are gone within 3 days); (2) the 24-month-old rats, but *not* the 3-month-old rats show a significant impairment in long-term hippocampus-dependent memory four days after the E. coli infection (Barrientos, Higgins et al. 2006); and (3) levels of IL-1 protein in the hippocampus are still significantly elevated in the 24-month-old rats, but not in the 3-month-old rats, 4-5 days after the infection (Barrientos et al., 2009). Experiments on hippocampi from young and aged, saline or *E. coli* injected animals were interleaved. Hippocampi were collected from rats following decapitation. Transverse hippocampal slices (400 μm) were prepared using conventional techniques (e.g. (Patterson, Grover et al. 1992; Patterson, Abel et al. 1996). Slices were maintained in an interface chamber at 28^{o}C, and perfused with an oxygenated saline solution (in mM: 124.0 NaCl, 4.4 KCl, 26.0 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose). Slices were permitted to recover for at least 90 minutes before recording. Field excitatory postsynaptic potentials (fEPSP) were recorded from Schaffer collateral–CA1 synapses by placing both stimulating

and recording electrodes in the *stratum radiatum*. All stimuli were delivered at intensities that evoked fEPSP slopes equal to 1/3 of the maximum in each slice. Test stimuli were delivered once every minute, and test responses were recorded for 15-30 minutes prior to beginning the experiment to assure stability of the response. Slices were tetanized using one of three protocols: one, 1 sec train at 100 Hz; four, 1 sec trains, at 100 Hz, delivered 5 min apart; or 12 bursts, of 4 pulses at 100 Hz, delivered 200 msec apart (theta frequency). The 1-Train protocol was used to induce E-LTP. The 4-Train and Theta Burst protocols were used to induce L-LTP. The same stimulus intensity was used for tetanization and evoking test responses.

Statistical Analysis

Data were analyzed using factorial ANOVA, followed by Fisher's PLSD *post hoc* tests.

Results

Input-output curves did not differ across groups

We began seeking functional deficits associated with aging and or a recent history of infection by examining basal synaptic transmission at the Schaffer collateral-CA1 synapse in hippocampal slices. To provide an initial indication of possible differences in the response to stimuli of a given intensity, we generated input-output curves from slices from young and aged rats injected 4 days earlier with either *E. coli* or saline. We found that these curves were not significantly

altered by aging, or infection. (Figure 2.1)

Figure 2.1

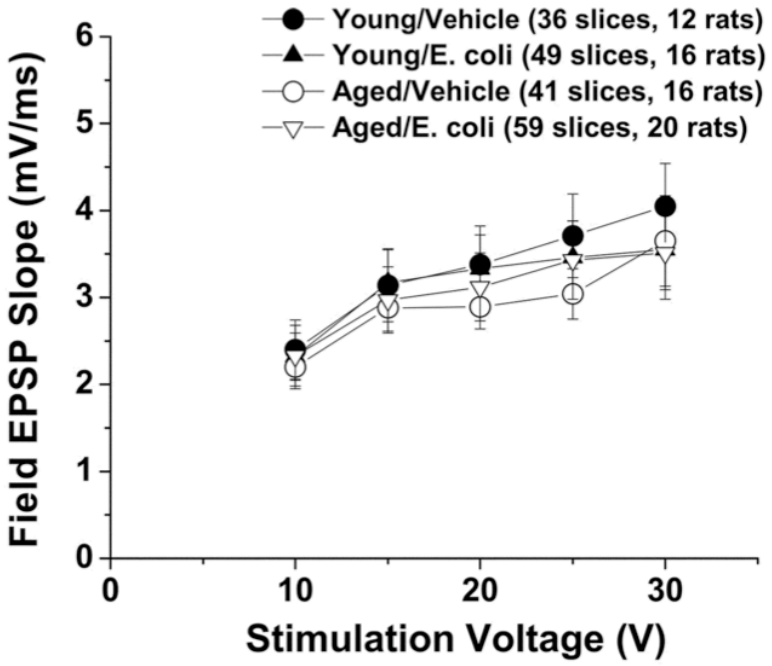

Figure 2.1 Stimulus-response curves are not altered by age or a history of infection. Plots of fEPSP slopes (mV/ms) at various stimulation intensities for hippocampal slices from young and aged rats with and without a recent history of infection (4 days after injection of E. coli or saline) show no significant differences in basal synaptic transmission in area CA1.

Paired-pulse facilitation was not significantly different across groups

We next examined paired-pulse facilitation. PPF is a presynaptic form of short-term plasticity in which the synaptic response to the second of a pair of closely spaced stimuli is increased. This is thought to reflect residual Ca^{2+} in the presynaptic nerve terminal from the first stimulus adding to the influx of Ca^{2+} evoked by the second stimulus, with a resulting increase in presynaptic neurotransmitter release (Katz and Miledi 1968). Neither aging nor a history of infection had a significant effect on PPF across a range of inter-stimulus intervals. (Figure 2.2)

Figure 2.2

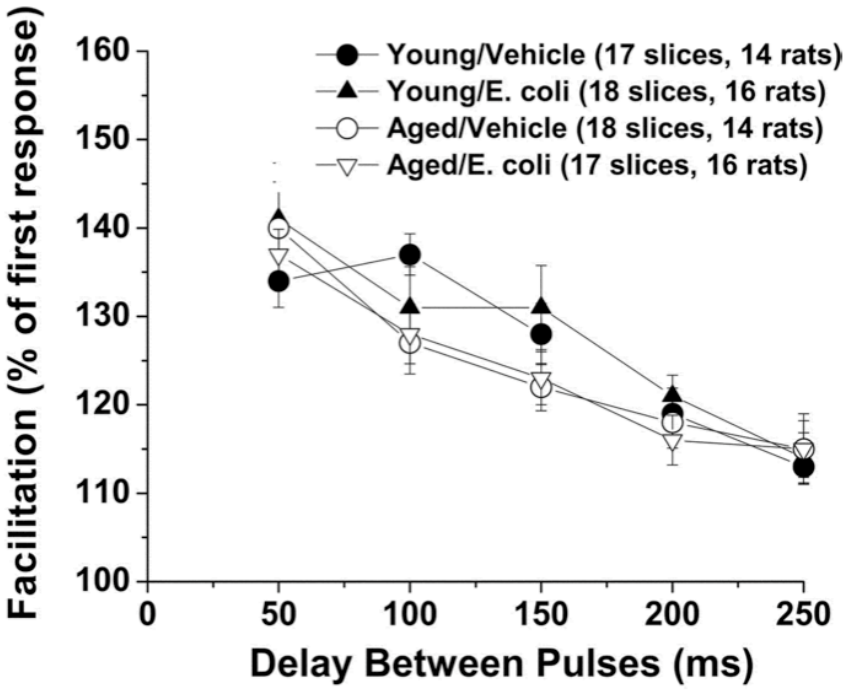

Figure 2.2 Paired-pulse facilitation is not significantly altered by age or a history of infection. Percent facilitation, calculated from the ratio of the second fEPSP slope to the first fEPSP slope, is shown at inter-pulse intervals ranging from 50 to 250 msec. No significant differences in PPF were observed across the groups at any of the inter-pulse intervals examined.

Short-term synaptic plasticity was not significantly altered by aging or a history of infection

In order to examine possible alterations in short-term synaptic plasticity, we tetanized the slices using one high frequency stimulus train: 1 sec, at 100 Hz - a protocol frequently used to induce early-phase LTP lasting approximately 1-2 hours in slices from naive rats. Neither age nor a history of infection had a significant effect on post-tetanic potentiation (% baseline, immediately after the stimulus train: young/vehicle = 235 ± 20%, young/E coli = 214 ±17%, aged/vehicle = 220 ± 24%, and aged/E coli = 223 ± 16%; $P_{(age)} = 0.581$, and $P_{(infection)} = 0.290$), or 1-Train E-LTP (% baseline, measured 90 minutes after the stimulus train: young/vehicle = 163 ± 23%, young/E coli = 134 ± 13%, aging/vehicle = 143 ± 13%, and aging/E coli = 141 ± 5%; $P_{(age)} = 0.373$, and $P_{(infection)} = 0.250$). (Figure 2.3)

Figure 2.3

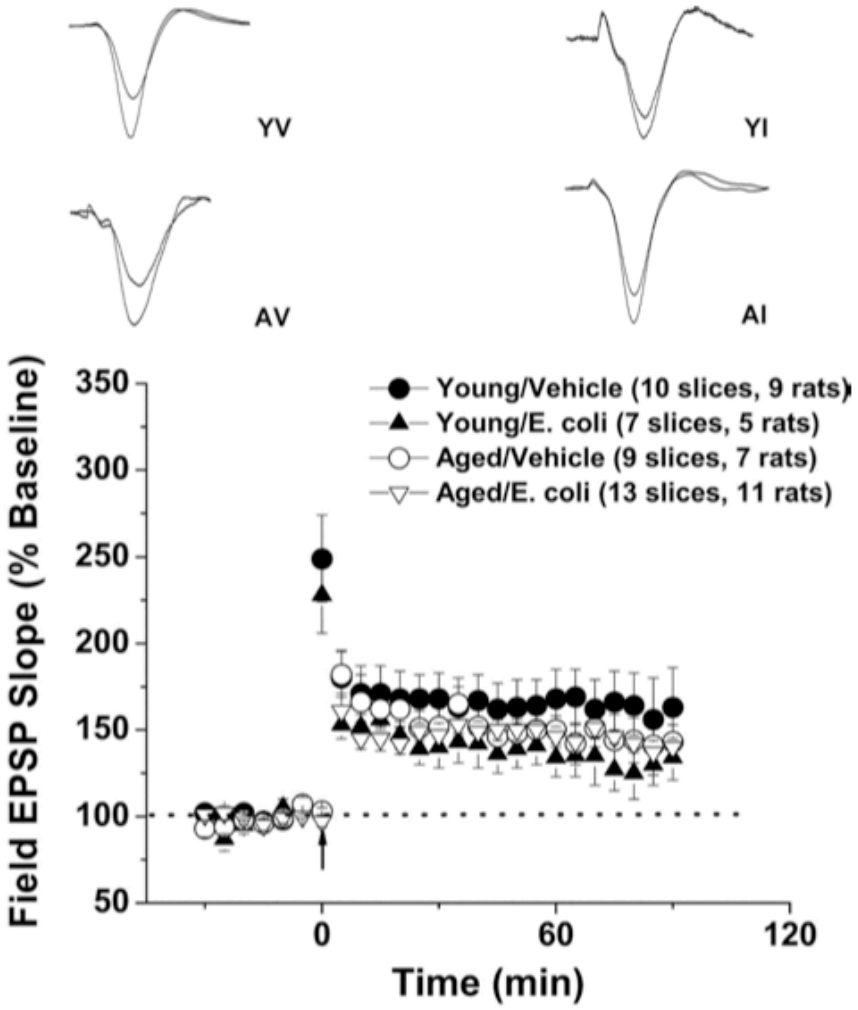

Figure 2.3 One-train E-LTP is not greatly altered by age or infection. Mean fEPSP slopes are plotted as a percent of pre-tetanus baseline values. E-LTP evoked by a relatively weak stimulus protocol (a single, 1 sec, 100 Hz stimulus train) at the Schaffer collateral-CA1 synapse in hippocampal slices from aged and young rats, with and without a prior history of *E. coli* infection. Slices were prepared from animals four days after injection of *E. coli* or vehicle. Neither age nor a history of infection had a significant effect on E-LTP in area

Four train L-LTP and theta burst L-LTP, to distinct types of long-lasting synaptic plasticity, are differentially affected by age and infection

Different stimulus protocols produce long-lasting forms of synaptic plasticity with somewhat different molecular requirements (e.g. (Kang, Welcher et al. 1997; Patterson, Pittenger et al. 2001). Thus, examining forms of long-lasting plasticity induced by different stimulus paradigms should ultimately provide useful clues about molecular mechanisms that may contribute to the infection-evoked memory deficits in the aged animals.

For the experiments reported here, slices were tetanized using one of two protocols: either four trains of high frequency stimulation, each 1 sec, at 100 Hz, delivered 5 min apart; or theta-burst stimulation consisting of 12 bursts, of 4 pulses at 100 Hz, delivered 200 msec apart (theta frequency). Both of these protocols induce late-phase LTP in animals of hybrid strain used here. The high frequency 4-train protocol produces a robust activation of many, though not all, plasticity-related signaling cascades (reviewed in (Bliss, Collingridge et al. 2007). The theta burst protocol is more naturalistic - designed to mimic the burst firing of CA1 pyramidal cells at theta frequency recorded *in vivo* from awake, behaving animals during spatial exploration (reviewed in (O'Keefe 2007) – and has proven to be a sensitive indicator of alterations in mnemonic processes associated with aging (reviewed in Lynch et al., 2006) or pharmacological or genetic manipulation of the substrates for memory.

When we examined the effects of aging and infection on the L-LTP evoked by

the 4-train protocol, we found no significant effects on post-tetanic potentiation (% baseline: young/vehicle = 247 ± 23%, young/E coli = 247 ± 18%, aged/vehicle = 253 ± 42%, and aged/E coli = 280 ± 27%; $P_{(age)} = 0.857$, and $P_{(infection)} = 0.420$) or 3 hours after tetanus (% baseline: young/vehicle = 204 ± 20%, young/E coli = 178 ± 13%, young/vehicle = 202 ± 32%, and young/E coli = 184 ± 16%; $P_{(age)} = 0.970$, and $P_{(infection)} = 0.408$). (Figure 2.4A)

Figure 2.4

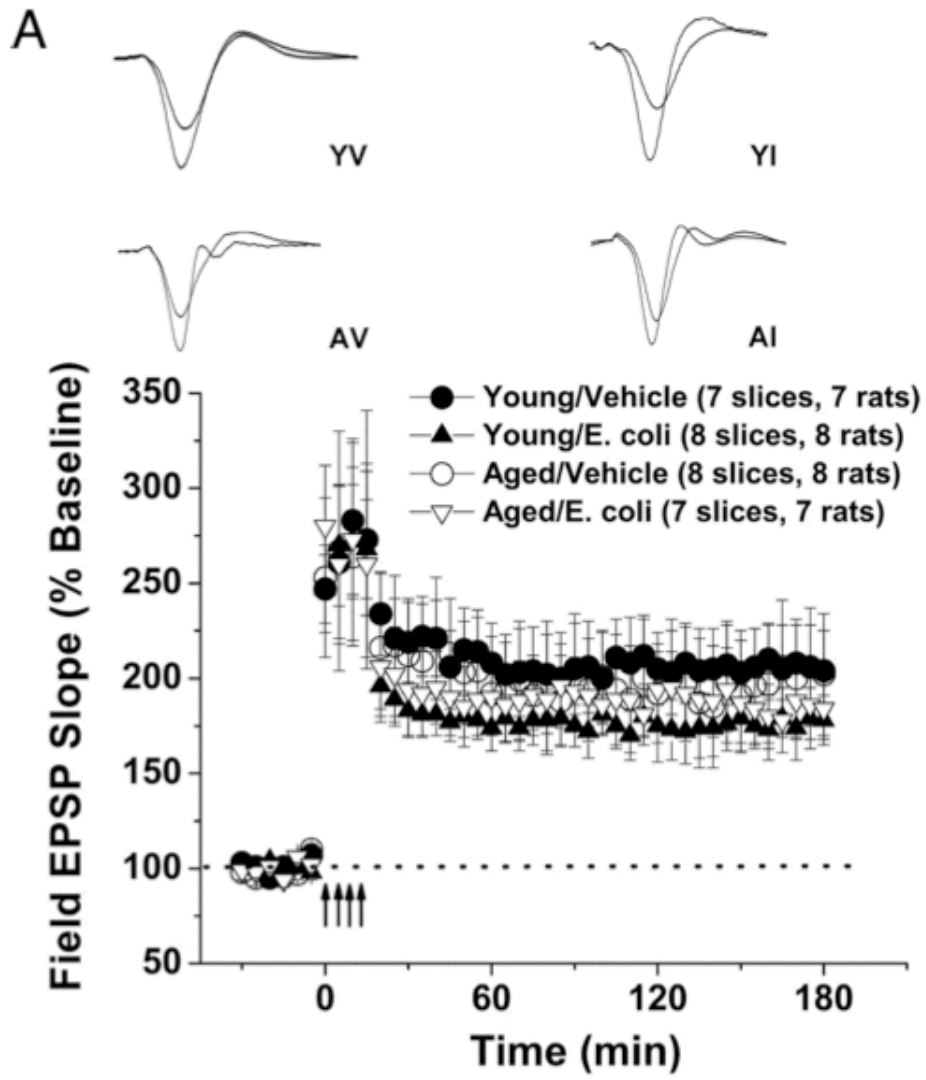
A
YV
YI
AV
AI
Young/Vehicle (7 slices, 7 rats)
Young/E. coli (8 slices, 8 rats)
Aged/Vehicle (8 slices, 8 rats)
Aged/E. coli (7 slices, 7 rats)
Field EPSP Slope (% Baseline)
350
300
250
200
150
100
50
0
60
120
180
Time (min)

Figure 2.4 continued

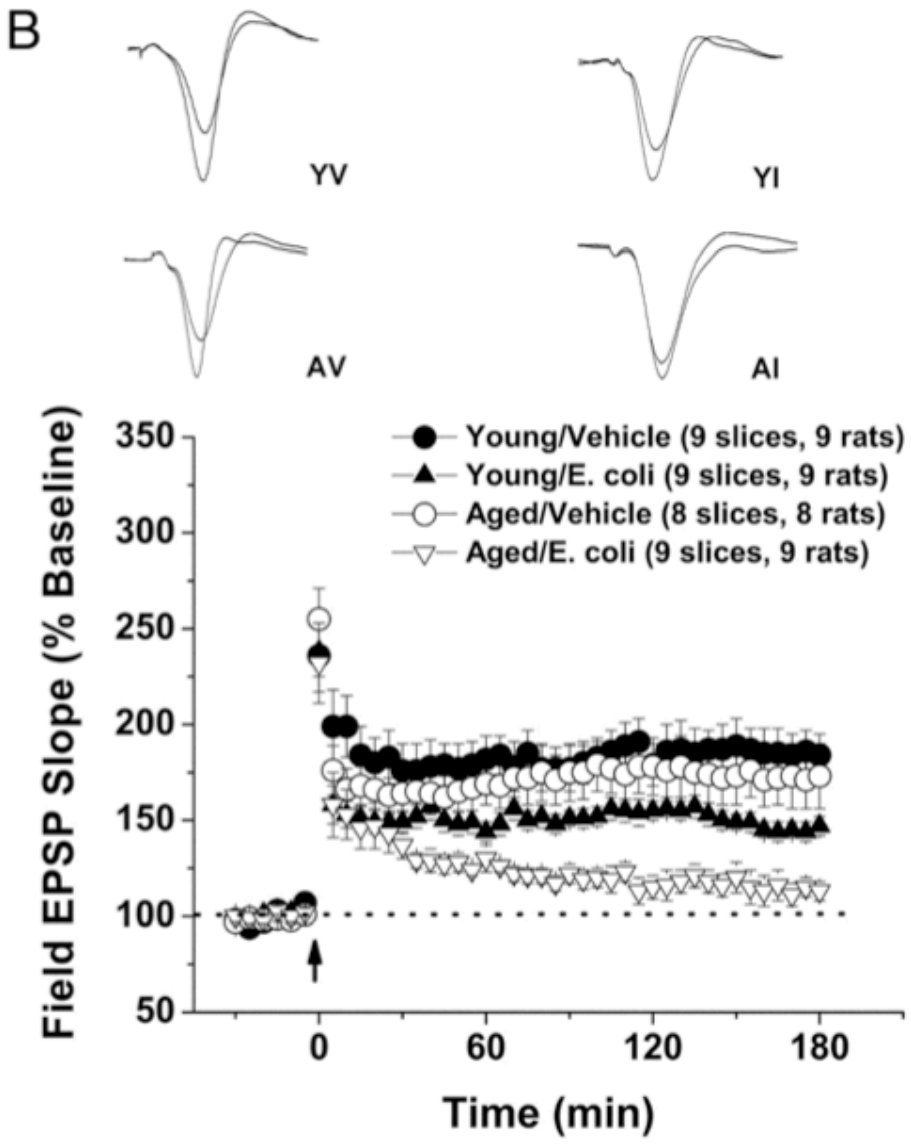

Figure 2.4 Age and infection differentially affect distinct types of L-LTP. Experiments examining two forms of L-LTP in aged and young rats, with and without a prior history of *E. coli* infection. Slices were prepared from animals four

days after the *E. coli* or vehicle injections. L-TLP was elicited at the Schaffer collateral-CA1 synapse using one of two distinct stimulus protocols: A 4-train protocol (Four 1 sec, 100 Hz stimulus trains, delivered 5 minutes apart); and a theta burst protocol (12 bursts of 4 pulses at a 100 Hz, delivered 200 msec apart). ***A***, The L-LTP evoked by the intense, second-long bouts of high frequency stimulation (the 4-train protocol) was not significantly affected by age or infection. ***B***, In contrast, infection suppressed the full expression of theta burst L-LTP, and aging greatly exacerbated this effect.

The effects of the theta burst stimulation were more complex (Figure. 2.4B). Under the conditions used, age did not have a significant effect on post-tetanic potentiation ($P_{(age)} = 0.703$) or L-LTP three hours after the tetanus ($P_{(age)} = 0.307$). In contrast, *E coli* infection had no effect on post-tetanic potentiation (% baseline: young/vehicle = 236 ± 19%, young/E coli = 238 ± 13%, aged/vehicle = 255 ± 14%, and aged/E coli = 232 ± 21%, $P_{(infection)} = 0.534$), but resulted in significantly smaller L-LTP in slices from young rats ($P_{(infection)} = 0.010$), and profoundly reduced L-LTP in slices from aged animals (% baseline, 3 hours after tetanus: young/vehicle = 184 ± 11%, young/E coli = 147 ± 5%, aged/vehicle = 169 ± 12%, and aged/E coli = 113 ± 5%; $P_{(infection)} = 0.006$).

Central administration of the anti-inflammatory cytokine IL-1Ra ameliorates the aging associated, infection-induced impairment of theta burst L-LTP

Interleukin-1beta (IL-1β) is a major mediator of inflammatory responses in the brain. We have previously found that IL-1β is elevated in the hippocampi of aged rats with a recent history of peripheral *E. coli* infection (Barrientos et al., 2006). This elevation parallels the *E. coli* evoked deficits in hippocampus-dependent long-term memory (Barrientos, Higgins et al. 2006), and blocking IL-1 signaling in the brain with the naturally occurring interlukin-1 receptor antagonist (Dinarello 1997) blocks the memory deficit (Frank et al., 2010). We therefore set out to determine if blocking hippocampal IL-1β signaling with IL-1Ra would also block the *E. coli* evoked deficit in theta burst L-LTP in aged animals.

As before, *E. coli* infection greatly reduced theta burst L-LTP in the aged animals ($P_{(\text{peripheral } \textit{E. coli})} = 0.002$), but we found that this reduction could be blocked by central administration of IL-1Ra ($P_{(\text{peripheral } \textit{E. coli} + \text{CNS IL-1Ra})} = 0.001$), which had no significant effect on L-LTP in the absence of infection ($P_{(\text{peripheral vehicle + CNS IL-1Ra})} = 0.69$; % baseline 3 hours after tetanus: vehicle/vehicle = 156± 15%, *E. coli*/vehicle = 104± 3%, E coli/IL-1Ra = 143 ± 3%, and vehicle/IL-1Ra = 166± 17%). We found no significant effects of *E. coli* or IL-Ra on post-tetanic potentiation (% baseline: *E. coli*/vehicle = 199± 13%, E coli/IL-1Ra = 237 ± 26%, vehicle/vehicle = 241 ± 16%, and vehicle/IL-1Ra = 228 ± 22%; $P_{(\text{peripheral injection})} = 0.417$, and $P_{(\text{CNS injection})} = 0.476$). (Figure 2.5)

Figure 2.5

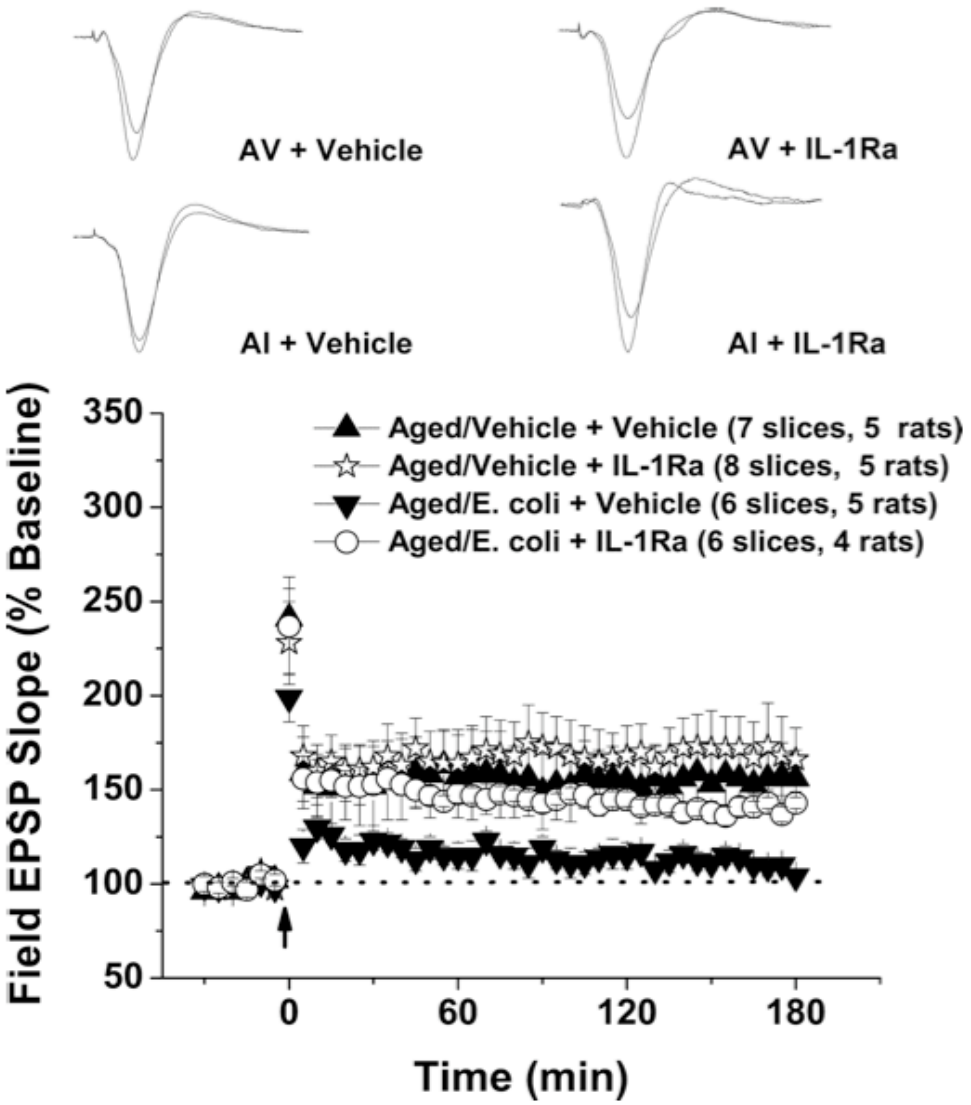

Figure 2.5 Blocking the actions of the IL-1β in the CNS ameliorates the effect of peripheral infection on theta burst L-LTP in aged rats. The anti-inflammatory cytokine IL-1Ra or vehicle was injected into the cisternae magna of aged rats and immediately afterwards the rats received an i.p. injection of either *E. coli* or vehicle.

Hippocampal slices were prepared from the animals four days after the injections, and Schaffer collateral-CA1 synapses were stimulated using the theta burst protocol. Infusion of exogenous IL-1Ra into the brain greatly reduced the *E. coli*-induced impairment in theta burst L-LTP. *Insets* show representative traces.

Discussion

In the experiments presented here, we examined for the first time the effects of aging *combined* with a secondary experimental insult - a peripheral immune challenge - on synaptic function in area CA1 of the hippocampus. Our principle findings are that the *E. coli* infection (1) had no significant effects on basal synaptic transmission or short-term synaptic plasticity in slices from young or aged rats; (2) had no significant effects on a form of late-phase LTP evoked by high-frequency stimulation in slices from young or aged rats; but (3) significantly reduced a form of L-LTP evoked by theta burst stimulation in slices from young rats and essentially abolished it in slices from aged rats. Interestingly, we were able to block the reduction in theta burst L-LTP in aged animals by blocking IL-1 signaling in the brain with the anti-inflammatory cytokine IL-Ra.

These results are consistent with the results of prior behavioral studies indicating that the *E. coli* infection does not compromise the initial learning of the test tasks, or the formation of short-term memories in any of the animals, but instead, produces profound deficits specific to the consolidation of hippocampus-dependent memory in aged, but not in young rats (Barrientos, Higgins et al. 2006). The physiology experiments presented here add support to the idea that the infection does not produce large-scale, non-specific disruptions in hippocampal function. Instead, they suggest that limited and relatively subtle synaptic deficits might give rise to the selective memory deficits associated with the combined effects of aging and infection.

A number of studies have examined the impact of aging alone on learning and memory and synaptic plasticity - often with mixed result. As noted earlier, the range of cognitive and synaptic function grows wider with increasing age - an observation consistent with the idea that age is not the only important variable in aging-associated cognitive decline. Aging is often, but not always, associated with some cognitive impairment, and with deficits in the induction and or maintenance of hippocampal LTP (Gage, Dunnett et al. 1984; Barnes and McNaughton 1985; Deupree, Turner et al. 1991; Gallagher, Bizon et al. 2003). At Schaffer collateral-CA1 synapses, the available data suggest that the basic mechanisms for producing LTP remain intact into old age but are somewhat less likely to be recruited by naturalistic patterns of stimulation or by patterns of afferent activity associated with normal behavior – in contrast, age-related impairments tend to be masked by high frequency stimulation protocols (reviewed in (Lynch, Rex et al. 2006).

Our results are largely in line with these earlier findings, but may also offer insight into secondary events that can interfere with production of long-lasting plasticity in aging. In the absence of immune challenge, the aging Fischer Brown Norway rats did not show overt cognitive deficits or impairments in synaptic function. This is not particularly surprising, as we elected to use the aging, but pre-senescent 24-month-old F344xBN rats in order to minimize basal differences in memory functions between young and aged rats. However, following the immune challenge, the aged animals showed dramatic deficits in consolidation of hippocampus-dependent memories (Barrientos, Higgins et al. 2006), and, as

reported here, in theta burst L-LTP. The association of these deficits is intriguing since it has been suggested that the formation of stable spatial memories may require selective strengthening of synapses in hippocampal area CA1 in response to short bursts of action potentials at theta frequency (Buzsaki, 2002) – an idea supported by the observation that deficits in theta-frequency LTP in area CA1 distinguish cognitively impaired from unimpaired aged Fischer 344 Rats (Tombaugh et al., 2002).

How might aging render hippocampal memory processes vulnerable to the deleterious effects of a peripheral infection? One possibility is suggested by the fact that products of peripheral immune activation also signal the brain, leading to a cascade of CNS effects including microglial activation and subsequent production of pro-inflammatory cytokines such as interleukin-1beta (IL-1β) (reviewed in (Maier, Watkins et al. 2001; Konsman, Parnet et al. 2002). Numerous studies have provided evidence that elevated levels of pro-inflammatory cytokines such as IL-1β may sometimes impair cognition and synaptic plasticity. Conditions or treatments likely to lead to aberrant increases in brain levels of pro-inflammatory cytokines are intermittently associated with problems in memory, learning and concentration. Experimentally elevated levels of IL-1β in the hippocampus impair the formation of long lasting memory in hippocampus-dependent tasks (Oitzl, van Oers et al. 1993; Gibertini, Newton et al. 1995; Pugh, Nguyen et al. 1999; Barrientos, Higgins et al. 2002; Yirmiya, Winocur et al. 2002), and inhibit LTP in several regions of the hippocampus (e.g. (Katsuki, Nakai et al. 1990; Bellinger, Madamba et al. 1993;

Coogan and O'Connor 1997)) in young adult animals. Thus, individuals with exaggerated brain inflammatory responses to peripheral immune challenge might be more vulnerable to challenge-evoked disruptions of hippocampal memory systems.

Interestingly, aging sensitizes the hippocampal inflammatory response to peripheral *E. coli* (Frank et al., 2006; Chen et al., 2008). Basal levels of IL-1β protein in the hippocampus are low in Fischer Brown Norway rats, and do not differ significantly between 3- and 24-month-old animals (Barrientos, Higgins et al. 2006). However, when levels of hippocampal IL-1β protein were examined 4 days after infection with *E. coli*, IL-1β was markedly increased in the older animals, but not in the younger (Barrientos, Higgins et al. 2006). This is not because the dose of *E. coli* used failed to induce an inflammatory response in the younger animals. Rather, both the magnitude and the duration of the inflammatory response were increased in the older animals (Barrientos, Frank et al. 2009). Not surprisingly, we have very recently found that blunting this response in the brain using the IL-1 receptor antagonist IL-1Ra largely prevents the *E. coli* induced impairment in hippocampus-dependent memory in the aged rats (Frank, Barrientos et al. 2010), and as shown here, blocks the deficit in theta burst L-LTP.

The aging and immune challenge model provides an excellent system for exploring these questions. The relatively physiological *E. coli* infection has been shown to produce selective deficits in hippocampus-dependent memory. Here we extend these results, demonstrating that the interaction between aging and

peripheral infection also produces selective effects on synaptic plasticity. Since these behavioral and physiological deficits occur in a predictable time frame, and are not cofounded by genetic manipulation, the aging induced vulnerability model may be especially tractable for examining the cellular and molecular basis of the initial events (e.g. early failures of synaptic plasticity) giving rise to a form of memory disruption that mimics many aspects of human pathology.

Chapter 3

Synaptic Plasticity is Shifted Toward Long-Term Depression In Aged Rats Following a Peripheral Infection

Abstract

Brain inflammation increases with age and may be an important contributor to the decline of memory processes. Older individuals are more likely to have exaggerated microglia activation and cytokine release in the brain following a peripheral immune challenge, which may interfere with synaptic plasticity. It was previously demonstrated that aged rats have a prolonged inflammatory response in the hippocampus following a peripheral injection of *Escherichia coli* (*E. coli*), which correlated with a specific deficit in LTP in area CA1 following a weak theta-burst tetanus but not after a more robust stimulation (4 x 100 Hz). Based on similar observations in other aged rodents, it has been proposed that normal aging raises the threshold for the induction of NMDA receptor-dependent LTP but does not interfere with mechanisms needed for maximal expression of LTP. To determine if a peripheral inflammation affects the induction of synaptic plasticity in a similar manner, we used low- to mid-frequency stimulations that are near the threshold for LTP and LTD. We found that at four and five days after a peripheral infection, the

threshold of plasticity was shifted toward LTD in CA1 of aged rats. This was correlated with increased expression of GluN2B subunits in crude hippocampal synaptoneurosomes, but did not increase the association of GluN2B with the postsynaptic density (PSD) in region CA1. These results indicate that prolonged, increased expression of Il-1β in the hippocampus following a peripheral infection alters synaptic plasticity in a metaplastic manner that may be dependent upon increased expression of GluN2B in synapses.

Introduction

Neuroinflammation is emerging as a major factor in the decline of cognition. Most neurodegenerative diseases have an inflammatory component; and general brain inflammation appears to increase with age (Glass, Saijo et al. 2010). Although dementia is commonly associated with Alzheimer's disease (AD), there is extensive evidence implicating inflammation in the sudden onset and progression of dementia (Cunningham 2011). Brain inflammation can be induced directly by neurodegeneration, or indirectly by systemic inflammation caused by a sickness or injury (Perry, Cunningham et al. 2007). During such an insult to the periphery, macrophages release pro-inflammatory cytokines, which communicate with the brain via neural and circulatory routes and cause additional release of cytokines from microglia, the major immune cell of the brain. Older individuals are more

likely to have an exaggerated inflammatory response in the brain when faced with a peripheral immune challenge, which could trigger and/or accelerate cognitive decline (Cunningham 2011). It has been demonstrated that microglia become sensitized, or 'primed' with age, possibly caused by other aspects of normal brain aging, including altered neuronal activity, increased oxidation, and disrupted vascularization; or as a simple result of intrinsic cell senescence (Barrientos, Frank et al. 2010; Freund, Orjalo et al. 2010; Norden and Godbout 2012). Nevertheless, when signaled by the periphery, primed microglia release larger amounts of cytokines in the aged brain for longer periods of time. Because cytokines like interleukin 1-beta (Il-1β) and tumor necrosis factor alpha (TNFα) directly affect neurons, exaggerated cytokine release is predicted to interfere with normal synaptic plasticity and plasticity-supported memory processes (Viviani, Gardoni et al. 2007; Lynch 2010).

Our lab has developed a rodent model that reflects the disruption of memory that the elderly can experience following a sickness or major injury (Barrientos, Higgins et al. 2006). F344 x Brown Norway rats (F344 x BN) are widely used for aging studies because they are genetically healthier and live longer (~34-39 months) than other inbred rat strains (~25-30 months). At 24 months, when other strains are clearly senescent and have spatial memory deficits, F344 x BN rats are considered pre-senescent and have only minimal cognitive impairments (Markowska and Savonenko 2002). However, following an intraperitonneal injection of *E. coli*, 24 month-old F344 x BN rats exhibit a robust neuroinflammatory

response specifically in the hippocampus. Although there is a brief (< 24 hrs) increase in Il-1β in young F344xBN rats (3 month), aged F344xBN have more than twice the basal amount of Il-1β for at least 8 days following an infection (Barrientos, Watkins et al. 2009). During this period, aged rats have a long-term memory deficit for a contextual fear-conditioning task (tested 3 days after conditioning), but not for an auditory-cued fear-conditioning task (Barrientos, Higgins et al. 2006). The specificity of this impairment implicated reduced memory consolidation the hippocampus; which could indicate changes in synaptic plasticity.

Our initial investigation into synaptic plasticity began by determining if impaired long-term memory could be correlated with a decrease in late phase long-term potentiation (L-LTP) in Schaffer collateral-CA1 synapses in aged and infected rats. We found that an infection did not affect L-LTP following fours trains of high frequency stimulation (4x 100 Hz, 1 sec, delivered 5 min apart), but it did sharply reduce L-LTP following a single theta burst (12 bursts of 4 pulses at 100 Hz, delivered 200 ms apart). This suggested that prolonged inflammation in aged rats affects at least some plasticity mechanisms necessary needed for long-term memory.

Interestingly, our results were similar to other studies in the aging hippocampus (CA1 in particular) that find that LTP impairments associated with age can be overcome using stronger stimulations (Burke and Barnes 2010; Foster 2012). For example, LTP elicited by weak paradigms (25-50 Hz, one theta burst, etc) are typically vulnerable to age, but more robust LTP paradigms (100, 200 Hz, etc) produce equivalent LTP in young and old rodents. In addition, weak stimulations

are more likely to induce long-term depression in aged rats (Kumar, Bodhinathan et al. 2009). Based on these observations, it has been proposed that aging does not impair the overall mechanisms of LTP, but rather increases the threshold to induce LTP, which is correlated with decreased NMDA receptor function (Foster 2012). This is because NMDA receptors act as the primary source of Ca^{2+} during the induction of weak LTP while stronger LTP recruits Ca^{2+} from additional sources (L-type Ca^{2+} channels and intracellular Ca^{2+} stores) (Grover and Teyler 1990). It is therefore possible that maximal LTP can still be achieved in aged rodents with impaired NMDA receptors because alternate sources of Ca^{2+} can compensate and activate downstream mechanisms necessary for LTP (Shankar, Teyler et al. 1998).

In regards to our original finding (Chapman, Barrientos et al. 2010) and in relationship to the experiments presented here, we hypothesized that a prolonged inflammation, as measured by increased l-1β, in the hippocampus specifically reduced single theta-burst LTP in aged animals because it altered NMDA receptor function. How inflammation might do this is not exactly clear. It has been repeatedly demonstrated that acute application of l-1β (minutes to hours) can reduce CA3-CA1 LTP and LTD in hippocampal slices and *in vivo* (Bellinger, Madamba et al. 1993; Ross, Allan et al. 2003; Lynch 2010). However, similar treatments have also been shown to enhance LTP (Nistico, Mango et al. 2013). And while there l-1β has been shown to reduce NMDA receptor currents (Coogan and O'Connor 1997), there is also evidence that acute l-1β actually enhances NMDA receptor Ca^{2+} influx and causes subunit phosphorylation changes that help stabilize

the NMDA receptor in the synapse (Viviani, Bartesaghi et al. 2003). Chronic inflammation (< 3weeks) in the hippocampus causes a more simple effect, generally reducing NMDA receptor-dependent LTP, including theta burst (Min, Quan et al. 2009). Due to this general confusion, it not straightforward how NMDA receptor function might be altered when we test LTP after 4-5 day of increased Il-1β expression in aged rats.

We decided to use weak stimulations that are the near the threshold of NMDA receptor-dependent LTP and LTD in CA1 to determine if the induction of synaptic plasticity, and possibly metaplasticity, is altered by prolonged inflammation in aged rats. Furthermore, we explored the synaptic content of NMDA and AMPA receptors in the whole hippocampus and isolated CA1 to for any correlative changes.

Materials and Methods

Animals

Rats were 3- and 24- month old male Fisher344/Brown Norway F1 hybrids from the NIA Aged Rodent Colony. They were allowed to acclimate to the animal facility for at least two weeks before experiments began. The animals were pair housed, on a 12-hr light dark cycle, with *ad libitum* access to food and water. All experiments

were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

E. coli Infection

Stock *E. coli* cultures (ATCC 15746; American Type Culture Collection, Manassas, VA) were thawed and cultured overnight (15-20 hours) in 40 ml of BHI (DIFCO) at 37°C. Growth of individual cultures was quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 3000 rpm, supernatant discarded, and bacterial pellets were resuspended in sterile PBS to give a final concentration of 1.0 $X10^{10}$ CFU/ml. All animals received an intraperitoneal (i.p.) injection of 250 μl of either *E. coli* or vehicle (PBS).

Electrophysiology

Physiology experiments were performed 4-5 days after the initial infection. This time point was chosen based on several observations: (1) all of the animals have completely recovered from the acute infection after four days (symptoms such as fever are gone within 3 days); (2) the 24-month-old rats, but *not* the 3-month-old rats show a significant impairment in long-term hippocampus-dependent memory four days after the *E. coli* infection and (3) levels of IL-1 protein in the hippocampus are still significantly elevated in the 24-month-old rats, but not in the 3-month-old rats, 4-5 days after the infection (Barrientos et al., 2009).

Experiments on hippocampi from young and aged, saline or *E. coli* injected animals were interleaved. Rat brain were removed from the skull following decapitation and placed in ice-cold and oxygenated artificial cerebrospinal fluid (ACSF, in mM: 124.0 NaCl, 4.4 KCl, 26.0 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose). Hippocampi were dissected from cortex and collected and transverse hippocampal slices (400 μm) were prepared using a McIlwain manual tissue chopper. Slices were maintained in an interface chamber at 28ºC, and perfused with an oxygenated ACSF. Slices were permitted to recover for at least 120 minutes before recording. Field excitatory postsynaptic potentials (fEPSP) were recorded from Schaffer collateral–CA1 synapses by placing both stimulating and recording electrodes in the *stratum radiatum*. All stimuli were delivered at intensities that evoked fEPSP slopes equal to 40% of the maximum in each slice. Test stimuli were delivered once every minute, and test responses were recorded for 30-60 minutes prior to beginning the experiment to assure stability of the response. Slices were tetanized by two episodes of 600 pulses, separated by 10 minutes. The pulse frequencies were set at 3 Hz, 10 Hz, or 30 Hz (Wang and Wagner 1999). The same stimulus intensity was used for tetanization and evoking test responses.

Crude Synaptoneurosome Preparation

All tissue was collected four days after infection. Rats underwent rapid decapitation and hippoacampi were extracted. Tissue was homogenized in 500 ul ice-cold sucrose buffer (320 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1mM EGTA,

supplemented with a cocktail of Sigma protease and phosphatase inhibitors) using a glass tissue grinder with a Teflon pestle. Homogenized samples were centrifuged (1,000g, 10 min, 4°C) to pellet nuclear material and unbroken cells (P1). The remaining supernatant (S1) was centrifuged (10,000g, 15 min, 4°C) to yield a P2 fraction containing enriched synaptic material. This crude synaptoneurosome pellet was washed with 100 ul sucrose buffer and homogenized in 100 ul sucrose buffer with 1% SDS using a plastic pestle and then sonicated. Protein concentration was quantified using a BCA protein assay (BioRad, Hercules, CA, USA)

CA1 tissue preparation and subcellular fractionation

All tissue was collected four days after infection. Hippocampi were removed after decapitation and transverse slices (1 mm) were prepared and dissected in ice-cold ACSF supplemented with Sigma protease and phosphatase inhibitors. Slices were dissected under 10X magnification using a scalpel and fine-tipped forceps. The CA3 was removed with a cut across the mossy fibers and Schaffer collaterals at end of the hippocampal fissure, and remaining CA1 was pulled away from dentate along the hippocampal fissure. CA1 tissue from multiple slices and from both hippocampi were aggregated and homogenized in 300 ul ice-cold sucrose buffer (320 mM sucrose, 10 mM HEPES, pH 7.4) using a glass tissue grinder with a Teflon pestle. Homogenized samples were centrifuged (1,000g, 10 min, 4°C) to pellet nuclear material and unbroken cells (P1). The remaining supernatant (S1) was centrifuged (12,000g, 20 min, 4°C) to yield a P2 fraction containing enriched synaptic material.

The P2 pellet was resuspended in 150 ul HEPES resuspension buffer (4mM HEPES, 1mM EDTA, pH 7.4) and centrifuged (12,000g, 20 min, 4°C). This step was repeated. The P2 pellet was solubilized in 200 ul Buffer A, (20mM HEPES, 100mM NaCl, 0.5% Triton X-100, pH 7.2) by pipetting followed by slow rotation for 20 min at 4°C, and then centrifuged (12,000g, 20 min, 4°C). The supernatant was collected (non-PSD enriched fraction) and the pellet was resuspended in 120 ul Buffer B (20mM HEPES, 0.15mM NaCl, 1% Triton-X, 1% deoxycholic acid, 1% SDS, 1mM DTT, pH 7.5) to serve as PSD enriched fraction. All buffers were kept ice cold and supplemented with a complete set of protease and phosphatase inhibitors (Sigma). Protein concentration for all fraction were quantified and normalized using a BCA protein assay (BioRad, Hercules, CA, USA)

Western Blot Analysis

Samples were prepared under reducing conditions in 4x Laemmli buffer and heated for 5 minutes at 70°C. Different amounts of total protein (10-20 ug for crude synaptoneurosomes, 3 ug for PSD enriched fraction, or 6 ug for non-PSD fraction) were loaded into pre-cast 4-12% NuPage Bis-Tris SDS polyacrylamide gels (Invitrogen) and subjected to electrophoresis at 150V at 4°C for 2 hrs. Separated protein was transferred onto polyvinylidene fluoride membranes (Millipore) and membranes were rinsed in phosphate buffered saline with 1% Triton (PBST) and blocked in 5% milk/PBST for one hour at room temperature. Membranes were cut into three strips based on molecular weight and incubated with primary antibodies

in PBST at 4°C overnight, followed by 3x10 minutes with PBST, and incubated with secondary antibodies in PBST for one hour at room temperature. The following primary antibodies (and dilutions) were used: GluN1 (1:2000, Millipore), GluN2A (1:2000, BD Bioscience), GluN2B (1:2000, BD Bioscience) p-GluN2B (1:2000, PhosphoSolutions), GluA1 (1:3000, Millipore), GluA2 (1:3000, Millipore), PSD-95 (1:3000, Millipore), Synaptophysin (1:2000, Millipore).

Statistical Analysis

Data from electrophysiology experiments were analyzed using independent t-tests, and data from western blotting was analyzed using a factorial ANOVA, followed by Tukey's HSD *post hoc* comparison when necessary.

Results

Peripheral *E. coli* infection in aged rats enhances LTD at 3 and 10 Hz, but does not affect LTP at 30 Hz.

Stimulation frequencies below 5 Hz can reliably induce NMDA receptor-dependent LTD in CA1 of some rodent strains, while higher frequencies induce NMDA receptor-dependent LTP (Yashiro and Philpot 2008; Kumar 2011). The approximate range between these frequencies that does not change basal synaptic

transmission is the crossover point between LTD and LTP, and is termed the modification threshold (Abraham 2008; Cooper and Bear 2012). According to theory, the modification threshold is not fixed, and can vary according to previous synaptic activity. For example, following a period of strong postsynaptic activity, the modification threshold shifts up, and it becomes harder to induce LTP but easier to induce LTD.

Here we used a protocol established by Wang *et al.* to determine whether prolonged hippocampal inflammation in aged rats can shift the modification threshold of synaptic plasticity. The extracellular fEPSPs were monitored in CA1 in response to stimulation of the Schaffer collaterals by three protocols that varied by frequency but kept the number of pulses and episodes constant (2 X 600 pulses delivered at 3, 10, and 30 Hz, 10 minute interval between episodes). Wang *et al.* had demonstrated that a priming stimulation (high frequency stimulation under NMDA receptor blockade to eliminate LTP) delivered 30 minutes prior to the varied frequency protocols facilitated LTD at 3 Hz, eliminated LTP at 10 Hz, and attenuated LTP at 30 Hz (Wang and Wagner 1999). Therefore, these stimulations served as an adequate method to observe a shift in synaptic plasticity. This has been further replicated and extended elsewhere (Hulme, Jones et al. 2012). In our study, we did not use a priming stimulation, but rather assumed that 4-5 days of inflammation served as the priming stimulation.

In aged rats injected with vehicle (n = 15 slices from 8 animals), a 3 Hz stimulation (Figure 1) caused a significant depression of the fEPSP slope 5 minutes

after the second episode (68.3 ± 5.9%,), which almost returned to baseline 60 minutes later (92.9 ± 6.1%). In aged rats injected with *E. coli* (n = 18 slices from 9 animals), a 3 Hz stimulation caused a similar depression 5 minutes after the second episode (62.5 ± 4.6%). However, fEPSPs in aged and infected rats remained depressed 60 minutes later (74.8 ± 5.0%). Therefore, a peripheral *E.coli* infection in aged rats significantly enhances LTD following a 3 Hz stimulation, t(31) = 2.40, p = .023. (Figure 3.1)

Figure 3.1

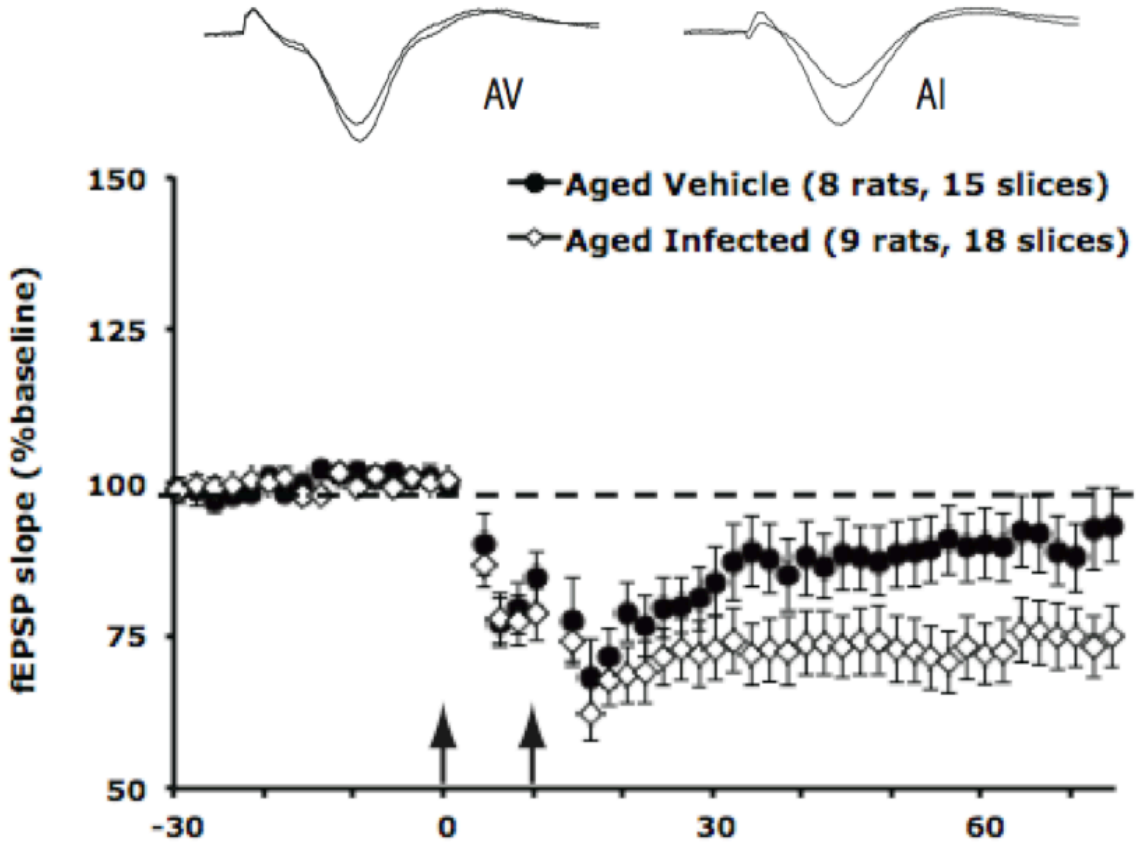

Figure 3.1 A peripheral infection enhances LTD in aged F344 x BN rats following a weak LTD paradigm. Hippocampal slices were prepared from young (3 months) and aged (24 months) rats four and five days following an i.p. injection of *E. coli* or saline.. Synaptic plasticity was evoked at Schaffer collateral – CA1 synapses by 2 episodes of 600 pulses at 3 Hz, with a 10-minute interval between bursts (indicated by arrows). Mean fEPSP slopes are plotted as a percentage of pretetanus baseline values. Aged rats given a peripheral infection have significantly more LTD after 60 minutes than aged rats given a vehicle injection (*t* test, $p < .05$). The inset shows superimposed representative traces 5 minutes before the first episode and 60 minutes after the second episode for each experimental group. Error bars indicate SEM.

In contrast to the depression that was induced by 3 Hz, a 10 Hz stimulation (Figure 2) did not induce any lasting significant change in fEPSP slope in hippocampal slices of aged vehicle rats (n = 13 slices from 7 animals). Immediately following the first episode, there was a small post-tetanic potentiation (143.0 ± 9.6%), which quickly returned to baseline; and there was even less post-tetanic potentiation following the second episode (116.2 ± 13.3%). There was a gradual and slight depression of fEPSPs that peaked 5 minutes after the second episode (90.3 ± 5.7%), but which quickly returned to baseline and remained there 60 minutes later (102.9 ± 8.9%). Hippocampal slices from aged and infected rats (n = 14 slices from 7 animals) also displayed a small post-tetanic potentiation (129.4 ± 9.8%) following the first episode, and less after the second (117.7 ± 9.3%). Similar to aged vehicle rats, there was also a gradual depression of fEPSPs five minutes after the second episode (83.9 ± 5.7%). However, the fEPSP slope did not immediately return to baseline and remained depressed 60 minutes later (80.3 ± 7.9%). Therefore, although a 10 Hz stimulation does not normally induce a change in baseline synaptic transmission in aged rats, it causes significant LTD in aged rats following a peripheral E. coli infection, $t(25) = 2.41$, $p = 0.24$. (Figure 3.2)

Figure 3.2

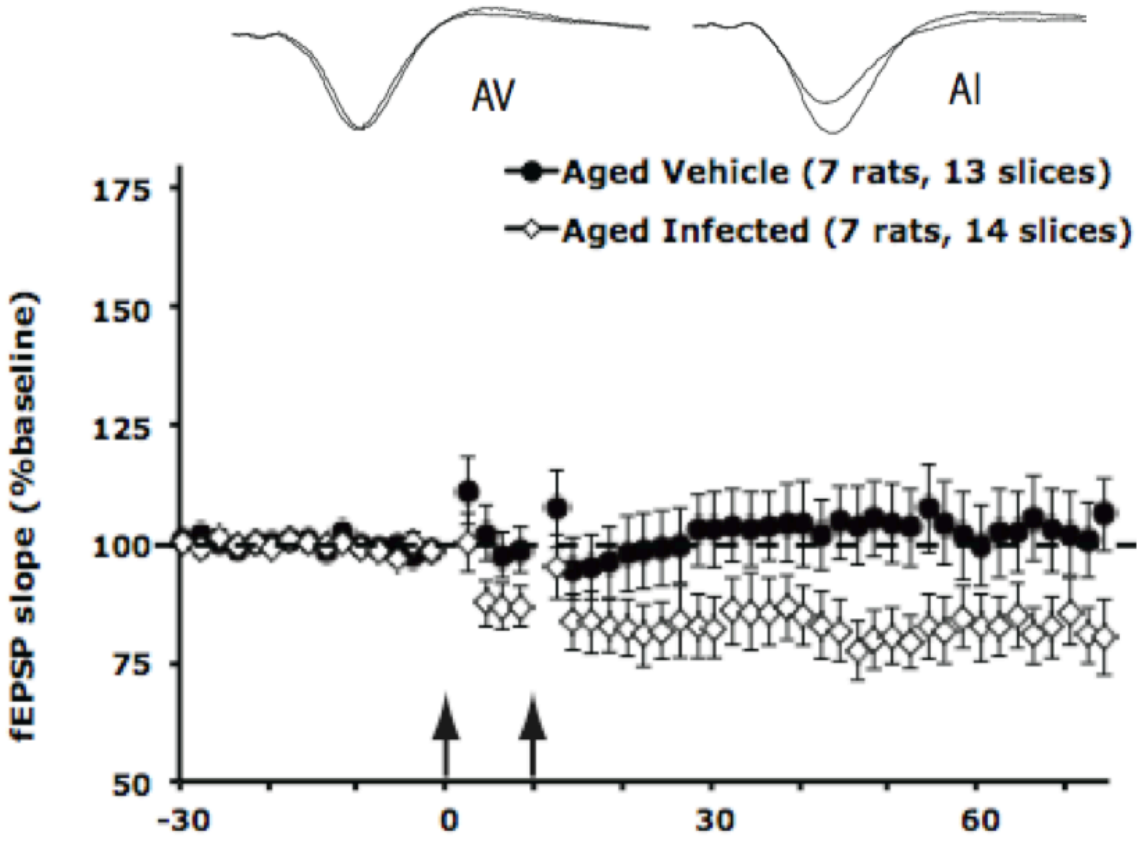

Figure 3.2 A peripheral infection in aged F344 x BN rats causes LTD following a stimulation paradigm that normally does not cause synaptic plasticity. Hippocampal slices were prepared from young (3 months) and aged (24 months) rats four and five days following an i.p. injection of *E. coli* or saline. Synaptic plasticity was evoked at Schaffer collateral – CA1 synapses by 2 episodes of 600

pulses at 10 Hz, with a 10-minute interval between bursts (indicated by arrows). Mean fEPSP slopes are plotted as a percentage of pretetanus baseline values. Although this stimulation does not cause any significant change from baseline synaptic transmission in aged/vehicle rats, a peripheral infection causes significant LTD after 60 minutes (*t* test, $p < .05$). The inset shows superimposed representative traces 5 minutes before the first episode and 60 minutes after the second episode for each experimental group. Error bars indicate SEM.

While 3 and 10 Hz stimulations on Schaffer collaterals of aged vehicle rats induced slight LTD and no change of fEPSPs, respectively, a 30 Hz stimulation induced a small potentiation (n = 11 slices from 8 animals). There was a significant post-tetanic potentiation (161.8 ± 9.2%) following the first episode and less potentiation following the second (141.3 ± 10.5%). After 5 minutes, the decline in potentiation leveled off, remaining fairly constant throughout the time of the recordings and still significantly potentiated after 60 minutes (118.4 ± 10.3%). In aged and infected rats (n = 16 slices from 9 animals), a 30 Hz stimulation also caused a post-tetanic potentiation that was larger after the first episode (170.3 ± 10.0%) than the second (156.3 ± 11.9%). After 60 minutes, there was still a significant potentiation above baseline (117.0 ± 7.4%). Therefore, a peripheral *E. coli* infection in aged rats did not alter LTP following a 30 Hz stimulation, $t(25) = .286$, $p = .78$. (Figure 3.3)

Figure 3.3

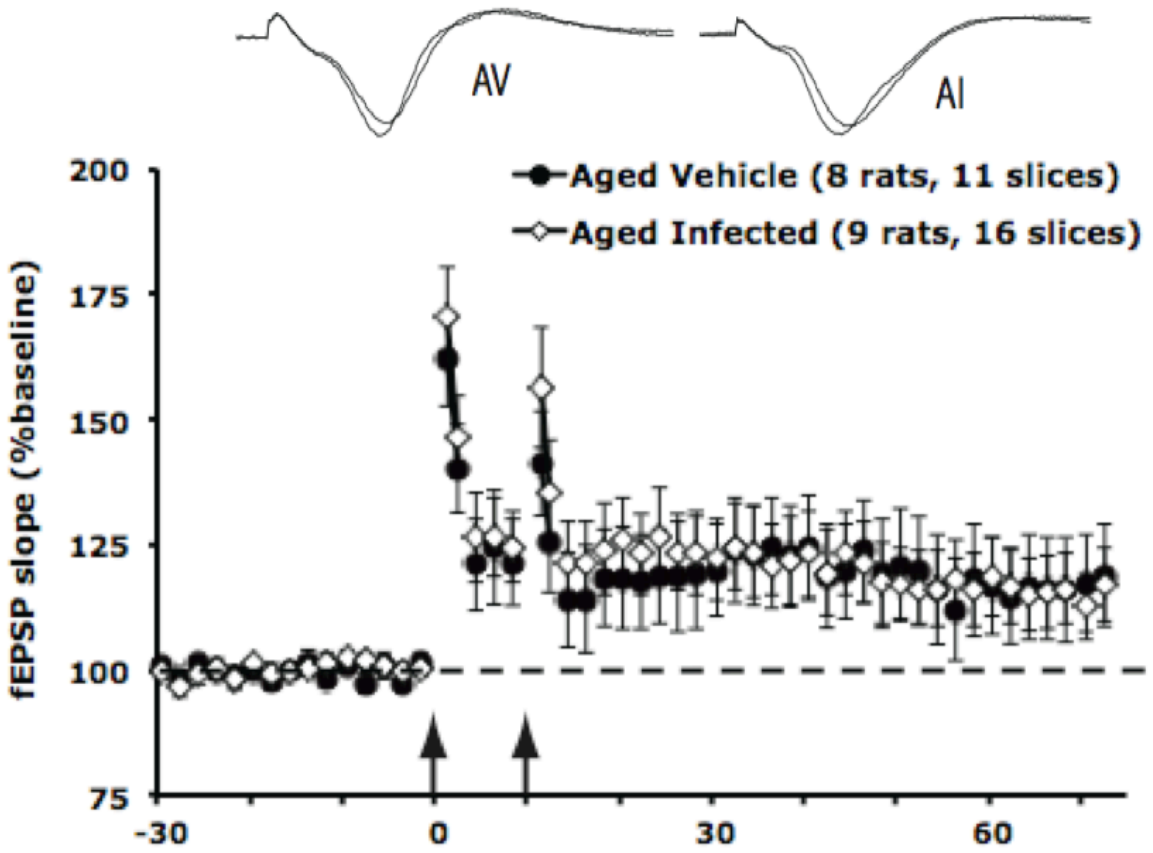

Figure 3.3 A peripheral infection does not alter LTP in aged F344 x BN rats following a weak LTP paradigm. Hippocampal slices were prepared from young (3 months) and aged (24 months) rats four and five days following either an an i.p. injection of *E. coli* or saline. Synaptic plasticity was evoked at Schaffer collateral – CA1 synapses by 2 episodes of 600 pulses at 30 Hz, with a 10-minute interval between bursts (indicated by arrows). Mean fEPSP slopes are plotted as a percentage of pretetanus baseline values. Aged rats given a peripheral infection

expressed the same amount LTP after 60 minutes as aged rats given a vehicle injection (*t* test, $p > .05$). The inset shows superimposed representative traces 5 minutes before the first episode and 60 minutes after the second episode for each experimental group. Error bars indicate SEM.

These results demonstrate that a peripheral E.coli infection in aged F344xBN rats facilitates LTD, but not LTP, following sub-threshold stimulations. (Figure 3.4)

Figure 3.4

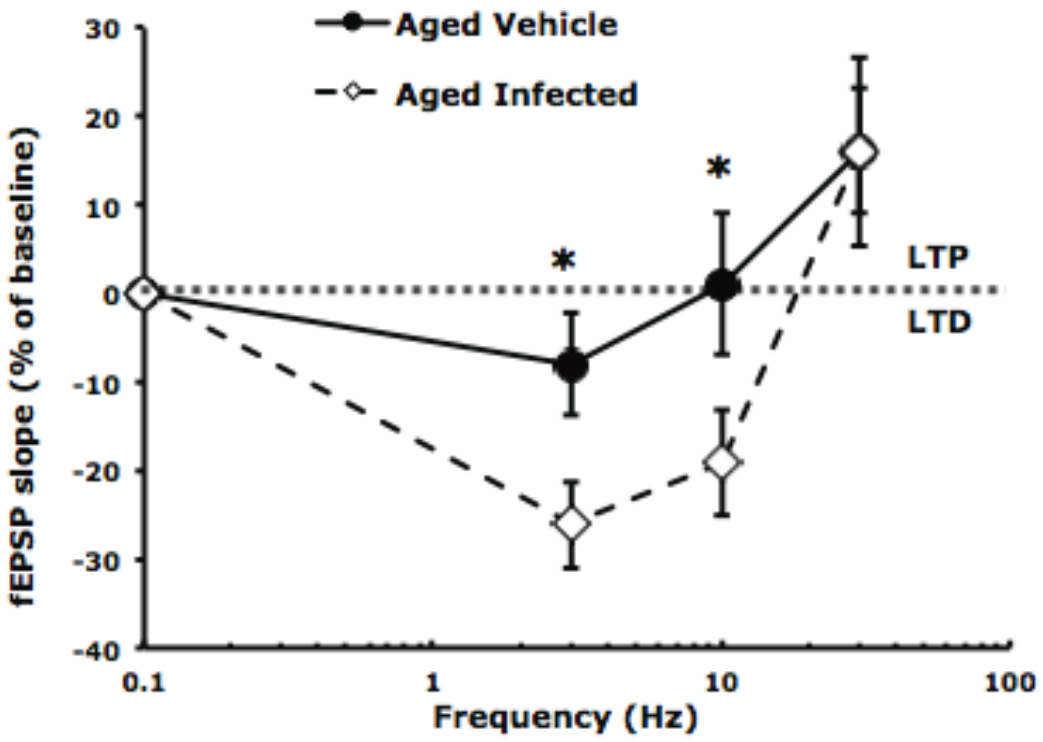

Figure 3.4 A previous infection shifts the induction of synaptic plasticity in aged F344 x BN rats toward LTD. A frequency response graph demonstrates percentage change in synaptic strength from baseline in uninfected and infected rats 60 minutes following stimulation at the indicated frequencies. At lower frequency

stimulations (3 Hz, 10 Hz), a peripheral infection significantly increases the depression of synaptic plasticity in aged rats. However, at a higher frequency (30Hz), potentiation is not affected. Mean + SEM derived from previous figures.

A peripheral infection in aged rats increases GluN2B content in crude hippocampal synaptoneurosomes.

We investigated whether altered LTP and LTD in aged and infected rats might be correlated with specific changes in synaptic NMDA receptor content. NMDA receptors are composed of two GluN1 subunits and two regulatory GluN2 subunits. In the hippocampus, GluN2A and GluN2B are the predominant regulatory subunit; and it has been theorized that GluN2A activation favors the induction of LTD while GluN2B activation favors LTP (Yashiro and Philpot 2008). Furthermore, a number of rodent studies indicate that NMDA receptor function is reduced with advanced aging, including in F344xBN rats, which demonstrate reductions in all three NMDA receptor subunit levels in hippocampal subfields by 18 months (Shi, Adams et al. 2007; Adams, Shi et al. 2008; Newton, Forbes et al. 2008; Magnusson, Brim et al. 2009).

We prepared crude synaptoneurosomes from hippocampus of young (3 months) and aged (24 months) rats, 4 days after being given a i.p. injection of vehicle or *E.coli*. Crude synaptoneurosomes contain resealed presynaptic terminals attached to resealed postsynaptic terminals, and are enriched for synaptic proteins, like NMDA receptors (Hollingsworth, McNeal et al. 1985; Villasana, Klann et al. 2006). We began by analyzing total protein levels of the three major NMDA receptor subunits and normalized them to β-actin. (Figure 3.5)

Figure 3.5

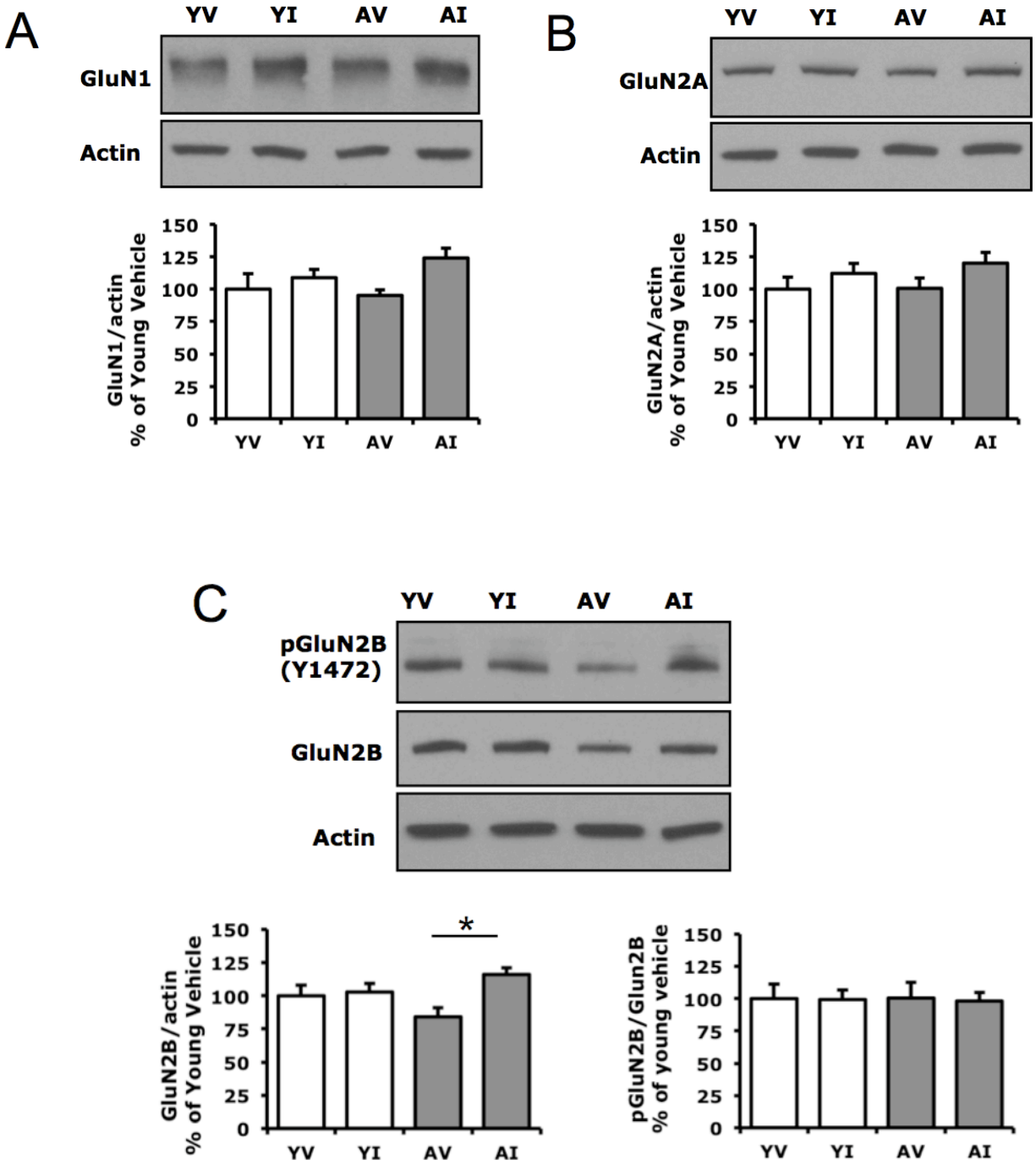

Figure 3.5 continued

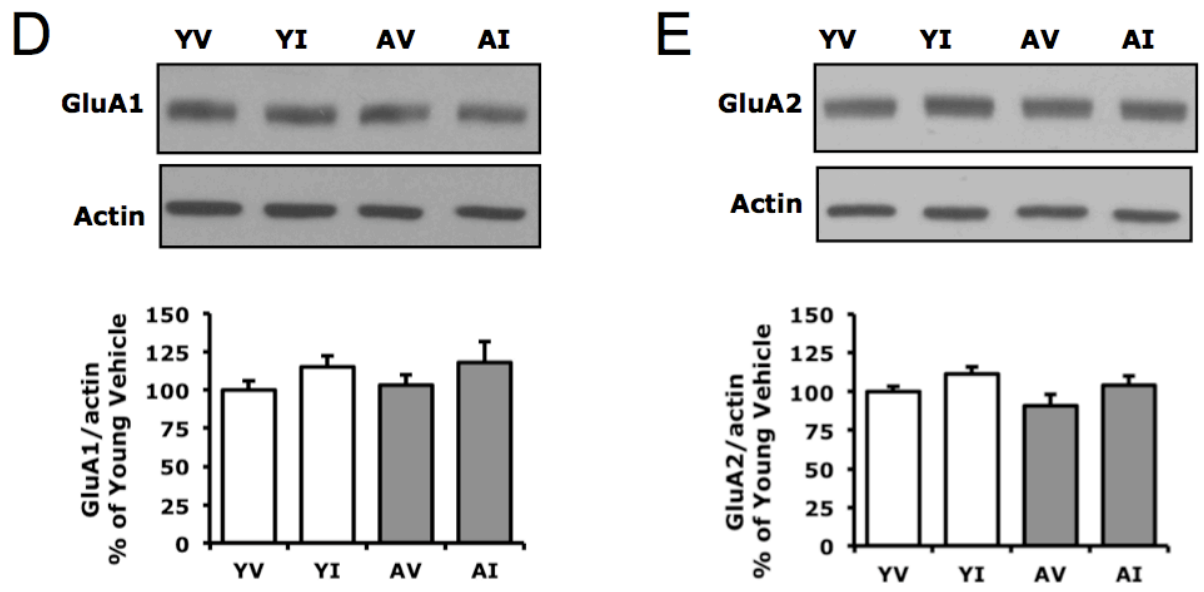

Figure 5. The GluN2B subunit of NMDA receptors is significantly enriched in crude hippocampal synaptoneurosomes from aged F344xBN rats following a peripheral infection. Crude synaptoneurosomes were prepared from hippocampus of young (3 months) and aged (24 months) rats four days following an i.p. injection of *E. coli* or saline. (A) There was no main effect of age on the obligatory NMDA receptor subunit, GluN1, but a main effect of infection approached significance ($p < .07$). (B) There was no main effect of age or infection on the alternative regulatory NMDA receptor subunit, GluN2A. (C) There was no main of age on the alternative regulatory GluN2B, but there was a significant main effect of infection ($F_{(1,31)} = 5.275$, $p < .05$). Tukey's *post hoc* test revealed that GluN2B was significantly increased in aged rats following a peripheral infection, ($p < .05$). Phosphorylation of GluR2B at tyrosine 1472 relative to total GluR2B was not significantly different

between groups. (E) There was not a main effect of age or infection on GluA1 subunits of AMPA receptors. (F) There was not a main effect of age on GluA2 subunits, but there was a significant main effect of infection ($F_{(1,31)} = 4.51$, $p = .043$), which did not cause a significant difference between groups. All protein was normalized against B-actin. N = 8 animals/group. Error bars indicate SEM. ∗ indicates $p < .05$.

We found that the obligatory NR1 subunit was not significantly affected by age (p = .606) nor infection (p = .069). We also found that the regulatory subunit, GluN2A, was not affected by age (p = .590) or infection, (p = .113). However, GluN2B, that other major regulatory subunit in the hippocampus, was significantly affected by infection, ($F_{(1,31)}$ = 5.275, p = .029), but not by age (p = .954); and there was not a significant interaction of age and infection (p = .094). A Tukey's HSD *post-hoc* comparison revealed that crude synaptoneurosomes from aged rats injected with *E. coli* had significantly more GluN2B than aged rats injected with vehicle (aged/vehicle, 99.3 ± 6.7%; aged/infected 118.1 ± 4.7, p = .038). We additionally explored a specific phosphorylation site on GluN2B that has been shown in culture to be increased following Il-1β treatment (Viviani, Bartesaghi et al. 2003). Increased phosphorylation of tyrosine 1472 on increases GluN2B binding to PSD-95, reduces clathrin-mediated endocytosis, and results in synaptic stabilization (Sanz-Clemente, Nicoll et al. 2012). Because it is known that a peripheral *E.coli* infection causes increased and prolonged expression of Il-1β in the hippocampus, we tested the possibility that the increased presence of GluN2B in synaptoneurosomes might be caused by increased Y1472 phosphorylation. We found that the levels of Y1472 phosphorylation by themselves were not significantly altered by age (p = .811) or infection (p = .062) (graph not shown); but did corresponded with increased GluN2B in aged and infected rat. In other words, there was no difference in groups when Y1472 phosphorylation was normalized to total GluN2B levels. It is not clear if

increased Y1472 causes or is simply a result of increased GluN2B in crude synaptoneurosomes.

We also examined total levels of two predominant AMPA receptor subunits in the hippocampus, GluA1 and GluA2. AMPA receptors are usually in the conformation of tetramers, typically composed of identical heterodimers, such as GluA1/GluA2. AMPA receptors are the main mediator of excitatory synaptic transmission and their trafficking in and out postsynaptic sites appears to be a major mechanism of LTP and LTD (Kessels and Malinow 2009). GluA1 content in hippocampal synaptoneurosomes were not significantly affected by age, ($p = .758$), or infection, ($p = .141$). We found that GluA2 was not significantly affected by age, ($p = .171$), but was significantly affected by infection, ($F_{(1,31)} = 4.51$, $p = .043$). However, there was not a significant interaction of aged and infection ($p = .855$), nor was there any significant difference between groups.

Neither age nor infection alters NMDA and AMPA receptor content in PSD-enriched or extrasynaptic membrane from isoloted CA1 tissue.

We hypothesized that we could gain more accurate information if we examined ionotropic glutamate receptor content only in region CA1 instead of the entire hippocampus. This assumption was based on multiple reasons. Region CA1 appears to be more susceptible to the effects of age and microglia. A study with particular relevance here found that aged F344xBN rats have greater expression of genes related to inflammation and oxidative stress in CA1 compared to CA3 and

dentate (Zeier, Madorsky et al. 2010). We have also observed a similar effect of aging on BDNF gene expression, which is important for synaptic plasticity and neuronal health. We found that aged F344xBN rats had decreased BDNF mRNA in CA1 and CA3, but not dentate (Chapman, Barrientos et al. 2011). Region CA1 also has a greater expression of activated microglia following excitotoxic NMDA treatment, further suggesting a greater vulnerability to inflammation (Vinet, Weering et al. 2012) . Finally, all of our electrophysiological data obtained thus far has been based in CA1.

We also wanted to improve the detection of glutamate receptors that are specifically expressed in the postsynaptic site. Although crude synaptoneurosomes are enriched for synapses, they contain presynaptic and extrasynaptic membranes that also have glutamate receptors inserted in them, as well as contaminating non-synaptic membrane such as endosomes. To avoid these issues, we further isolated the postsynaptic membrane by virtue of insolubility of the postsynaptic density (PSD) in Triton detergent (Goebel-Goody, Davies et al. 2009; Milnerwood, Gladding et al. 2010). The PSD is disc-like structure that forms beneath the postsynaptic membrane and contains scaffolding proteins that recruit and organize glutamate receptors, ion channels, intracellular signaling molecules, and cytoskeletal components. When crude synaptoneurosomes are digested with 0.5% Triton, the PSD and associated proteins, such as glutamate receptors, become insoluble and can be separated by simple centrifugation, as outlined in Figure 3.6.

Figure 3.6

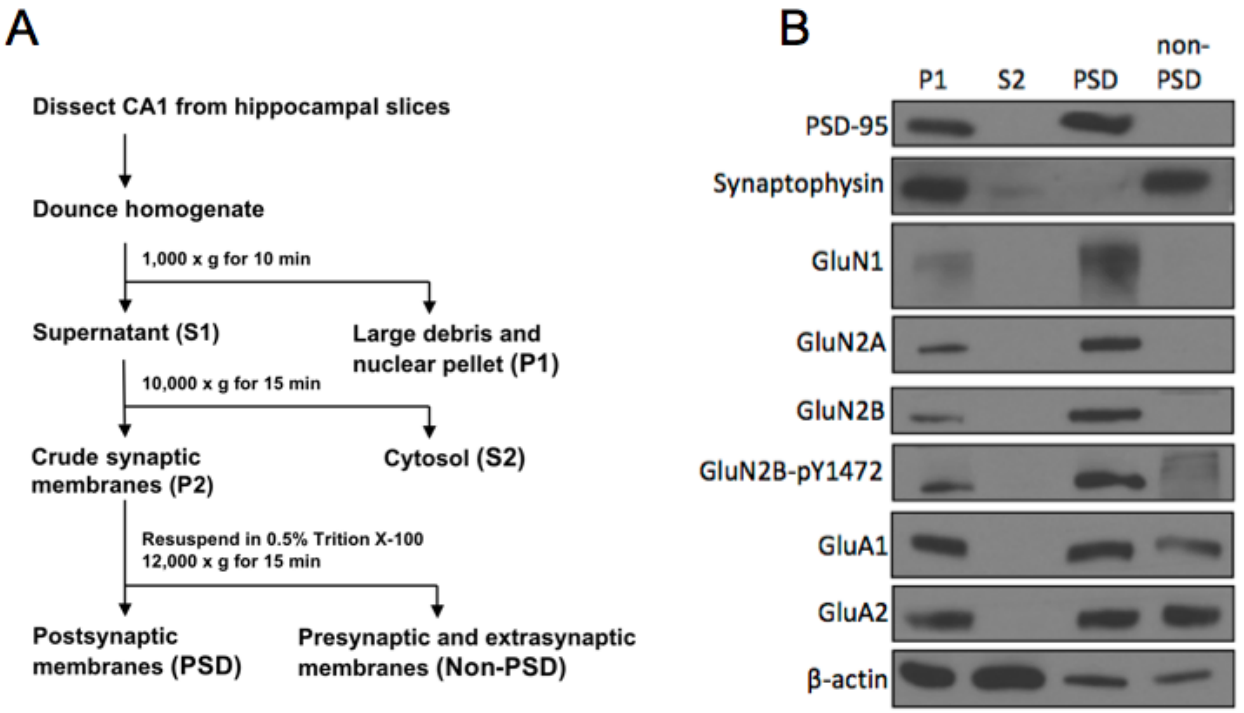

Figure 3.6 Characterization of PSD-enriched and non PSD-enriched membrane fractions from isolated CA1 tissue. (A) Schematic of the subcellular fractionation method for deriving proteins associated with the PSD. Briefly, crude synaptic membranes (P2) are further fractionated based into postsynaptic membranes and pre-and extrasynaptic membranes based on the insolubility of the PSD in Triton-X 100 detergent. (B) Characterization by western blot of proteins found in P1 (nuclear fraction, unbroken cells), S2 (cytosolic proteins), PSD enriched, and non-PSD enriched fractions from CA1 of a young F344 x BN rat. Equal amounts of total protein (5 ug) for each fraction were loaded. PSD-95 and synaptophysin

demonstrate specific enrichment to the appropriate PSD and non-PSD fraction. NMDA receptor subunits demonstrate a particular enrichment in the PSD fraction, while AMPA receptor subunits are abundant in both PSD and non-PSD fractions. B-actin is observed in all fractions.

Our characterization of dissected CA1 tissue clearly demonstrates a separation of the postsynaptic and presynaptic membrane by the distribution of PSD-95 and synaptophysin. In regards to glutamate receptors, NMDA receptor subunits were enriched in the PSD-membrane fraction while AMPA receptor subunits were roughly equivalent in both PSD and non-PSD fractions. The abundance of AMPA receptors in the non-PSD fraction might be indicative of a high rate of synaptic turnover, as they are inserted into extrasynaptic membranes before lateral diffusion into synapses and later removed to endosomes via clathrin-mediated exocytosis (Kessels and Malinow 2009). Both extrasynaptic and endosomal membranes are most likely solubilized in the non-PSD fraction.

We prepared PSD-enriched membrane from dissected CA1 tissue of young (3 months) and aged (24 months) rats, 4 days after being given an i.p. injection of vehicle or *E.coli*. *(*Figure 3.7)

Figure 3.7

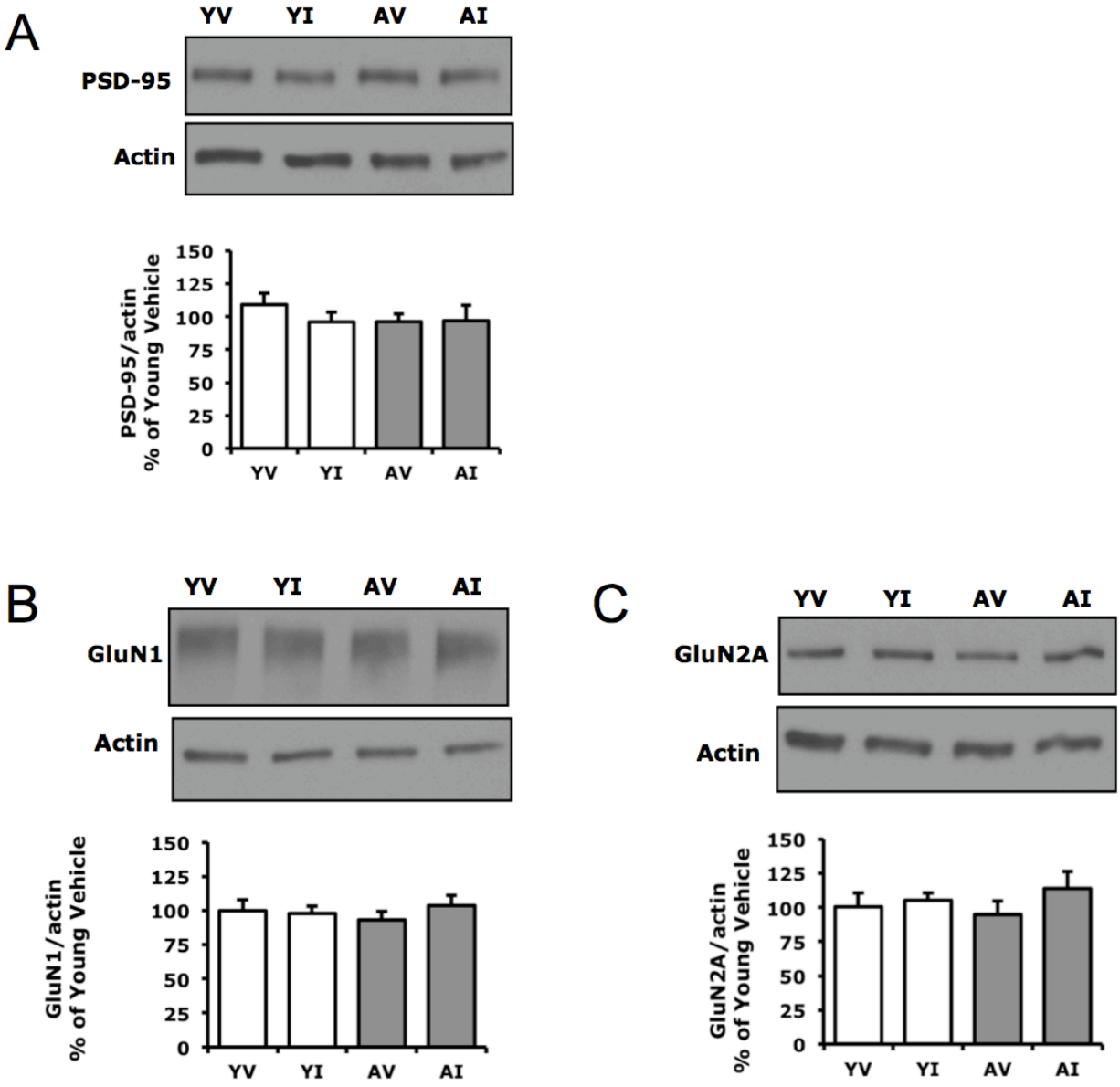
A
YV
YI
AV
AI
PSD-95
Actin
PSD-95/actin
% of Young Vehicle
150
125
100
75
50
25
0
YV
YI
AV
AI
B
YV
YI
AV
AI
GluN1
Actin
GluN1/actin
% of Young Vehicle
150
125
100
75
50
25
0
YV
YI
AV
AI
C
YV
YI
AV
AI
GluN2A
Actin
GluN2A/actin
% of Young Vehicle
150
125
100
75
50
25
0
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Figure 3.7 continued

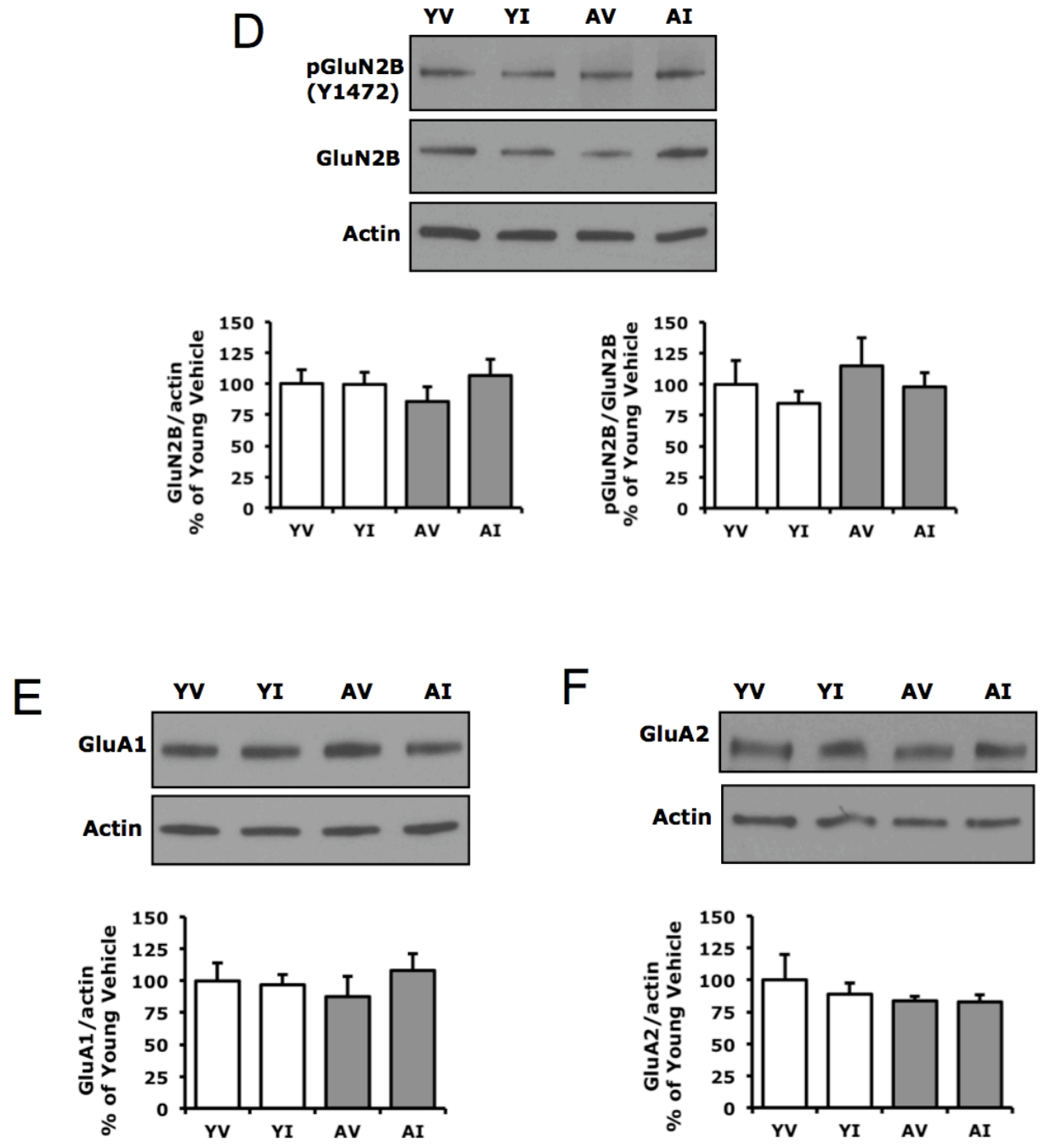

Figure 7. NMDA and AMPA receptor composition in PSD-enriched membrane from CA1 tissue is not altered by age or infection. PSD-enriched membrane fractions

were prepared from CA1 tissue of young (3 months) and aged (24 months) rats four days following an i.p. injection of *E. coli* or saline. There was not a significant main effect of age or infection on (A) PSD-95, (B) GluN1, (C) GluN2A, (D) GluN2B, (E) GluNB tyrosine 1472 phosphorylation, (F) GluA1, or (G) GluA2. All protein was normalized against B-actin. N = 4 animals/group. Error bars indicate SEM

We first determined that there was no effect of age (p = .751) or infection (p = .888) on the amount of PSD-95 when normalized to B-actin (p_{age} = .751, $p_{infection}$ = .888). We then found that there was no significant effect of age or infection on NMDA receptor subunits, or GluN2B Y1472 phosphorytion, in PSD-enriched membrane from isolated CA1 tissue (GluN1, p_{age} = .957, $p_{infection}$ = .537; GluN2A, p_{age} = .903, $p_{infection}$ = .274; GluN2B, p_{age} = .751, $p_{infection}$ = .381; p-Glu2B, , p_{age} = .386, $p_{infection}$ = .321) Importantly, while GluN2B in hippocampal synaptoneurosomes was significantly increased in aged rats following infection, it was significantly increased PSD-enriched membrane from CA1. We also found that there was no significant effect of age or infection on AMPA receptor subunits GluA1 or GluA2 (GluA1, p_{age} = .970, $p_{infection}$ = .487; GluA2, p_{age} = .274, $p_{infection}$ = 553). Therefore, prolonged inflammation in the hippocampus in aged rats does not change the expression of NMDA and AMPA receptors in CA1.

Neither age nor infection alters NMDA or AMPA receptor content in extrasynaptic membrane from isolated CA1 tissue.

NMDARs are also located in extrasynaptic and perisynaptic membranes near synapses and on the dendritic shaft and are recognized for their roles in synaptic plasticity, excitotoxicity, and neurodegeneration (Hardingham and Bading 2010; Liu, Yang et al. 2013). It is believed that while synaptic NMDARs activate signaling cascades that are neuroprotective, extrasynaptic NMDARs activate cascades that cause neurodegeneration. Furthermore, while activation of synaptic NMDA

receptors causes LTP, there is evidence that activation of extrasynaptic NMDA receptors causes LTD (Liu, Yang et al. 2013). Extrasynaptic receptors can be activated during glutamate spillover in synapses, or by glutamate release from astrocytes, which has been shown to occur during microglia activation and heightened inflammation (Wong 2013). We used the non-PSD fraction, which is theoretically enriched for extrasynaptic membrane, to determine if the expression of NMDA receptors is altered by age or infection (Milnerwood, Gladding et al. 2010). Although the amount of NMDA receptor subunits in non-PSD membrane fraction is drastically reduced compared to the PSD membrane fraction, we were able to reliably detect GluN1 and GluN2B, but not GluN2A. Again, we found no significant changes in either GluN1 or GluN2B by age or infection (GluN1, $p_{age} = .091$, $p_{infection} = .741$; GluN2B, $p_{age} = .527$, $p_{infection} = .660$). We also examined AMPA receptor subunits in the non-PSD membrane fraction and found no significant changes (GluA1, $p_{age} = .970$, $p_{infection} = .487$; GluA2, $p_{age} = .325$, $p_{infection} = .518$). (Figure 3.8)

Figure 3.8

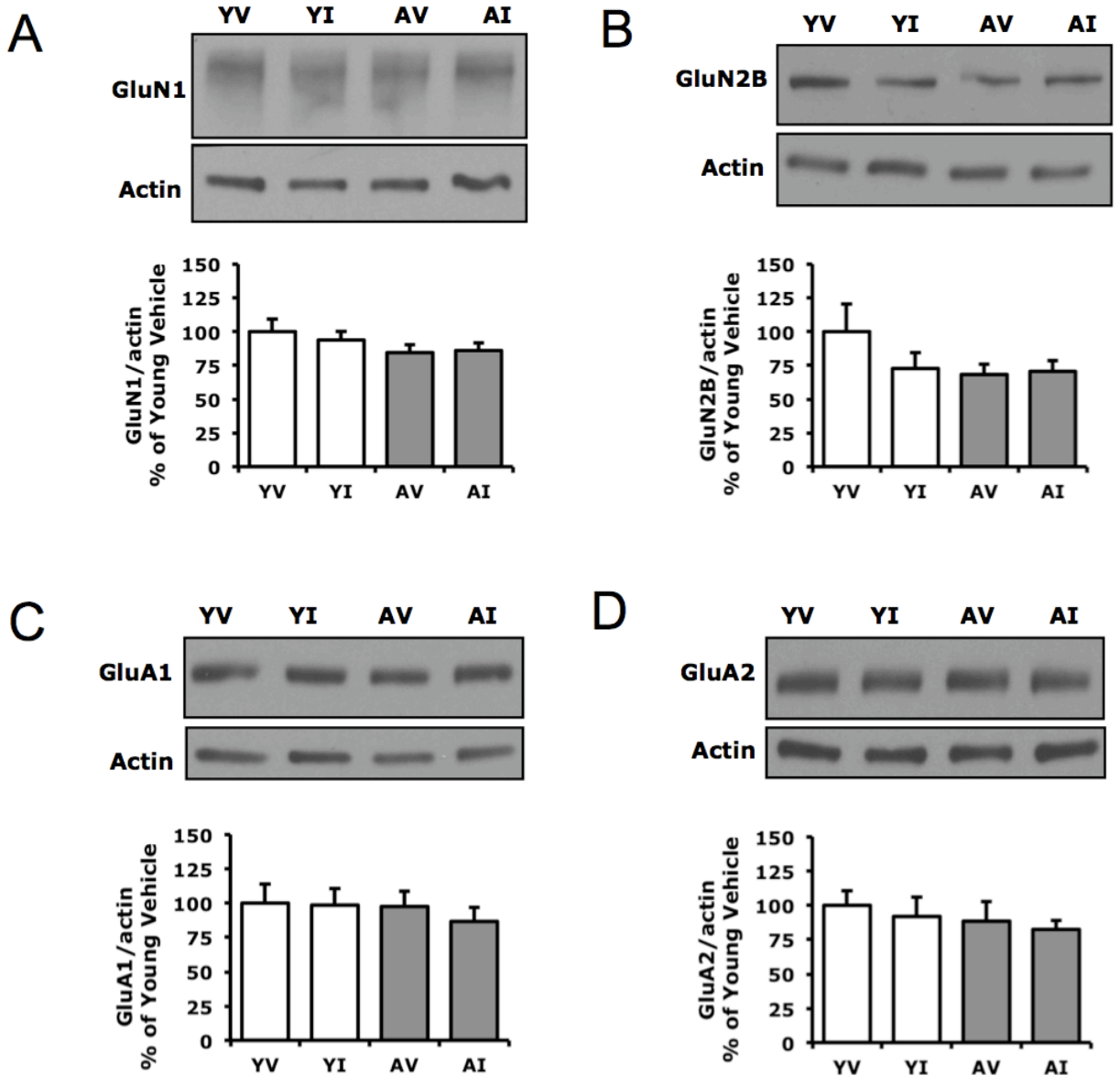

Figure 8. NMDA and AMPA receptor composition in extrasynaptic membrane from CA1 tissue is not altered by age or infection. Non PSD-enriched membrane fractions were prepared from CA1 tissue of young (3 months) and aged (24 months) rats four

days following an i.p. injection of *E. coli* or saline. There was not a significant main effect of age or infection on (A) GluN1, (B) GluN2B, (C) GluA1, or (D) GluA2.All protein was normalized against B-actin. N = 4 animals/group. Error bars indicate SEM.

Discussion

The experiments presented here indicate that a peripheral infection facilitated LTD, but did not alter LTP, in CA3→CA1 synapses of aged rats following sub-threshold stimulations. Specifically, LTD was enhanced when low frequency (3 Hz) stimulation was used, and manifested following an intermediate frequency (10 Hz) that did not alter baseline synaptic transmission in uninfected, aged rats. However, higher frequency (30 Hz) caused the same magnitude of LTP in infected and uninfected aged rats. A peripheral infection also caused a small increase in NMDA receptor subunits in crude synaptoneurosomes from whole hippocampus of aged rats, but only the increase in the GluN2B subunit was significant. However, there were not corresponding increases in any NMDA receptor subunits in PSD membrane fractions from CA1 specifically, including GluN2B.

A feature of the rodent model used here is that a peripheral *E. coli* infection provokes a prolonged inflammatory response in the hippocampus (Barrientos, Higgins et al. 2006). At the time that we conduct our electrophysiology experiments, brain inflammation, as measured by Il-1β, has been increased 2-3 fold for at least four days. We feel that the duration of inflammation above baseline, and not just inflammation itself, has an important effect on synaptic plasticity The simplest conclusion for our experiments is that prolonged inflammation in hippocampus reduces NMDA receptor function so that is observable with weak tetani, but masked by a stronger tetanus. This would be in accordance with a number of

studies indicating that aging preferentially reduces NMDA receptor function in the hippocampus, including NMDA receptor-dependent LTP (Norris, Korol et al. 1996; Magnusson, Brim et al. 2009). It is not exactly known why NMDA receptors diminishes with age, but it has been linked to oxidative stress, a process that is associated with chronic inflammation (Norden and Godbout 2012). In fact, it has been shown in the dentate of hippocampal slices that Il-1β by itself depresses isolated NMDA receptor-dependent excitatory postsynaptic potential (NMDA-EPSP) amplitude without altering AMPA-EPSP amplitude (Coogan and O'Connor 1997). Furthermore, F334xBN rats already have reduced total protein levels of NMDA receptor subunits in the hippocampus by 18 months. The direction (LTP or LTD) and magnitude of most synaptic plasticity depends on concentration of postsynaptic Ca^{2+} influx . Whereas the induction of LTP requires a brief and rapid rise of intracellular Ca^{2+}, LTD requires a modest but prolonged influx (Malenka and Bear 2004). Although LTD is blocked by complete Ca^{2+} sequestration, reducing the amount of Ca^{2+} influx during low frequency stimulation facilitates LTD (Harney, Rowan et al. 2006). Therefore, prolonged inflammation in aged rats could limit the influx of Ca^{2+} via NMDA receptors following low frequency stimulations used here (3 and 10 Hz). This could facilitate LTD.

A decrease in NMDA receptor function might also explain results from our previous study in which we found that a peripheral infection specifically reduced single theta-burst LTP in aged animals, but did not affect LTP following a stronger stimulus (4 x 100 Hz). Similar to the weak stimulation paradigms used here, a

single theta burst activates NMDA receptors as the primary source of Ca^{2+}; and NMDA receptors contribute more to the postsynaptic response during theta-burst stimulation than during continuous, high frequency stimulation, like the commonly used 100 Hz tetanus (Grover, Kim et al. 2009). Furthermore, there was a significant decline in LTP immediately following theta burst; further implicating reduced NMDA receptor function during LTP induction. However, in this study, a peripheral infection did not alter LTP in aged rats following a 30 Hz tetanus, which is a considerately weak stimulation that likely depends on NMDA receptors. We cannot explain this discrepancy.

An alternative view of the results presented here is that prolonged inflammation, and Il-1β in particular, induces metaplasticity in Schaffer collateral – CA1 synapses. In fact, the stimulation protocols used in this study were chosen because they have been previously used to observe metaplasticity. The theory of metaplasticity states that prior neural activity at a synapse affects how it responds to later activity; and is best explained by the Bienenstock, Cooper, Munro computational model (BCM Model) of synaptic plasticity (Abraham 2008; Cooper and Bear 2012). According to the theory, there is a level of postsynaptic activity, termed the modification threshold, that does not change basal synaptic transmission. However, if postsynaptic activity is greater than or less than the modification threshold, the synapse undergoes potentiation or depression, respectively. For example, once a synapse is potentiated, a new modification threshold is set; and it requires a greater level of activity to undergo further

potentiation. But because the new modification threshold is higher, the synapse can undergo depression at a higher level of activity as well.

Our results in Figure 3.1 and 3.2 suggest that a peripheral infection reduces the threshold needed to induce LTD. We used a simplified version of protocols created by Wagner *et al.* to observe metaplastic changes in region CA1. They observed that a priming stimulation that did not cause synaptic plasticity itself still altered subsequent synaptic plasticity following 3, 10, and 30 Hz stimulations. The authors speculated that their protocol caused prior neural activity that raised the modification threshold, decreasing future LTP and increasing future LTD. Rather than using a priming stimulation to manipulate metaplasticity, we assumed that 4-5 days of hippocampal inflammation acted as a priming stimulation. Interestingly, whereas a 10 Hz stimulation did not cause a lasting change in the baseline synaptic response aged vehicle animals, it caused significant LTD following prolonged inflammation. If 10 Hz is assumed to be the relative modification threshold in aged rats, then prolonged inflammation raises it. In support of this, LTD at 3 Hz was also enhanced. Unfortunately, LTP was not reduced following 30 Hz as it was in Wagner *et al.*, so our results might not completely conform to the theory of metaplasticity. However, another lab using a similar priming protocol as Wagner *et al.* found that shifting the modification threshold altered plasticity at 10 Hz and 50 Hz, but not at 20 Hz (Hulme, Jones et al. 2012). It is therefore possible that prolonged inflammation raises the modification threshold for synaptic plasticity, causing depression of synapses at higher levels of activity. An effect like this could explain

the contradiction of how acute inflammation increases neural excitability yet chronic inflammation reduces synaptic potentiation (Viviani, Gardoni et al. 2007; Lynch 2010; Galic, Riazi et al. 2012).

Although it has been demonstrated that acute and chronic inflammation reduces LTP in the dentate and CA1, there is substantial evidence demonstrating that pro-inflammatory cytokines facilitate neuronal excitability and excitotoxicity. Cytokines are commonly viewed as pro-convulsants in epilepsy; and peripheral inflammation can enhance seizure generation and reduce the threshold needed to induce seizures (Galic, Riazi et al. 2012). Although acute Il-1β application (15-30 minutes) has been shown to reduce LTP in hippocampal slice (Bellinger, Madamba et al. 1993), it has been observed in neuronal cultures that Il-1β immediately enhances NMDA receptor Ca^{2+} currents (Yang, Liu et al. 2005). In addition, Viviana *et al*. found that greater Ca^{2+} influx by Il-1β treatment was the result of enhanced GluN2B phosphorylation at tyrosine 1472 via a Src family kinase. Phosphorylation at this site increases GluN2B binding to PSD-95 and stabilizes GluN2B-containing receptors in the synapse. We also found a significant increase in GluN2B, and insignificant increases in GluN1 and GluN2A, in hippocampal synaptoneurosomes from aged rats following an infection. This may have a role in the altering metaplasticity. Recent derivations of the BCM model have theorized that bidirectional synaptic plasticity is regulated by synaptic NMDA receptor composition, in which more GluN2B in the synapse favors the induction of LTP, and more GluN2A favors the induction of LTD (Yashiro and Philpot 2008). This is based

on specific properties of each subunit. For example GluN2B channels influx more Ca^{2+} and recruit CamKII, two necessary events for LTP (Lisman, Yasuda et al. 2012) . Although our results (increased synaptic GluN2B following infection) would suggest that aged animals should have enhanced LTP following infection, it is possible that at the time of our plasticity studies, after 4 and 5 days of increased Il-1β levels, synapses have already undergone some form of potentiation, possibly unspecific and cell-wide. This would make it harder to induce further LTP, but easier to induce LTD. In addition, one study found that aged mice with the poorer spatial memory had higher levels of NR2B (Zhao, Rosenke et al. 2009), Finally, the theory that that the direction of plasticity is related to NMDA receptor composition is controversial as both GluN2A and GLuN2B have been shown to support both LTP and LTD (Yashiro and Philpot 2008).

It has been consistently demonstrated that NMDA receptor function, and memory and plasticity functions that depend on NMDA receptors, decrease with age (Foster 2012). Aging also leads to an increase in systemic and brain inflammation (Perry, Cunningham et al. 2007), but whether this precedes, proceeds, or co-ccurs occurs with NMDA receptor function loss is not known. Here we demonstrate a peripheral infection enhances LTD in the hippocampus of aged animals, which may be related to a mechanism of metaplasticity. If chronic neuroinflammation directly decreased NMDA receptor function, then it is possible that age-related losses could actually be the caused by increased levels of brain cytokines. Alternatively, it has been theorized that the age-related decreases in NMDA receptor activity might

functionally serve as a neuroprotective mechanism to reduce excitotoxicity (Kumar 2011). If chronic inflammation continually enhanced neural excitability through NMDA receptors, then neurons in the aged brain could undergo homeostatic changes that reduced NDMA receptor function to preserve Ca^{2+} homeostasis. Finally, chronic inflammation might simply overwhelm the aged brains' ability to regulate Ca^{2+}. Any of these possible scenarios could result in decreased synaptic potentiation, increased synaptic depression, and a decline in cognition in advanced age.

Chapter 4

A Peripheral Infection Occludes Reduction of Long-Term Potentiation by L-type Calcium Channel Antagonism

Abstract

Neuroinflammation is emerging as a major factor in the decline of cognition. Most neurodegenerative diseases have an inflammatory component; and general brain inflammation appears to increase with age. Pyramidal neurons in the aging hippocampus demonstrate well-characterized alterations that are believed to cause a 'dysregulation' of Ca^{2+} homeostasis. Specifically, a decline in *N*-methyl-D-aspartate receptors (NMDA receptors) and an increase in L-type voltage-dependent Ca^{2+} channels may reduce overall neuronal excitability and synaptic plasticity. Although chronic inflammation has been demonstrated to reduce long-term potentiation (LTP) in the hippocampus, it is not known if age-related changes in Ca^{2+} homeostasis are related to an increase in brain inflammation. It has been demonstrated in aged rodents that a peripheral immune challenge with *Escherichia*

coli (E. coli) triggers a prolonged inflammatory response in the hippocampus, which impairs long-term memory consolidation. We previously found that this correlated with a specific deficit in LTP in area CA1 following a single theta-burst tetanus but not after a strong stimulation (4 x 100 Hz). Here, we used an even stronger stimulation (200 Hz) reliably activates NMDA receptors and L-type Ca^{2+} channels to determine effects of age or peripheral infection. We found that a peripheral infection reduces robust LTP in region CA1 of young rats, but not aged rats. Furthermore, blocking L-type Ca^{2+} channels during induction equally reduced LTP in young and aged, control rats; but was occluded following an infection. This effect could not be replicated by a chemical LTP protocol that uses tetraethylammonium (TEA) to activate L-type Ca^{2+} channels; nor were there any effects on synaptic expression of $Ca_V1.2$, the major pore-forming subunit of L-type Ca^{2+} channels.

Introduction

Advanced age can bring cognitive decline that is exemplified by memory loss. The hippocampus is critical for memory formation and is particularly vulnerable to the effects of age. Although late stages of severe dementia involve neurodegeneration, memory loss begins much earlier. Furthermore, even normal aging, free of any obvious pathology (i.e. Alzheimer's disease) includes memory decline and discernible volume loss in the hippocampus. A major theory of age-

related memory decline, supported by observations in humans and rodents, posits that a 'dysregulation' of Ca^{2+} homeostasis caused by changes in Ca^{2+} channel function alters synaptic plasticity in hippocampal pyramidal neurons. What causes Ca^{2+} dysregulation is not exactly known, but it has been linked with other features of general aging, including oxidative stress and mitochondrial dysfunction. Another major feature of aging is increased inflammation in both the periphery and central nervous system. Brain inflammation alone can induce learning and memory problems in the elderly; and there is now extensive evidence that pro-inflammatory cytokines like (interleukin-1beta) Il-1β and (tumor necrosis factor alpha) TNFα directly affect synaptic plasticity. Therefore, it is possible that increased inflammation could cause the change in Ca^{2+} homeostasis that is commonly associated with the aging hippocampus.

Brain inflammation can be induced directly by neurodegeneration, or indirectly by systemic inflammation caused by a sickness or injury. Cytokines in the periphery communicate with the brain via neural and circulatory routes and cause additional release of cytokines from microglia, the major immune cell of the brain. Older individuals are more likely to have an exaggerated inflammatory response in the brain following a peripheral immune challenge, possibly due to 'primed' microglia. It has been demonstrated in rodents that microglia become sensitized with age, releasing excessive amounts of cytokines when activated. When aged, but pre-senescent rats are given a peripheral challenge, like an *E. coli* infection or sham surgery, they exhibit enhanced inflammation in the hippocampus, including

elevated levels of Il-1β that last longer than a week. During this period, aged rats have more difficulty forming long-term memories for a contextual fear-conditioning task.

This memory deficit also corresponds with a reduction in a specific form of LTP in region CA1, where synaptic plasticity is thought to be important for the consolidation of memories from the hippocampus to the neocortex. We found that LTP induced by a brief theta burst stimulation was similar in young and aged rodents; but following a peripheral infection, LTP was reduced only in aged animals. However, LTP induced by high frequency stimulation (4x100Hz) was not affected. Interestingly, others have demonstrated a similar effect of age on LTP, and it believed that the age-related change in Ca^{2+} homeostasis is responsible. When LTP is elicited by weak paradigms (25-50 Hz, one theta burst, etc), NMDA receptors act as the primary source of Ca^{2+}. However, stronger LTP paradigms (200 Hz, multiple theta bursts) recruit additional Ca^{2+} from L-type voltage-gated Ca^{2+} channels and intracellular Ca^{2+} stores. Activation of L-type Ca^{2+} channels requires strong membrane depolarization, but when opened, Ca^{2+} influx triggers further Ca^{2+} influx via ryanodine receptors (RyR) on the endoplasmic reticulum. Multiple lines of evidence demonstrate that while NMDA receptor function is reduced with age, L-type Ca^{2+} channel function and intracellular Ca^{2+} release are actually enhanced. Consequently, LTP following a weak stimulation is vulnerable to age, which could be theoretically masked by enhanced L-type Ca^{2+} channel function and intracellular Ca^{2+} release when a stronger stimulation is used. Indeed, an early study

demonstrated that although young and aged rodents have the same magnitude of LTP in region CA1 following a 200 Hz stimulation, a pharmacological dissection revealed that aged rats have a reduced NMDA receptor component which is compensated for by a larger L-type Ca^{2+} channel component. (shankar).

It is therefore conceivable that our previous finding, which demonstrated that a peripheral infection in aged rats reduces LTP following a theta-burst stimulation but not following high frequency stimulation, is the result of reduced NMDA receptor function by inflammation, but enhanced L-type Ca^{2+} channel function by age. For this study, we hypothesized that L-type Ca^{2+} channel antagonism during high frequency stimulation (200 Hz) would cause a greater LTP reduction in aged rats compared to young, and would further reveal a decreased NMDA receptor component in aged rats following a peripheral infection. On the contrary, we found that L-type Ca^{2+} channels contribute equally to LTP in young and aged rats. Even more unexpected, a peripheral infection reduced LTP in young rats, but not aged rats. This led us to further explore possible reductions in L-type Ca^{2+} channel function by inflammation.

Materials and Methods

Animals

Rats were 3- and 24- month old male Fisher344/Brown Norway F1 hybrids from the NIA Aged Rodent Colony. They were allowed to acclimate to the animal facility for

at least two weeks before experiments began. The animals were pair housed, on a 12-hr light dark cycle, with *ad libitum* access to food and water. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

E. coli Infection

Stock *E. coli* cultures (ATCC 15746; American Type Culture Collection, Manassas, VA) were thawed and cultured overnight (15-20 hours) in 40 ml of BHI (DIFCO) at 37°C. Growth of individual cultures was quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 3000 rpm, supernatant discarded, and bacterial pellets were resuspended in sterile PBS to give a final concentration of 1.0 $X10^{10}$ CFU/ml. All animals received an intraperitoneal (i.p.) injection of 250 μl of either *E. coli* or vehicle (PBS).

Electrophysiology

Physiology experiments were performed 4-5 days after the initial infection. This time point was chosen based on several observations: (1) all of the animals have completely recovered from the acute infection after four days (symptoms such as fever are gone within 3 days); (2) the 24-month-old rats, but *not* the 3-month-old rats show a significant impairment in long-term hippocampus-dependent memory four days after the *E. coli* infection and (3) levels of IL-1 protein in the hippocampus

are still significantly elevated in the 24-month-old rats, but not in the 3-month-old rats, 4-5 days after the infection (Barrientos et al., 2009).

Experiments on hippocampi from young and aged, saline or *E. coli* injected animals were interleaved. Rat brain were removed from the skull following decapitation and placed in ice-cold and oxygenated artificial cerebrospinal fluid (ACSF, in mM: 124.0 NaCl, 4.4 KCl, 26.0 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose). Hippocampi were dissected from cortex and collected and transverse hippocampal slices (400 μm) were prepared using a McIlwain manual tissue chopper. Slices were maintained in an interface chamber at 28ºC, and perfused with an oxygenated ACSF. Slices were permitted to recover for at least 2 hours before recording. Field excitatory postsynaptic potentials (fEPSP) were recorded from Schaffer collateral–CA1 synapses by placing both stimulating and recording electrodes in the *stratum radiatum*. All stimuli were delivered at intensities that evoked fEPSP slopes equal to 40% of the maximum in each slice. Test stimuli were delivered once every minute, and test responses were recorded for 20-40 minutes prior to beginning the experiment to assure stability of the response. L-type calcium channel-dependent LTP was induced by either a 200 Hz stimulation (4 x 200 Hz, 0.5 s, delivered at .2 Hz) or by bath application of 25 mM tetraethylammonium chloride, (TEA-Cl, Sigma) for 15 minutes. For 200 Hz-LTP, slices were treated by bath application with 10 uM nifedipine (Sigma) with .01% dimethylsulfoxide (DMSO) for 15 minutes before and 30 minutes following tetanization, and recordings were carried out in the dark to reduce

photodegradation of nifedipine. For TEA-LTP, slices were treated by bath application of 50uM D-(-)2-amino-5-phosphonopentanic acid (D-APV, Tocris) for 15 minutes before and 60 minutes following TEA treatment.

CA1 tissue preparation and subcellular fractionation

All tissue was collected four days after infection. Hippocampi were removed after decapitation and transverse slices (1 mm) were prepared and dissected in ice-cold ACSF supplemented with Sigma protease and phosphatase inhibitors. Slices were dissected under 10X magnification using a scalpel and fine-tipped forceps. The CA3 was removed with a cut across the mossy fibers and Schaffer collaterals at end of the hippocampal fissure, and remaining CA1 was pulled away from dentate along the hippocampal fissure. CA1 tissue from multiple slices and from both hippocampi were aggregated and homogenized in 300 ul ice-cold sucrose buffer (320 mM sucrose, 10 mM HEPES, pH 7.4) using a glass tissue grinder with a Teflon pestle. Homogenized samples were centrifuged (1,000g, 10 min, 4°C) to pellet nuclear material and unbroken cells (P1). The remaining supernatant (S1) was centrifuged (12,000g, 20 min, 4°C) to yield a P2 fraction containing enriched synaptic material. The P2 pellet was resuspended in 150 ul HEPES resuspension buffer (4mM HEPES, 1mM EDTA, pH 7.4) and centrifuged (12,000g, 20 min, 4°C). This step was repeated. The P2 pellet was solubilized in 200 ul Buffer A, (20mM HEPES, 100mM NaCl, 0.5% Triton X-100, pH 7.2) by pipetting followed by slow rotation for 20 min at 4°C, and then centrifuged (12,000g, 20 min, 4°C). The supernatant was collected

(non-PSD enriched fraction) and the pellet was resuspended in 120 ul Buffer B (20mM HEPES, 0.15mM NaCl, 1% Triton-X, 1% deoxycholic acid, 1% SDS, 1mM DTT, pH 7.5) to serve as PSD enriched fraction. All buffers were kept ice cold and supplemented with a complete set of protease and phosphatase inhibitors (Sigma). Protein concentration for all fraction were quantified and normalized using a BCA protein assay (BioRad)

Western Blot Analysis

Samples were prepared under reducing conditions in 4x Laemmli buffer ( ) and heated for 5 minutes at 70°C. Total protein (5-10 ug of PSD and non-PSD fraction membrane fraction) were loaded into pre-cast 4-12% NuPage Bis-Tris SDS polyacrylamide gels (Invitrogen) and subjected to electrophoresis at 150V at 4°C for 2 hrs. Separated protein was transferred onto polyvinylidene fluoride membranes (Millipore) and membranes were rinsed in phosphate buffered saline with 1% Triton (PBST) and blocked in 5% milk/PBST for one hour at room temperature. Membranes were cut into three strips based on molecular weight and incubated with primary antibodies in PBST at 4°C overnight, followed by 3x10 minutes with PBST, and incubated with secondary antibodies in PBST for one hour at room temperature. The following primary antibodies (and dilutions) were used: PSD-95 (1:3000, Cell Signaling), Synaptophysin (1:2000), and CaV1.2 (1:250, Millipore)

Statistical Analysis

Data from electrophysiology experiments and western blotting was analyzed using a factorial ANOVA, followed by Fisher's LSD *post hoc* comparison when necessary.

Results

Peripheral *E. coli* infection in young and aged F344xBN rats occludes further reduction of LTP by L-type Ca2+ channel antagonism.

Most forms of LTP require NMDA receptor activation, but LTP induced by very robust, high frequency stimulations, like a 200 Hz tetanus, activates both NMDA receptors and L-type Ca^{2+} channels. This has been termed 'compound LTP'. When animals are young, the majority of synaptic potentiation is due to Ca^{2+} influx from NMDA receptors. As animals age though, potentiation becomes more dependent on L-type Ca^{2+} channels. This has been demonstrated in 24 month-old Fisher 344 rats, 24 month-old Long-Evan rats, and 16 month-old C57Bl/6 mice. We decided to explore the contribution of L-type Ca^{2+} channels to LTP in 24 month-old F344xBN rats and what effects, if any, prolonged brain inflammation might have on them.

As in our previous study, LTP in 3 and 24 month-old F344xBN rats was examined four and five days after a peripheral *E. coli* infection, when Il-1b levels are still elevated in the hippocampus of aged rats, but not young. LTP was induced

using a tetanus (4x 200 Hz, 0.5 s, delivered 5 s apart) that has been previously demonstrated to reliably activate both NMDA receptors and L-type Ca^{2+} channels. During baseline recording, we bath applied an L-type Ca^{2+} channel antagonist, nifedipine (10 uM, with .01% DMSO) or .01% DMSO as treatment control, and continued for 30 minutes following tetanus. We expected that aged rats would have less LTP than young rats during L-type Ca^{2+} channel blockade, and even less following an infection. However, we obtained unexpected results. (Figure 4.1)

Figure 4.1

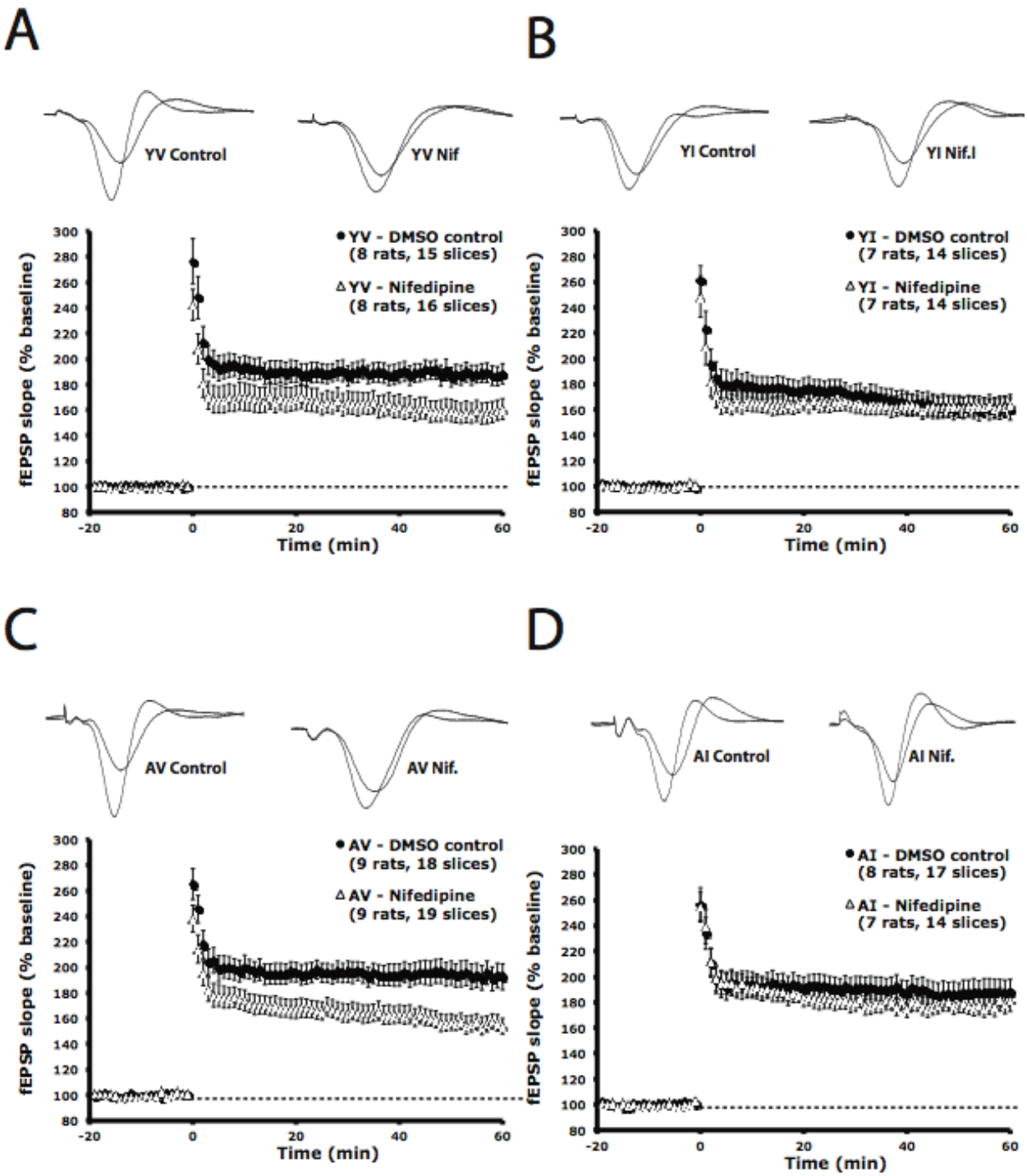
A
YV Control
YV Nif
YV - DMSO control
(8 rats, 15 slices)
YV - Nifedipine
(8 rats, 16 slices)
fEPSP slope (% baseline)
Time (min)
B
YI Control
YI Nif.I
YI - DMSO control
(7 rats, 14 slices)
YI - Nifedipine
(7 rats, 14 slices)
fEPSP slope (% baseline)
Time (min)
C
AV Control
AV Nif.
AV - DMSO control
(9 rats, 18 slices)
AV - Nifedipine
(9 rats, 19 slices)
fEPSP slope (% baseline)
Time (min)
D
AI Control
AI Nif.
AI - DMSO control
(8 rats, 17 slices)
AI - Nifedipine
(7 rats, 14 slices)
fEPSP slope (% baseline)
Time (min)

Figure 1. A peripheral *E. coli* infection reduces high frequency LTP in young rats and blocks LTP reduction by nifedipine in both young and aged rats. Hippocampal slices were prepared from young (3 months) and aged (24 months) rats four and five days following an i.p. injection of *E. coli* or saline. Synaptic plasticity was evoked at Schaffer collateral – CA1 synapses by a 200 Hz tetanus (4x 200 Hz, 0.5 s, delivered 5 s apart) in the presence of the L-type Ca^{2+} channel antagonist, nifedipine or 0.01 % DMSO vehicle. Mean fEPSP slopes are plotted as a percentage of averaged pretetanus baseline values. (A,C) Nifedipine significantly reduced LTP in young rats ($p_{nifedipine}$ = .03) and aged rats ($p_{nifedipine}$= .009) after 60 minutes following tetanus. There was not a significance difference in LTP in the presence of nifedipine between young and aged rats ($p_{age/nifedipine}$ = .81). (B,D) A peripheral infection significantly reduced LTP in young rats ($p_{infection}$ = .04), but not aged rats ($p_{infection}$ = .62). However, nifedipine did not further reduce LTP in young rat ($p_{nifedipine}$ = .87) or aged rats ($p_{nifedipine}$= .70) following a peripheral infection. The inset shows superimposed representative traces 5 minutes before the first episode and 60 minutes after the second episode for each experimental group. Gray bar along x-axis indicate bath perfusion of nifedipine. Error bars indicate SEM.

We found the magnitude of post-tetanic potentiation following a 200 Hz tetanus was not significantly affected by age ($F_{(1,126)}$ = .10, p = .75) or infection ($F_{(1,126)}$ = 0, p = .98), but there was a main effect of nifedipine treatment that trended toward significance ($F_{(1,126)}$ = 3.25, p = .07). However, the magnitude of LTP one hour later was significantly effected by nifedipine treatment ($F_{(1,126)}$ = 5.76, p = .02), and although there was not a main effect of infection ($F_{(1,126)}$ = 0, p = .985), there was a significant interaction of infection and nifedipine treatment ($F_{(1,126)}$ = 4.62, p = .03). We used Fisher's LSD *post hoc* test to reveal significant group differences. Both young and aged vehicle-injected rats have the same relative magnitude of post-tetanic potentiation following a 200 Hz tetanus (young/vehicle: 276.2 ± 17.7%; aged/vehicle: 265.3 ± 12.2%, p_{age} = .58) and also have the same magnitude of LTP after one hour (young/vehicle: 186.5 ± 7.4%; aged/vehicle: 193.7 ± 9.8%, p_{age} = .76). Nifedipine treatment similarly affected LTP in young and aged, vehicle-injected rats, slightly reducing post-tetanic potentiation (young/vehicle/nifedipine: 242.3± 12.1%, $p_{nifedipine}$ = .09; aged/vehicle/nifedipine: 238.3 ± 10.5%, $p_{nifedipine}$ = .15); but significantly reducing LTP (young/vehicle/nifedipine: 159.6 ± 12.1%, $p_{nifedipine}$ = .03; aged/vehicle/nifedipine: 156.6 ± 10.5%, $p_{nifedipine}$= .009). However, a peripheral infection differently affected young and aged rats. We found that an infection, when compared to vehicle-injected rats, did not significantly affect post-tetanic potentiation in either young or aged rats (young/infected: 261.1 ± 11.7% $p_{infection}$ = .37; aged/infected: 256.1 ± 13.4%, $p_{infection}$ = .62); but it did significantly reduce LTP in young rats while not affecting

LTP in aged rats (young/infected: 162.2 ± 8.5%, $p_{infection}$ = .04; aged/infected: 187.2 ± 9.9%, p_{age} = .80). In contrast to the effect of nifedipine on vehicle-injected animals, nifedipine treatment did not reduce LTP in young and aged, infected rats. (young/infected/nifedipine: 162.4 ± 7.7%, $p_{nifedipine}$ = .87; aged/infected/nifedipine: 179.1 ± 8.1%, $p_{nifedipine}$= .70)

In summary, a peripheral *E. coli* infection reduced LTP following a robust, high frequency stimulation in region CA1 of young rats, but not aged rats. This is despite the fact that a peripheral infection only induces a brief inflammatory response in the hippocampus of young animals, but prolonged inflammation in aged animals. Furthermore, although blocking L-type Ca^{2+} channels reduces LTP in vehicle-injected rats, it does not have an effect on infected rats.

Neither age nor a peripheral infection alters pre- or post-synaptic expression of the L-type Ca^{2+} channel subunit, $Ca_V1.2$ in isolated CA1 tissue.

L-type Ca^{2+} channels have one of two α1 subunits, $Ca_V1.2$ and $Ca_V1.3$, which reside in the membrane and form the pore of the channel. $Ca_V1.2$ is predominant subunit (80%) in the hippocampus and is found in clusters on the soma, along dendrites, and on pre- and postsynaptic terminals. We hypothesized that the failure of nifedipine to reduce LTP after a peripheral infection could be due to a reduction in $Ca_V1.2$ membrane expression in synapses, which might be selective to region CA1. Aging leads to enhanced L-type Ca^{2+} currents and increased membrane

expression in CA1 pyramidal neurons. But CA1 also has more activated microglia following insults such as ischemia, which was shown to induce a selective down-regulation of L-type Ca^{2+} channel activity region CA1, but not in other areas of the hippocampus. Treating cultured neurons with Il-1β for 24 hrs reduces both total homogenate and plasma membrane expression of $Ca_V1.2$ protein by ~40%. Finally, a previous study from our lab demonstrated that BDNF gene expression, which is dependent upon Ca^{2+} influx from L-type Ca^{2+} channels, was reduced in CA1, but not CA3 or dentate, in young rats following a peripheral infection.

To examine the synaptic membrane expression of $Ca_V1.2$, we prepared pre- and postsynaptic-enriched membrane fractions from dissected CA1 tissue of 3 and 24 month-old rats, 4 days after receiving an i.p. injection of saline vehicle or *E.coli*. After homogenization and subcellular fractionation, crude synaptic membrane was digested with 0.5% Triton. In this detergent, the postsynaptic density (PSD), surrounding membrane, and associated proteins, such as glutamate receptors, become insoluble and can be separated from the soluble non-PSD membrane fraction, which includes presynaptic and extrasynaptic membranes. Our characterization of dissected CA1 tissue demonstrates a clear separation of the postsynaptic and presynaptic membrane based on the distribution of PSD-95 and synaptophysin, which respectively serve as markers for pre- and post-synaptic terminals based on their high abundance (Figure 4.2).

Figure 4.2

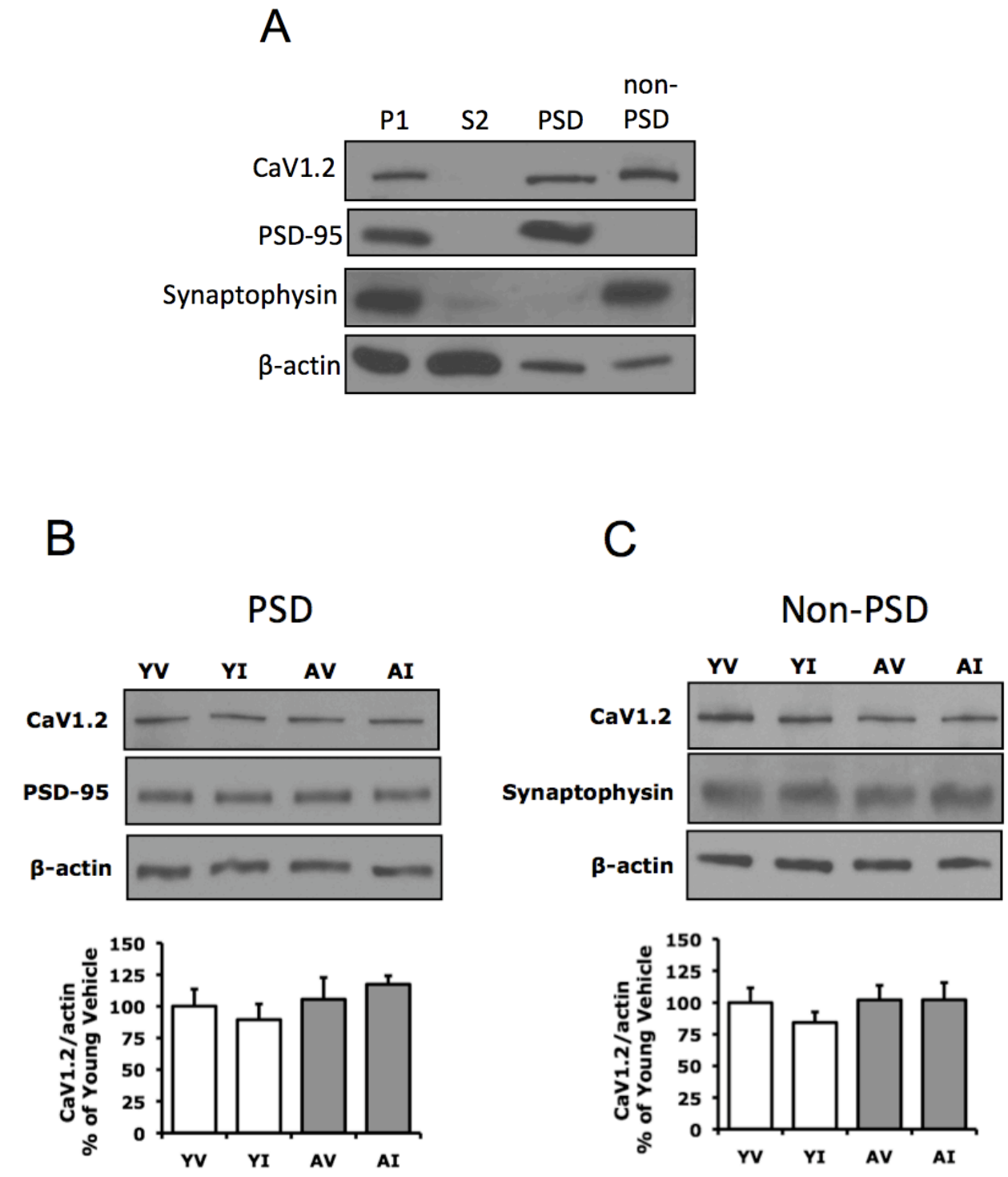
A
P1
S2
PSD
non-PSD
CaV1.2
PSD-95
Synaptophysin
β-actin
B
PSD
YV
YI
AV
AI
CaV1.2
PSD-95
β-actin
CaV1.2/actin
% of Young Vehicle
150
125
100
75
50
25
0
C
Non-PSD
Synaptophysin

Figure 4.2 Pre- and postsynaptic expression of $Ca_V1.2$, the main pore-forming subunit of L-type Ca^{2+} channels, in region CA1 is not altered by age or infection. PSD-enriched and non-PSD membrane fractions were prepared from CA1 tissue of young (3 months) and aged (24 months) rats four days following a peripheral infection. (A) Characterization of subcellular fractionation by western blotting. P1 consists of nuclei and unbroken cells; S2 consists of cytosolic proteins; PSD consists postsynaptic proteins, and non-PSD consists of presynaptic and extrasynaptic proteins. Equal amounts of total protein (8 ug) for each fraction were loaded. PSD-95 and synaptophysin demonstrate specific enrichment to the appropriate PSD and non-PSD fraction, and $Ca_V1.2$ is enriched is both fraction. B-actin is enriched in P1 and S2 fraction. (B) There was not a significant main effect of age or infection on PSD-95 or $Ca_V1.2$ expression in the postsynaptic membrane fraction of CA1. (C) There was not a significant main effect of age or infection on synaptophysin or $Ca_V1.2$ expression in the presynaptic and extrasynaptic membrane fraction of CA1. All protein was normalized against B-actin. N = 4 animals/group. Error bars indicate SEM

Interestingly, the distribution of $Ca_V1.2$, detected as a single band at ~240 kDa, was similarly enriched in the PSD and non-PSD membrane fractions (Figure 4.2A). An important caveat is that although non-PSD membrane fraction is enriched for pre-synaptic membranes, it also contains extrasynaptic membranes, making it impossible to determine if $Ca_V1.2$ expression is presynaptic or extrasynaptic.

In the PSD-enriched membrane fraction, we determined that there was no effect of age ($p = .751$) or infection ($p = .888$) on PSD-95 protein levels when normalized to B-actin (data not shown). However, there was also no significant effect on $Ca_V1.2$ protein levels either ($p_{age} = .225$, $p_{infection} = .947$) (Figure 4.2B). In non-PSD membrane fraction, we determined that there was no effect of age ($p = .751$) or infection ($p = .888$) on synaptophysin protein levels; but again, there was also no effect on Cav1.2 protein levels either ($p_{age} = .970$, $p_{infection}$) (Figure 4.2C). Therefore, F344xBN rats have similar synaptic expression levels of $Ca_V1.2$ in region CA1 of the hippocampus at 3 and 24 months, and these levels are not altered by a peripheral *E. coli* infection.

Neither age nor a peripheral infection alters TEA-induced LTP.

Based on the results positive results obtained from a 200 Hz LTP and the negative results from western blotting, we decided to further test the possibility that a peripheral infection reduces L-type Ca^{2+} channel activity by using a alternative chemical LTP that has an NMDA receptor and L-type Ca^{2+} channel component. Tetrethylammonium (TEA) acts a potassium channel blocker and increases the general excitability of neurons, causing depolarization, action potential broadening, and significant Ca^{2+} influx. A brief bath application of TEA (7-15 minutes) while blocking NMDA receptors with D-APV has been repeatedly demonstrated to induce a synaptic potentiation that is dependent upon L-type Ca^{2+} channels. If a peripheral infection does reduce L-type Ca^{2+} channel activity, then TEA-LTP during NMDA receptor blockade should also be reduced.

We examined TEA-LTP in 3 and 24 month-old F344xBN rats four and five days after a peripheral *E. coli* infection (Figure 4.3).

Figure 4.3

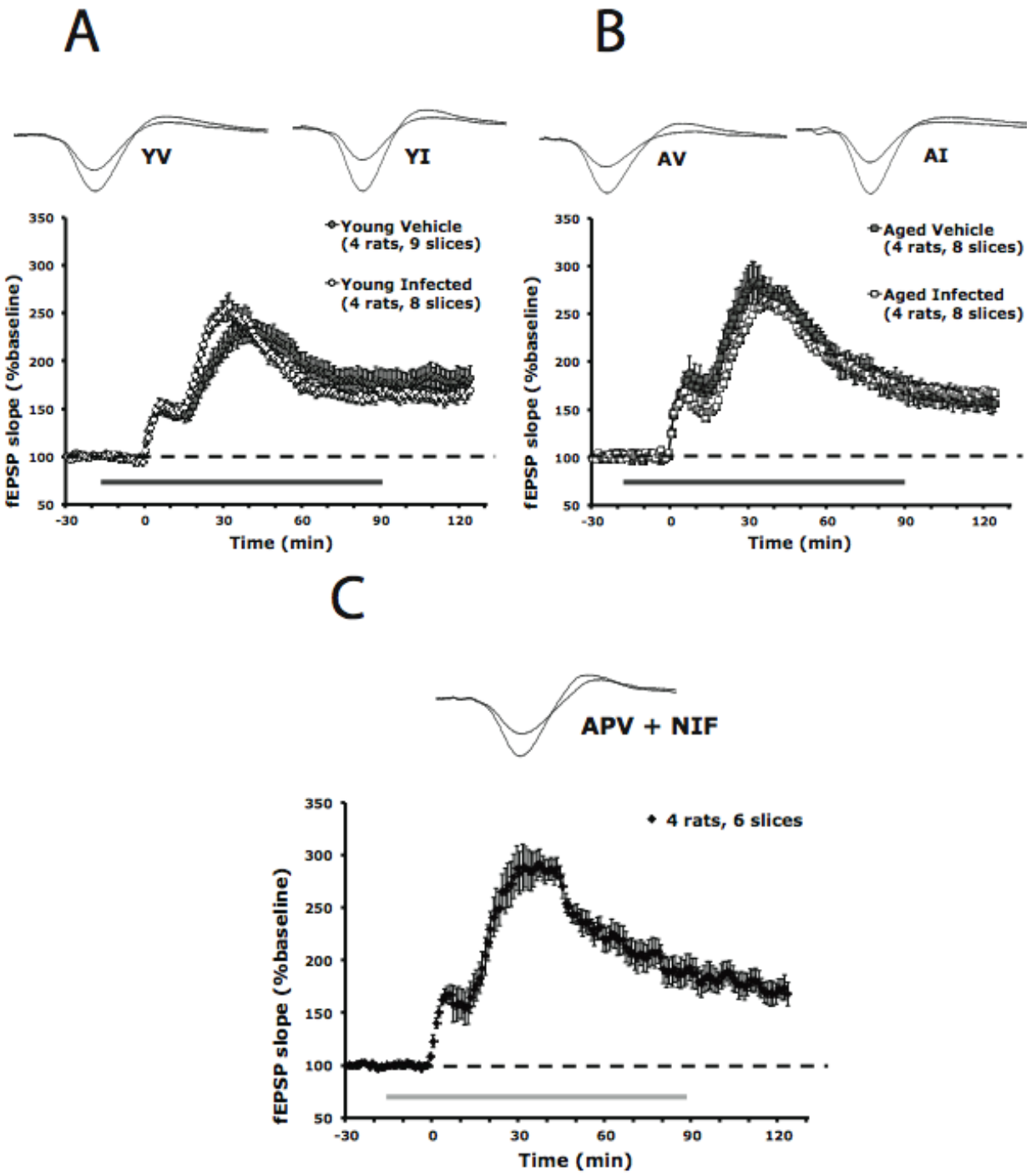
A
YV
YI
Young Vehicle
(4 rats, 9 slices)
Young Infected
(4 rats, 8 slices)
fEPSP slope (%baseline)
Time (min)
B
AV
AI
Aged Vehicle
(4 rats, 8 slices)
Aged Infected
(4 rats, 8 slices)
fEPSP slope (%baseline)
Time (min)
C
APV + NIF
4 rats, 6 slices
fEPSP slope (%baseline)
Time (min)

Figure 4.3 Chemical LTP (25 mM TEA) is not altered by age or a peripheral infection. Hippocampal slices were prepared from young (3 months) and aged (24 months) rats four and five days following an i.p. injection of *E. coli* or saline. Synaptic plasticity was evoked at Schaffer collateral – CA1 synapses by bath application of 25 mM TEA, a K^+ channel blocker for 15 minutes in the presence of D-APV. Synaptic potentiation following this treatment has been demonstrated to be L-type Ca^2 channel dependent. Mean fEPSP slopes are plotted as a percentage of averaged pretetanus baseline values. (A) The magnitude of TEA-LTP in young rats after 120 minutes was not significantly different following by a peripheral infection ($p_{nifedipine}$= .009). (B) The magnitude of TEA-LTP in aged rats after 120 minutes was not significantly different following by a peripheral infection ($p_{nifedipine}$= .009. (A, B) There was not a significant effect of age on TEA-LTP. (C) The addition of nifedipine and D-APV did not significantly reduce LTP in control hippocampal slices from 3-6 month old F344 x BN rats. The inset shows superimposed representative traces 5 minutes before the first episode and 60 minutes after the second episode for each experimental group. Black and gray bars along x-axis indicate bath perfusion of D-APV and nifedipine, respectively. Error bars indicate SEM.

Bath application of 25mM TEA for 15 minutes during NMDA receptor blockade induced a robust and sustained increase in synaptic transmission in all groups. When LTP was measured 2 hours following TEA treatment, there was not a significant effect of age or infection (age: $p = .923$; infection: $p = .37$), nor was there significant differences between experimental groups rats (young/vehicle: 178.4 ± 11.5%; young/infected: 159.6 ± 7.5; aged/vehicle: 158.8 ± 11.3%; aged/infected: 165.5 ± 9.6%). Importantly, we did not observe a significant reduction of TEA-LTP by infection in either young or aged rats. One effect that we did observe that trended toward significance was that aged rats had increased fEPSPs during the peak of TEA treatment ($p_{age} = .064$). Furthermore, a Fisher's LSD *post hoc* analysis determined that this effect was significant between young and aged rats that did not received infections (young/vehicle: 230.4 ± 9.9%; aged/vehicle: 284.2 ± 19.6%).

We do not believe the resulting potentiation was due to residual TEA causing increased cell excitability based on previous reports. Huber *et al*. observed that TEA washes out within 20 minutes when delivered by a 2.0 ml/min flow rate, and Aniksztejn *et al*. demonstrated TEA has only a transient effect on cell excitability (< 30 min). Although we used a slower flow rate (1.0-1.5 ml/min), we kept slices under NMDA receptor blockade by D-APV for 60 minutes following TEA application to eliminate the possibility of NMDA receptor activation. To ensure that the remaining portion of synaptic potential under D-APV was due to L-type Ca^{2+} channel activity, we induced TEA-LTP during bath application of both d-APV and nifedipine (Figure 1C). However, we did not observe any further reduction in synaptic potentiation

with the addition of nifedipine when compared to any experimental group. (D-APV + Nif: 168.3 ± 13.1% at 2 hours). This is in contrast to Huber *et al.*, which demonstrated that the combination of D-APV and nifedipine reduced TEA-LTP by more than half of either drug alone. We do not believe that the failure to reduce TEA-LTP is a result of reduced drug efficacy because D-APV blocked 80-90% of tetanus-induced LTP (200 Hz, data not shown) and nifedipine significantly attenuated LTP in prior experiments. Others have found varying degrees of NMDA receptor and L-type Ca^{2+} channel participation in TEA-LTP; and one conflicting study found that TEA-LTP in region CA1 was dependent of T-type Ca^{2+} channels, not L-type (song d, berger tw 2002). Although we cannot explain the discrepancies of our work and others, it nevertheless seems that TEA-LTP may have not been a valid or appropriate assay of L-type Ca^{2+}channel activity.

Discussion

It is widely accepted that inflammation increases in the brain with age and contributes to memory loss and cognitive decline. The hippocampus is particularly sensitive to age, and its principle neurons undergo a number of changes that might be related to inflammation. Many rodent strains demonstrate an age-related decline in LTP following weak stimulation protocols but not following stronger ones. A leading explanation for these discrepancies proposes that the three main sources of

Ca^{2+} (NMDA receptors, L-type Ca^{2+} channels, and intracellular Ca^{2+} stores) are altered with age. This changes intrinsic membrane excitability and downstream synaptic plasticity. More specifically, NMDA receptor activity, including mediated currents and protein expression has been consistently shown to be reduced with age. Conversely, aging causes increased membrane expression of L-type Ca^{2+} channels and enhanced L-type Ca^{2+} currents in CA1 pyramidal neurons. Shankar *et al.* previously demonstrated that young and aged rats had similar LTP in CA1 following a robust LTP protocol (200 Hz), but individual blockade of NMDA receptors and L-type Ca^{2+} channels revealed that aged animals had less contribution from NMDA receptors and more contribution from L-type Ca^{2+} channels.

We had earlier found that an infection selectively reduced theta-burst LTP in aged rats, but did not affect LTP following a more robust stimulation (4 x 100 Hz). We assumed that an infection might have selectively reduced NMDA receptor activity, which was obscured during robust LTP by a compensatory age-related increase in L-type Ca^{2+} channels. Using the same robust protocol as Shankar *et al.*, we unexpectedly found that a peripheral infection reduced LTP in young rats, but did not in aged rats. And while nifedipine (L-type Ca^{2+} channel antagonist) reduced LTP in young and aged, uninfected rats, it did not reduce LTP following an infection. This suggests that an infection reduced L-type Ca^{2+} channel activity. However, it does not do so by reducing pre-, post-, or extrasynaptic expression of the

major pore-forming subunit, $Ca_V1.2$, as demonstrated by subcellular fractionation and western blotting.

These experiment raised many questions that we tried to answer by using an alternative measurement of NMDA receptor and L-type Ca^{2+} channel activity during synaptic potentiation. As with a 200 Hz LTP, a brief treatment of TEA also induces a chemical LTP that can be pharmacologically divided into a NMDA receptor and L-type Ca^{2+} channel component. We tried to isolate this L-type Ca^{2+} channels component of TEA-LTP in the presence of D-APV to determine in an infection reduces L-type Ca^{2+} channel activity. We found that the L-type Ca^{2+} channel component was not reduced by infection in young or aged rats. However, we were not able to significantly reduce TEA-LTP when both NMDA receptors and L-type Ca^{2+} channels were blocked, as others have demonstrated. It is possible that another source of Ca^{2+} that we did not consider contributed to LTP during TEA treatment. In fact, one study determined that TEA-LTP in region CA1 was dependent of T-type Ca^{2+} channels, not L-type Ca^{2+} channels. The possible lack of specificity of TEA-LTP for L-type Ca^{2+} channels makes it difficult to conclude how infection might be interfering with this channel.

There were many surprising findings from our 200 Hz LTP experiment. While Shankar et al. found L-type Ca^{2+} channels and NMDA receptors contributed more and less, respectively, to LTP in aged rats, we did not. This could be due to the different type of rat strains used. Shankar *et al.* studied F344 rats, which demonstrate cognitive impairments at 24 months, when LTP was examined. In this

study, we used F344xBN rats, which are genetically healthier and live longer (~34-39 months) than other rat strains (~25-30 months). At 24 months, when other rat strains have spatial memory deficits, F344xBN rats have only minimal impairments. This might be related to why we did not observe any age-related in L-type Ca^{2+} channels by electrophysiology or western blotting. Even more surprising though was that not only did a peripheral infection block further reduction of LTP by nifedipine in young rats, it did so in aged rats even though LTP was not reduced by infection.)

If we accept that LTP induction following a 200 Hz tetanus is entirely dependent upon Ca^{2+} influx from NMDA receptors and L-type Ca^{2+} channels, then we must conclude that a peripheral infection reduces L-type Ca^{2+} channel activity, regardless of age, but enhances NMDA receptor activity in aged rats. Treatment of cultured neurons with either Il-1β or Il-6 decreases L-type Ca^{2+} channel membrane expression, although this did not likely occur in our experiment as neither age or infection changes synaptic expression of $Ca_V1.2$. However, if inflammation does reduce L-type Ca^{2+} channel, its effects could be more pronounced in aged animals whose memory and synaptic plasticity depend more the channel. Using the same 200 Hz LTP protocol as here, a recent study demonstrated that aged rats with a larger L-type Ca^{2+} channel component performed better on a spatial memory task than aged rats with a smaller component, despite both groups having reduced a NMDA receptor component. The authors concluded that an age-related

compensatory mechanism that preserves Ca^{2+} homeostasis as NMDA receptor activity fades helps preserve memory.

It is also possible that an infection interferes with RyRs and reduces intracellular Ca^{2+} release, which is downstream of L-type Ca^{2+} channel influx (Foster 2012). In fact, decreased activity in either channel could paradoxically increase NMDA receptor activity in aged rats. This is because they jointly trigger a large rise in intracellular Ca^{2+}, which activates SK channels and K^+ efflux, causing hyperpolarization. Following a burst of action potentials, this Ca^{2+}-dependent, K^+ efflux mediates a slow afterhyperpolarization (sAHP). Because NMDA receptors are voltage-gated and require sufficient depolarization to open, dendritic hyperpolarization and a larger sAHP is thought to reduce NMDAR activity. Treatments that block L-type Ca^{2+} channels, RyRs, or SK channels in aged animals increase NMDA receptor activity.

Many of the age-related changes that alter Ca^{2+} homeostasis and change synaptic plasticity may be part of larger function to preserve neuronal viability. As neurons age, decreases in NMDA receptor activity and increases in L-type Ca^{2+} channel activity are thought to reduce excitotoxicity. As a consequence, synaptic plasticity in aged animals may depend less on NMDA receptors and more on L-type Ca^{2+} channel. (Magnusson, Brim et al. 2009). However, the effects of neuroinflammation, may act counter to aging, increasing NMDA receptor activity and decreasing L-type Ca^{2+} channel activity. This might increase neural excitability

and facilitate inappropriate strengthening of synapses, which would eventually reduce LTP.

Finally, although a peripheral infection only causes a brief inflammatory response (< 24 hrs) in the hippocampus of young rats, they demonstrated the largest reduction in LTP when tested 4-5 days later. A reduction in L-type Ca^{2+} channel activity in young rats could explain a previous unexpected result pertaining to brain-derived neurotrophic factor (BDNF) gene expression (Chapman, Barrientos et al. 2011). In *Chapman et al.* we found that a peripheral infection significantly reduced BDNF mRNA in CA1 of young rats. Interestingly, even though an infection reduced basal levels , it did not prevent increased transcription one hour after contextual fear conditioning. Because activity-dependent BNDF transcription is regulated by Ca^{2+} from NMDA receptors and L-type Ca^{2+} channels, reduced activity at one, but normal activity at the other following an infection could explain why young rats had lower basal BDNF transcription, but retained a transcriptional response following fear conditioning.

Chapter 5

Conclusion

The rate of dementia is predicted to soar over the next 25-50 years as an aged population continues to grow with the support of improved healthcare (brayne). Although the most common cause of dementia is Alzheimer's disease (AD), there are obvious changes occurring in the brain well before the substantial build-up of amyloid beta plaques and neurofibrillary tangles (small 2006). In fact, almost all cases (95%) of AD arise spontaneously; and while there are genetic risk factors, age is still the best indicator. It is likely that earlier changes in healthy brain, possibly decades before, triggers a slow cascade that eventually leads to neurodegeneration. As a healthy brain reaches advanced age, changes can be detected in all of its cell types. Neurons have altered Ca^{2+} homeostasis and synaptic plasticity, astrocytes produce less 'gliotransmiters' and uptake less neurotransmitters, and microglia become sensitized and release more cytokines. It is not known how these changes interact, or if one is causing the others. Chronic neuroinflammation has been demonstrates to affect both neurons and astrocytes, and there is evidence that it may both trigger and accelerate AD. It has been hypothesized by others that an injury or sickness in the elderly initiates a chronic inflammatory response in the brain that strains already weakened neurons, accelerating synaptic dysfunction and

cognitive decline (herrup k 2010, perry vh 2007) The experiments in this dissertation support this hypothesis.

All of the experiments used a rodent model that mimics the learning and memory loss that can occur in the elderly after a challenge to the peripheral immune system. These aged rats have prolonged inflammation in the hippocampus, which impairs long-term memory consolidation when tested 4 days later. Young animals only have a brief inflammatory response and are not cognitively affected. Importantly, short-term memory and memories that consolidated outside the hippocampus are fine. To remain consistent, all electrophysiology and molecular biology experiments were performed at 4-5 days following infection.

In Chapter 2, we set out to determine if a peripheral inflammation reduced LTP in region CA1 of the hippocampus, as this is believed to be critical for long-term memory consolidation (Bartsch, Schonfeld et al. 2010). In parallel with our LTP studies, we determined that a peripheral infection did not alter general synaptic transmission, as measured by input/output curves and pair-pulse facilitation (Figure 2.1). We used three different LTP protocols with the intention of mapping early E-LTP and L-LTP. We found that E-LTP induced by a brief high frequency stimulation (HFS, 100 Hz) and L-LTP induced by four trains of HFS (4 x 100 Hz) were *not* affected by age or infection (Figure 2.2, 2.3). However, another form of L-LTP induced by a theta-burst stimulation was severely reduced in aged rats, and moderately reduced in young animals (Figure 2.3), Furthermore, this

specific LTP deficit was blocked by the administration of Il-RA, implicating the inflammatory response.

In Chapter 3, I hypothesized that the selective reduction of theta-burst LTP might be correlated with changes in metaplasticity and NMDA receptors. This was based on multiple reasons: (1) the overall mechanism of LTP did not seem impaired, because aged/animals were still able to obtain maximal LTP using a robust stimulation; (2) the largest decline in theta burst-LTP occurred almost immediately, suggesting impairment(s) in the induction phase; (3) LTP induction by theta-burst is mainly mediated by Ca^{2+} influx via NMDA receptors; (4) many aged rodent species demonstrated declines in NMDA receptor function, and dependent LTP. It was also possible that effect we observed in Chapter 2 might be result of metaplasticity. This is because inflammation, and Il-1β in particular, has been shown to both acutely enhance and chronically reduce NMDA receptor activity. Metaplasticity, which regulates the threshold of synaptic plasticity, has also been shown to depend of NMDA receptor activity. According to Abraham et al., one must consider the history of activity at synapses before judging their response to a given stimulus. Therefore, I assumed that inflammation has an acute, cell-wide excitatory effect on NMDA receptors, but that after four days, the modification threshold would be shifted, making it harder to induce LTP, but easier to induce LTD. Using protocols designed to observe metaplasticity, LTD was found to be enhanced and the modification threshold was shifted toward LTD in aged rats following an infection, agreeing with the hypothesis (Figure 3.1, 3.2). However, LTP was not reduced

(Figure 3.3). Increased GluN2B in crude synaptoneurosomes might be further evidence that inflammation leads to excitatory changes (Figure 3.5).

In Chapter 4, I hypothesized that aged rodents had pre-existing, age-related changes in Ca^{2+} homeostasis that concealed the effect of inflammation on LTP following a robust stimulation. Specifically, an age-related increase in L-type Ca^{2+} channel function might compensate for an infection-related decrease in NMDA receptor function. However, inflammation had a greater effect on robust, 200 Hz LTP in young rat and occluded a further reduction of LTP by L-type Ca^{2+} channel antagonism (Figure 4.1). Furthermore, the combination of an infection and L-type Ca^{2+} channel antagonism did not reduce LTP in aged animals. If the induction of a 200 Hz LTP activates Ca^{2+} influx via NMDA receptors and L-type Ca^{2+} channels, then this tenuously suggests that an inflammation reduces L-type Ca^{2+} channel activity in young and aged rats, but enhanced NMDA receptor activity in aged rats.

It is generally believed that aging causes a progressive decline in brain activity, neuronal excitability, and synaptic plasticity, which reduces learning and memory in the elderly. This has been correlated with a decrease in NMDA receptor activity and an increase in L-type Ca^{2+} channel activity and intracellular Ca^{2+} release. It has also been proposed that altered Ca^{2+} dynamics and reduced synaptic plasticity may be part of a homeostatic function to reduce excitotoxicity and preserve neuronal viability. However, the effects of neuroinflammation may act counter to aging, increasing NMDA receptor activity and decreasing L-type Ca^{2+} channel activity. This might increase neural excitability and facilitate inappropriate

synaptic strengthening. According to the rules of metaplasticity, this would increase LTD and decrease LTP, which might explain the contradiction of how acute inflammation increases neural excitability yet chronic inflammation reduces synaptic potentiation.

Another possibility is that chronic inflammation causes the age-related alterations in Ca^{2+} homeostasis. In this case, the reducing effect of chronic inflammation on L-type Ca^{2+} channels and its enhancing effect on NMDA receptors could trigger a homeostatic mechanism in neurons to scale up L-type channel Ca^{2+} channel activity, and scale down NMDA receptor activity. This would serve as neuroprotective mechanism against chronic inflammation and hyperexcitability. Furthermore, by shifting away from NMDA receptors and toward L-type Ca^{2+} channels, older animals may paradoxically preserve memory by reducing excitability. In fact, studies have found that when aged rodents are grouped by performance on memory tasks, better performers have more L-type Ca^{2+} channel activity and less NMDA receptor activity.

There is data from humans and rodents that suggests that hyperactivity in the hippocampus occurs in the very early stages of cognitive decline, before an eventual reduction in activity. Human patients with mild MCI have been observed to have increased hippocampal activity as measured by high resolution fMRI. This transitions to hypoactivity in later stages of MCI and AD. (Ewers M 2010 Trends Neuro). Similarly, aged rodents who have increased activity in the hippocampus also have impaired spatial memory and more “rigid” place cell activity. (Wilson ia

2006 trends in neuro, also a 2005), In humans, place cell "rigidity" has also been demonstrated by a memory task that requires subjects to discriminate similar objects. (bakker a 2012, yassa ma 2011 PNAS). (Kanak, dl 2011) By treating aged rats with very low doses of the anti-epileptic drug, levetiracetam, *Koh et al.* reduced hyperexcitability and improved spatial memory performance. In humans, a similar low dose treatment of levetiracetam also reduced hippocampal hyperexcitability and improved memory recall. Although it has been proposed that increased activity in the early stages of cognitive decline could serve as a compensatory mechanism for a failing network, that seems unlikely, given that reducing activity corrected memory problems. It is possible that hyperactivity in the hippocampus is triggered by a sudden onset of brain inflammation; as clinical evidence demonstrates increased levels of inflammatory markers in serum before the diagnosis of dementia and Alzheimer's disease.

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