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Immediate Early Gene Expression in Medial Prefrontal Cortex and Hippocampus as a Function of Aging

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IMMEDIATE EARLY GENE EXPRESSION IN MEDIAL PREFRONTAL CORTEX AND HIPPOCAMPUS AS A FUNCTION OF AGING

by

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ABSTRACT
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Normal aging is accompanied by cognitive decline that differs from other aging-related pathological states like Alzheimer's disease. With an increasing proportion of the world population falling in an age group of 65 years and above, a preventive gerontological approach would improve the quality of life in the elderly. Especially important in this regard is the early detection of cognitive decline, so that appropriate measures can be taken to prevent development of cognitive deficits. Impairment in cognitive flexibility, the ability to modify a previously learnt behavior, is one such measure of impairment across species in aged animals. Previous work from our lab has demonstrated that a cognitive flexibility deficit, as measured by extinction of conditioned fear, first emerges in middle-aged animals. Extinction of conditioned fear requires coordinated activity of infralimbic (IL) and prelimbic (PL) subregions of prefrontal cortex, dorsal and ventral hippocampus, and various amygdala sub nuclei. Of these, prefrontal cortex- and hippocampus-dependent behaviors are impaired during aging, indicating that aging-related impairments within these structures could underlie extinction deficits during

aging. One way to measure region-specific neuronal activation is through analysis of immediate early gene (IEG) expression. IEG expression at rest is not random but reflects ongoing memory consolidation. The role of IEGs as markers of neuronal plasticity and their critical role in memory consolidation make them ideal markers for investigating early cognitive decline. The current study investigated aging-related changes in the expression of IEGs, Zif-268 and Arc in the IL and PL subregions of the mPFC in addition to dorsal and ventral hippocampus. Specifically, the current study used western blotting and immunohistochemistry to investigate region-specific expression of the IEGs Zif-268 and Arc in naive adult, middle-aged and aged animals. We found that Zif-268 expression was reduced in IL, PL, CA1 and DG of dorsal hippocampus and DG of ventral hippocampus starting middle-age. In addition, Arc expression was reduced in IL but not PL in aged rodents. Within hippocampus, Arc expression was reduced within dorsal but not ventral subregion starting middle age. These data indicate that IEG expression changes are region-specific, can be evident starting middle age and may contribute to behavioral deficits during the aging process.

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Introduction

According to the U.S. Census Bureau, middle-aged and aged individuals will constitute 43 percent of the US population by the year 2050, drastically increasing the socio-economic impact of aging-associated cognitive decline (U.S. Census Bureau, 2004). Cognitive decline in the elderly can be viewed along a continuum and means more than just absence of neurological disorders like Alzheimer's disease (AD). In the absence of aging-related pathological conditions, normal aging leads to cognitive decline that compromises the quality of life and independent functioning. While AD is characterized by profound cell loss and neuropathological changes such as neurofibrillary tangles, normal aging is accompanied by more subtle changes in the functional output of these neurons. These differences are also consistent with the finding that memory impairments due to normal aging are quite different from those observed during early stages of AD (see Morrison and Hof, 1997 for review). These data emphasize the need to examine normal aging as a distinct etiology for cognitive decline.

Neurobiology of normal aging

Normal aging leads to a decline in many cognitive functions, but two brain regions consistently implicated in these functional alterations are the medial temporal lobe (MTL) and the prefrontal cortex (PFC) (Burke and Barnes, 2006; reviewed later). Aging-related changes in these structures, specifically the hippocampus and PFC, are briefly reviewed below.

Aging-related behavioral deficits: Hippocampus

The hippocampus is a MTL structure related to learning and memory, and its involvement in a variety of learning and memory paradigms is well documented (Squire,

2004). Perhaps the most well-known form of hippocampal-dependent learning is spatial learning. Spatial learning tasks such as the Morris water maze require intact hippocampal function (Schenk and Morris, 1985) and such spatial navigation abilities decline across species as a function of normal aging. For example, healthy aged humans, compared to young adults are consistently poorer at remembering the location of a landmark in a room filled with contextual cues (Newman, 2000). The above task is the human analogue for a well-accepted spatial navigation task, the Morris water maze in rodents. Aged rodents exhibit impaired performance on the Barnes maze, a spatial navigation task adapted to control for the motor deficits seen in aged rodents (Barnes, 1979). Similar spatial learning deficits are observed among dogs and non-human primates (Head et al., 1995; Rapp et al., 1997). Thus, hippocampus-dependent spatial memory tasks are impaired across species during the course of normal aging.

Another hippocampus-dependent task that is consistently impaired as a function of normal aging is trace eye blink conditioning (Moyer et al., 1990; Thompson et al., 1996; Moyer et al., 2000). Trace eye blink conditioning is a classical conditioning paradigm, in which a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US), such as an air puff to the eye. After many paired presentations of the CS and US, animals learn that the CS predicts the US. Thus, the CS will elicit a conditioned response (CR), the eyeblink. Trace eye blink conditioning is distinct from delay conditioning as the CS and the US presentations are separated by a short time interval, the trace interval. Aged animals across species, including rodents (Kishimoto et al., 2001; Knuttinen et al., 2001), rabbits (Solomon and Groccia-Ellison,

1996; Thompson et al., 1996) and humans (Finkbiner and Woodruff-Pak, 1991) display impaired acquisition of trace eye blink conditioning.

Trace fear conditioning is another example of a trace conditioning paradigm. The introduction of a trace interval in auditory fear conditioning necessitates the involvement of other brain structures, including the hippocampus and PFC (McEchron et al., 1998; Kholodar-Smith et al., 2008; Quinn et al., 2008; Gilmartin and Helmstetter, 2010), in addition to the amygdala (Kwapis et al., 2011). The hippocampus is also necessary for contextual fear conditioning, in which a CR is elicited due to the presentation of a US-paired context (Kim et al., 1992). Trace and contextual fear conditioning are impaired in middle-aged and aged rats (Moyer and Brown, 2006; Kaczorowski and Disterhoft, 2009). Thus, normal aging causes deficits in hippocampus-dependent learning, which are likely due to aging-related hippocampal dysfunction.

Aging-related behavioral deficits: PFC

In addition to hippocampus-dependent tasks, normal aging also results in changes in PFC-dependent functions. The PFC is critical for working memory and executive function (Funahashi et al., 1993; Mair et al., 1998). An example of this is the non-matching-to-sample (DNMS) task, a measure of working memory in humans, non-human primates and rodents. In this task, the subject is presented with a stimulus, which is followed by a variable delay period. Next, the subject is trained to pick a different stimulus than the one originally presented. Impairments in learning the DNMS task are observed across species with aging (Dunnett et al., 1988; Moss et al., 1988; Moss et al., 1997; Lyons-Warren et al., 2004), indicating that PFC-dependent working memory declines with age.

The PFC is also critical for executive function and cognitive flexibility, which is an ability to change behavior appropriately when a particular learned rule is no longer valid. The Wisconsin Card Sorting Task (WCST) is one way to measure set-shifting or cognitive flexibility. In this task, subjects are given a group of stimulus cards and learn to sort them into different sets on the basis of color, shape or quantity. The subjects are not explicitly informed about the correct stimulus property but are given feedback whether a particular sorting arrangement is correct or not. Once the matching rule for sorting is learnt, a different rule for sorting is introduced. Performance is analyzed on the basis of the time taken to learn the new rule and the number of errors made in the process.

Aged humans are impaired on the WCST and display more perseverative errors when a new rule is introduced (Rhodes, 2004). Similar results have been obtained on animal analogues of WCST with normal aging (Moore et al., 2003). Thus, normal aging impairs an animal's ability to adapt to changing environmental contingencies (cognitive flexibility), as measured by increasing number of preservative errors.

Behavioral Extinction, Cognitive Flexibility and Aging

Behavioral extinction can be viewed as a task requiring cognitive flexibility. Cognitive flexibility reflects an animal's ability to change its behavior in order to adapt to new environmental contingencies. Extinction training requires an animal to learn that a previously predictive CS no longer predicts an US. Classically conditioned subjects are given repeated presentations of the CS in the absence of the US. The subjects eventually learn that the CS no longer predicts the US and cease responding to the CS. Hence, extinction training requires that subjects learn that the old rule (CS predicts US) is no longer valid. This is consistent with the finding that extinction training leads to the

formation of a new 'extinction' memory (new rule) while leaving the old 'conditioning' memory (old rule) intact (Quirk and Mueller, 2008).

In rodents, extinction of trace fear conditioning is impaired with aging. These deficits first emerge in middle-aged animals (Kaczorowski et al., 2012), which contrasts with hippocampal-dependent memory decline (Moyer and Brown, 2006; but see Kaczorowski and Disterhoft, 2009). Interestingly, aged but not middle-aged rats exhibit deficits in context fear conditioning (Kaczorowski et al., 2012). Hence, fear extinction, a mPFC-dependent behavior is impaired beginning in middle age but context fear conditioning, a behavior dependent on hippocampus (Kim et al., 1992) shows impairment only in aged animals. This supports the frontal hypothesis of aging that suggests PFC-dependent behavioral deficits emerge early during normal aging (Greenwood, 2000). Consistent with this hypothesis, aging-related cellular and physiological changes believed to underlie behavioral deficits observed in the elderly are also more severe in PFC. These aging-related morphological and physiological changes are inter-related and interact to contribute to the aging-related cognitive decline.

Mechanisms of aging-related cognitive decline

Historically, the etiology of aging-related behavioral deficits has been attributed to massive cell loss that accompanies aging. However, with improved stereological methods that provide unbiased cell counts, it has become clear that normal aging does not lead to a profound cell loss in hippocampus or neocortex (Morrison and Hof, 1997). Aging-related changes in brain morphology appear to be more subtle, involving region-specific changes in dendritic branching and spine density.

Although PFC and hippocampus are known to be brain regions most susceptible to aging-related alterations, the impact of normal aging differs between and within these structures. For example, PFC neurons are highly susceptible to aging-related morphological changes. Male rats undergo a 15% reduction in ventromedial prefrontal cortex (vmPFC, includes IL and PL subregions in this study) volume during normal aging (Yates et al., 2008). This is paralleled by a 15% decrease in layer V/VI, but not II/III vmPFC neuronal numbers. Apical dendritic branching of layer II/III vmPFC (IL and PL) neurons is also decreased during normal aging (Kaczorowski et al., 2012). Structural changes in aging neurons such as altered dendritic branching and spine density may underlie aging-related changes in the biophysical properties of the neurons.

Normal aging also results in changes in the electrophysiological properties of dorsolateral PFC (area 46) neurons in primates. Specifically, aging increases input resistance, decreases action potential amplitude and fall time, and increases action potential firing rate in layer II/III dorsolateral PFC neurons (Chang et al., 2005). Working memory tasks in primates are dependent upon alterations in action potential firing rates in dorsolateral PFC (Goldman-Rakic, 1995) and are impaired beginning with middle age (Moore et al., 2006). In aged monkeys, performance on working memory tasks like DNMS has a U-shaped quadratic relationship with firing rate of layer II/III dorsolateral PFC neurons with low or very high firing rates predicting poor performance (Chang et al., 2005). Layer V neurons within dorsolateral PFC also undergo aging-related changes such as decreased single action potential amplitude, duration, and fall time along with an increased slow afterhyperpolarization (sAHP). However, there is no change in the firing rate of layer V neurons, and these aging-related changes are not correlated with

behavioral deficits. Thus, aging leads to a variety of cellular changes, some of which are behaviorally relevant (i.e. these changes correlate with behavioral deficits) and some of which are not (i.e., they do not predict cognitive changes).

Changes in gene expression: Immediate early genes

It is well established that new learning as well as morphological and electrophysiological changes in cells require changes in gene transcription and translation (Hernandez and Abel, 2008). Thus, it is not surprising that the behavioral and neuronal changes reviewed thus far are also accompanied by changes in gene expression during normal aging. Especially pertinent to the current study are the changes in immediate early gene (IEG) expression during aging. IEGs are defined as genes that are immediately and transiently transcribed in a cell following synaptic stimulation in the absence of any other responsive gene transcription or *de novo* protein synthesis. IEGs are also transcribed in a region-and cell-specific manner following a relevant environmental stimulus (Guzowski et al., 1999). Synaptic stimulation leads to activation of a constitutive regulatory transcription factor such as CREB (cAMP response element binding protein) which can trigger transcription of IEGs (Guzowski, 2002).

IEGs can be broadly divided into two classes: transcription factors and effector proteins. IEGs that act as a transcription factor include c-Fos (cellular-FBJ osteosarcoma oncogene) and Zif-268 (zinc finger binding protein clone 268), while Narp (Neuronal activity regulated pentraxin) and Arc (activity-regulated cytoskeletal gene) are examples of effector IEGs. Transcription factor IEGs such as c-Fos can be involved in driving another wave of transcription for "delayed onset effector genes" that mediate stabilization of the ongoing memory processes. Alternatively, effector genes can have a wide variety

of functions by being directly involved in neuronal plasticity and may not necessarily act by recruiting other proteins. Analysis of IEG expression has been used to identify cells that are active during new learning and are necessary for the successful retrieval of that memory at a later time (Reijmers et al., 2007). IEG expression is also critical for the consolidation of long term memory and maintenance of LTP (Guzowski et al., 2000; Jones et al., 2001). Thus, IEGs are important for behavioral and neural plasticity.

Among these, transcription factor Zif-268 (also known as EGR-1, NGFI-A or Krox-24) is rapidly transcribed following new learning and LTP induction (Richardson et al., 1992). Zif-268 knockout mice express the early phase of LTP in DG but lack the late phase of LTP, 24 and 48 hours after stimulation (Jones et al., 2001). Zif-268 null mice are also impaired for long-term memory of water maze, novel object discrimination, conditioned taste aversion, and olfactory discrimination tasks. Interestingly, the acquisition or short-term memory for these tasks was unaffected (Jones et al., 2001) indicating that Zif-268 is important for the consolidation of these memories.

Arc, another IEG, has also been implicated in learning and memory. Arc (also known as Arg3.1) transcription is under very tight regional and temporal control. Arc is rapidly transcribed within minutes of behavioral stimulation and *arc* mRNA is localized within the nucleus during the first 2-15 minutes after which it is translocated to the cytoplasm and can be detected there for 20-45 minutes following behavioral activity (Guzowski and Worley, 2001). Also, *arc* mRNA is found to be enriched at activated synapses, suggesting that *arc* mRNA is translocated to the dendrites and captured by the recently active synapses where it is translated into protein (Steward et al., 1998). Within the synapse, Arc protein interacts with the endocytic machinery regulating AMPA

receptor trafficking by causing AMPA receptor endocytosis and downregulation (Chowdhury et al., 2006). *In vivo*, infusions of Arc antisense oligonucleotides (ODNs) in the rat hippocampus and lateral amygdala block consolidation of spatial learning and fear conditioning respectively (Guzowski et al., 2000; Ploski et al., 2008). *In vitro*, Arc antisense ODNs in the hippocampus disrupt the maintenance phase of LTP (Guzowski et al., 2000). Thus, the IEG Arc is critical for long-term memory formation and synaptic potentiation.

Normal aging leads to changes in basal IEG expression. IEG expression at rest could recapitulate recent experience and may not be a result of spontaneous synaptic activity or noise of the system (Marrone et al., 2008). Gene microarray analysis has been used to reveal aging-related changes in the resting level expression of a wide variety of genes, including Arc and Narp which are down-regulated in the CA1 sub-region of rat hippocampus (Blalock et al., 2003). When basal *arc* mRNA expression in the whole hippocampus was analyzed, no change was found between adult and aged rats (Frank et al., 2010). However, another study measured the basal levels of *arc* mRNA in CA1 of dorsal hippocampus and found a decrease during aging with no change in the number of CA1 pyramidal neurons transcribing *arc*. This suggests that some or all aged CA1 cells make less Arc protein. No change was found in basal *arc* mRNA levels or number of cells transcribing *arc* in DG (Penner et al., 2010). These changes in Arc transcription correlate with increased DNA methylation levels of *arc* promoter and intragenic regions in aged CA1 neurons indicating aging could alter gene expression through epigenetic mechanisms (Penner et al., 2010). Spatial exploration leads to robust expression of *arc* mRNA in CA1 and CA3 pyramidal cells of hippocampus in adult (9 mo.), middle-aged

(15 mo.) and aged (24 mo.) rats with no significant effect of age. Such behaviorally induced transcription of Arc is decreased in granule cells in DG of middle-aged and aged rats (Small et al., 2004). Thus, aging leads to region-specific changes in arc transcription.

Aging decreases *zif-268* mRNA expression in the CA1 but not CA2, CA3 or DG subregions of rat dorsal hippocampus, and *zif-268* mRNA expression predicts behavioral performance on water maze learning (Yau et al., 1996). No change is found in the expression of c-Fos mRNA in CA1 of hippocampus (Bucci et al., 1998). Thus, while Arc and zif-268 expression decline in aging CA1, c-Fos expression remains unchanged suggesting that aging differentially impacts the expression of IEGs (Yau et al., 1996; Bucci et al., 1998; Penner et al., 2010).

Analysis of aging-related changes in the expression of protein products of these genes has found similar results. Number of Zif-268-positive neurons in absence of behavioral stimulation in CA1 of dorsal hippocampus decreases in aged rats but is not correlated with behavioral performance. No difference was found in the number of c-Fos-positive cells in dorsal hippocampus, and very low levels of basal expression detected (Desjardins et al., 1997). However, this study also found a decrease in the total number of cells in CA1 sub-region of dorsal hippocampus, a finding that contradicts the extensive literature on lack of aging-related neuronal death in hippocampus. Interestingly, high frequency stimulation of the perforant pathway in awake, behaving adult and memory-impaired aged rats leads to similar induction of IEG Zif-268 but not c-Fos in adult and aged rats (Lanahan et al., 1997).

The above review of the literature on gene expression changes during normal aging reveals a few important issues. First, aging-related changes in gene expression are

sub-region specific. For instance, these changes may occur in DG but not CA1 neurons limiting the effectiveness of a study that fails to distinguish between distinct subregions of the hippocampus. Second, aging-related changes may be evident in one IEG but not another. This necessitates the use of multiple IEGs to establish aging-related cognitive decline as evidenced by aberrant gene expression within a structure. Third, these changes may result from an altered proportion of cells expressing the protein or the same proportion of cells expressing reduced levels of the protein. Thus, in addition to population level analysis of gene expression, a determination the number of cells expressing the protein is also important for an accurate understanding of aging-related gene expression changes. Fourth, most studies investigating aging-related gene expression changes focus on dorsal hippocampus or fail to make any distinction between dorsal and ventral hippocampus. As a result, aging-related changes in ventral hippocampus are poorly understood. Lastly, there are no published reports investigating gene expression changes within the mPFC during normal aging.

Aging-related extinction deficits

Extinction circuit

Studies in rodents (Morgan et al., 1993; Quirk et al., 2000) and humans (Milad et al., 2005; Milad et al., 2007) have implicated the mPFC as important for learning extinction of conditioned fear. Specifically, two subregions of the mPFC, the infralimbic cortex (IL) and the prelimbic cortex (PL) act in opposing directions during extinction learning. While increased activity in the IL facilitates extinction learning, activity in the PL has the opposite effect (Milad and Quirk, 2002; Burgos-Robles et al., 2007; Quirk and

Mueller, 2008). This differential regulation of fear expression is mediated by the distinct roles of the IL and PL inputs to the amygdala as well hippocampal modulation of the mPFC and/or the amygdala function (Quirk and Mueller, 2008). The role of the IL and PL subregions of the mPFC, hippocampus and amygdala in fear extinction is reviewed below.

PL in fear expression: Inactivation of PL prevents fear expression (Blum et al., 2006; Corcoran and Quirk, 2007; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011) and PL microstimulation leads to increased fear expression (Vidal-Gonzalez et al., 2006). PL neurons show sustained conditioned response to the tone following auditory fear conditioning, and these tone responses in the PL are correlated with freezing behavior. Sustained conditioned tone responses in PL neurons following extinction also predict extinction failure (Burgos-Robles et al., 2009). Although PL promotes fear expression, temporary inactivation of PL during extinction training has no effect on extinction recall during a subsequent drug-free test (Sierra-Mercado et al., 2011). Thus, PL activity is not necessary for fear extinction. Accordingly, fear conditioning and extinction do not modulate the intrinsic excitability of PL neurons (Santini et al., 2008). In summary, Pl activity promotes fear expression but is not required for extinction of conditioned fear.

IL in fear extinction: In contrast to the PL, IL inactivation with muscimol or tetrodotoxin prior to extinction learning impairs extinction recall of cued and contextual fear (Sierra-Mercado et al., 2006; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). Similarly, pre-extinction IL infusions of an NMDA receptor antagonist (Burgos-Robles et al., 2007), β-adrenergic receptor antagonist (Mueller et al., 2008); PKA

inhibitor (Mueller et al., 2008), MAPK antagonist (Hugues et al., 2004), protein synthesis inhibitor (Santini et al., 2004; Mueller et al., 2008) also impair extinction memory.

Fear conditioning and extinction also modulate the intrinsic excitability of the IL neurons differentially. While conditioning decreases intrinsic excitability of the IL neurons, extinction restores excitability to naive levels (Santini et al., 2008). Pharmacological modulation of intrinsic excitabilty in the IL before extinction learning can modulate extinction recall (Mueller et al., 2008; Santini and Porter, 2010; Santini et al., 2012). Enhancing IL intrinsic excitability with M-type potassium channel blocker facilitates extinction recall, while decreasing IL intrinsic excitability with M-type potassium channel agonist impairs extinction (Santini and Porter, 2010). Similarly, β-adrenergic receptor blockade prevents norepinephrine mediated changes in intrinsic excitability in the IL neurons and, pre-extinction infusions of propranolol impair extinction recall (Mueller et al., 2008). Thus, plasticity in the IL is critical for extinction learning, and modulation of intrinsic excitability in the IL before extinction can bidirectionally modulate extinction memory. These data support the hypothesis that decreased intrinsic excitability of the IL neurons with age could lead to extinction deficts.

Accordingly, recent reports have suggested that a lack of mPFC activation underlies extinction deficits. In rodents, failure to learn or recall extinction is accompanied by a lack of neuronal activation as measured by IEG expression in the IL subregion of the mPFC (Herry and Mons, 2004). Freezing during extinction recall is negatively correlated with Zif-268 and c-Fos expression in IL (Hefner et al., 2008). In humans, extinction failure is believed to underlie post traumatic stress disorder (PTSD). PTSD patients not only have deficits in extinction learning, but these deficits are also

correlated with lack of vmPFC activation (Milad et al., 2009). Thus, IL dysfunction could result in extinction deficits during normal aging.

Hippocampus. In contrast to the inactivation of IL, reversible inactivation of the ventral hippocampus with muscimol leads to impaired fear expression during extinction training and subsequent deficits in extinction recall (Sierra-Mercado et al., 2011). Extinction learning induces LTP-like changes in the ventral hippocampus-mPFC pathway and manipulations that prevent these LTP-like changes also prevent subsequent extinction recall (Hugues et al., 2006). These data indicate that activity and plasticity within the ventral hippocampus is critical for successful extinction.

Similarly, inactivation of dorsal hippocampus with muscimol prior to extinction learning leads to impaired acquisition and subsequent recall of fear extinction, irrespective of whether the recall test is in the extinction context or another context (Corcoran et al., 2005). These findings indicate that dorsal and/or ventral hippocampal function is important for successful fear extinction, and these structures may provide context specificity to extinction learning.

Amygdala. In addition to the IL and the hippocampus, muscimol infusions into the basolateral nucleus of the amygdala (BLA) also disrupt fear extinction (Sierra-Mercado et al., 2011). The role of synaptic transmission from the IL to BLA or GABAergic intercalated cells (ITCs) in extinction has been under much focus recently. The BLA-ITC synaptic pathway is facilitated following fear extinction in an IL-dependent manner. Further downstream, a similar facilitation is observed in the afferent synapses of ITC neurons and neurons of the central medial nucleus (ACE_m), the primary output nucleus of the amygdala (Amano et al., 2010). Hence, following extinction, IL dependent

mechanisms lead to an increase in inhibition of ACE_m output via the BLA-ITC-ACE_m pathway. Further supporting this idea, targeted ablation of as low as ~30% ITC cells following extinction leads to impairment in extinction recall (Likhtik et al., 2008). Thus, plasticity of inhibitory transmission within amygdala is critical for inhibition of fear expression.

Stimulation of mPFC leads to increased firing of BLA interneurons (Grace and Rosenkranz, 2002) and has been implicated as a mechanism for mPFC-dependent inhibition of fear expression. Also, extinction deficits have been correlated with lack of Zif-268 and c-Fos immunoreactivity following extinction recall in BLA neurons (Hefner et al., 2008). Within ITCs, fear expression is positively correlated with increased Zif-268 immunoreactivity of medial paracapsular ITC neurons (IMPs) and fear extinction with main ITC nucleus (IN) Zif-268 immunoreactivity (Hefner et al., 2008; Busti et al., 2011). Interestingly, stimulation of IMPs leads to IPSPs in IN neurons (Busti et al., 2011) suggesting IMPs inhibit IN neurons to enhance fear expression. These findings demonstrate that the IL mediates inhibitory signaling in BLA and ITC leading to inhibition of ACE_m output, thus decreasing fear expression.

Proposed model for aging-related extinction deficits

Normal aging leads to impaired extinction of trace fear conditioning with extinction deficits first emerging in middle-aged animals (Kaczorowski et al., 2012). Also beginning in middle age, there is a parallel redistribution of intrinsic excitability within the mPFC, with the IL and PL neurons undergoing region-specific changes in distinct neuronal populations. With age, intrinsic excitability of layer II/III regular-spiking neurons decreases in the IL but not in the PL, and intrinsic excitability of layer II/III burst

spiking neurons increases in the PL but remains unchanged in the IL. These aging-induced changes result in a reversal of intrinsic excitability within the mPFC. Specifically, within the adult mPFC, the IL neurons are more excitable than PL neurons, but this balance in intrinsic excitability is reversed by middle age. In middle-aged and aged animals, PL neurons are more excitable than the IL neurons. Given the distinct role of the PL and IL neurons in fear expression and extinction, respectively, it is probable that aging-related changes in the mPFC intrinsic excitability underlie extinction deficits observed with normal aging.

Based on the role of mPFC, hippocampus and amygdala in extinction of fear conditioning (Quirk and Mueller, 2008) a model of aging-related extinction deficits can be proposed. In adult animals, following fear conditioning PL inputs to the BLA increase fear expression to the CS. Extinction leads to IL-dependent potentiation of inhibitory circuits in amygdala which results in decreased fear expression. Hippocampal inputs to the mPFC or amygdala (or both) provide contextual specificity to the fear responses. In both middle-aged and aged animals, IL intrinsic excitability decreases, which can contribute to an inability to activate inhibitory circuits within amygdala, thereby disrupting extinction. Increased PL activity with aging could also result in enhanced fear expression. Finally, aging-related changes in hippocampus may also contribute to extinction-deficits. The differential role of region-specific aging-related changes within the mPFC and hippocampus in extinction deficits is unknown.

Proposed Study

The emerging view regarding the extinction of emotional memories is that successful extinction requires coordinated activity between the mPFC (IL and PL), the hippocampus, and the amygdala. The emergence of extinction deficits in middle age is in accordance with the early onset of PFC-dependent cognitive decline, making decreased intrinsic excitability within the IL in middle age a highly suitable candidate mechanism underlying these extinction deficits. However, hippocampal function is also susceptible to aging-related changes and could contribute to extinction deficits during normal aging. Hippocampal contribution to aging-related behavioral deficits is likely, since deficits in hippocampal tasks are evident during middle age. However, aging-related changes in hippocampal function during middle age have not received much attention. Thus, these conclusions come with the caveat that most studies have focused on the differences between adult and aged animals with few studies investigating the age of onset of hippocampal deficits.

In the present study, we investigated the changes in basal IEG expression in the IL, PL, dorsal and ventral hippocampus in naive adult, middle-aged and aged rats (*Figure 1*). IEGs are markers of recent neuronal activity and critical for neuronal plasticity and memory consolidation. IEG expression at rest reflects recent experience and could indicate ongoing memory consolidation. Thus, IEG expression is an ideal marker for investigating the onset of aging-related cognitive decline. Understanding aging-related changes in these structures will allow us to better comprehend the contribution that these changes make to aging-related extinction deficits as well as other behaviors impaired during normal aging.

Basal IEG expression during normal aging will be quantified in two aims:

Aim 1: To quantify basal IEG expression in the IL, PL, dorsal and ventral hippocampus in adult, middle-aged and aged F344 rats using western blot analysis.

Aim 2: To quantify the number of IEG-immunoreactive neurons in the IL, PL and; CA1, CA3 and dentate gyrus subregions of the dorsal and ventral hippocampus in adult, middle-aged and aged F344 rats using immunohistochemistry.

Methods

Subjects

Male adult (n = 12, 3.38 ± 0.06 months), middle-aged (n = 13, 16.02 ± 0.26 months) and aged (n = 20, 25.35 ± 0.38 months) F344 rats were individually housed in clear plastic cages. Rats were maintained on a 14 hour light/10 hour dark cycle (lights on at 7am) and had unlimited access to both water and standard laboratory rat chow (Harlan Laboratories). All procedures were conducted in accordance with the University of Wisconsin-Milwaukee animal care and use committee (ACUC) and NIH guidelines.

Tissue collection

Rats were anesthetized with an overdose of isoflurane, brains were removed and rapidly frozen on dry ice, and stored at -80°C for western blot analysis. A subset of the rats (n = 15; 6 adult and 5 middle-aged and aged each) was given an intracardiac infusion with ice cold 0.1 M phosphate-buffered saline (PBS), the brain quickly removed and hemisected. One hemisphere (counter-balanced) was rapidly frozen on dry ice (for western blots) and the other hemisphere was put into 4% paraformaldehyde (PFA) for use in immunohistochemistry.

Western blot analysis

From each rat brain the dorsal hippocampus (Bregma –3.14 to –4.16 mm), ventral hippocampus (Bregma –5.20 to –6.04 mm), infralimbic cortex (Bregma 3.20 to 2.20 mm), and prelimbic cortex (Bregma 3.20 to 2.20 mm) (Paxinos and Watson, 1998) was dissected out and homogenized separately. Dorsal and ventral hippocampus boundaries were based previous literature (Moser and Moser, 1998). Samples were centrifuged at 4000 rpm for 20 min at 4 °C and the supernatant removed and measured using a Bradford

protein assay kit (Bio-Rad, Hercules, CA). Protein samples were normalized (equal amount of protein per lane) and loaded for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 9 %). A molecular weight standard (Bio-Rad) was added to one lane per gel and gel-loading buffer (120 mM Tris-HCl, 4% SDS, 2% glycerol, 0.01% bromophenol blue, and 5% mercaptoethanol) was added to the protein samples to indicate migration along the gel. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using a semidry transfer apparatus (Bio-Rad). Membranes were incubated for 1 hour in blocking buffer (3% nonfat dry milk, 0.05% Tween-20 in 1 mM tris-buffered saline (TBS)), primary antibody (overnight at 4 °C; 1:1000 rabbit anti-EGR1 or 1:200 mouse anti-Arc or 1:1000 rabbit β-actin), and for 90 min in secondary antibody (goat anti-mouse, Santa Cruz Biotechnology, Santa Cruz, CA; or goat anti-rabbit, Invitrogen, Carlsbad, CA). Membranes were then washed (0.05% Tween-20 in TBS), placed in a chemiluminescence solution (Santa Cruz Biotechnology), and exposed to autoradiographic film (Hyperfilm MP). The films were scanned using a Canon scanner and densitometry performed using NIH Image J Software. Alternatively, a subset of membranes was analyzed using Genesys imager and Syngene quantification software. For each brain region, a percentage of control score was derived for each animal by dividing the relative optical density score by the mean optical density scores of the adults.

Immunohistochemistry

Brains were fixed in 4% PFA for at least 24 hours, cryoprotected with 30% sucrose for a minimum of 2 days, and 25 μm thick coronal sections through the mPFC, dorsal and ventral hippocampus were cut on a cryostat. Sections were collected using a

systematic and random-sampling technique (Glaser et al., 2007). For each animal, every sixth section was systematically processed for Zif-268 immunoreactivity. Five such sections from each brain structure were labeled for Zif-268. A sixth section from each brain structure served as a control slice and was treated the same as other five sections, except it was not incubated in primary antibody. Another set of sections (every sixth section) were processed for Arc immunoreactivity. Slices were incubated in 0.3 % H_2O_2 for 10 min to catalyze endogenous peroxidase activity, and washed three times with 0.1M PBS for 5 min. Slices were then incubated in 10% NGS, 0.3% triton-X for blocking for 30 min followed by primary antibody (1:500 rabbit anti-EGR-1 and 1:100 mouse anti-Arc, diluted in blocking buffer) overnight at 4˚C. The following day, slices were washed in 0.1M PBS (two times, 5 min each) and incubated for one hour in biotynylated secondary antibody solution (1:200 goat biotinylated anti-rabbit or 1:200 goat bitotinylated anti-mouse secondary antibody in 3% NGS in PBS). Following secondary antibody incubation, the slices were washed with 0.1M PBS two times (10 min each) and incubated in the ABC Vectastatin mix for 60 mins. The slices were then washed twice in 0.1M PBS (5 min each). Following this slices were incubated with DAB (3,3'–Diaminobenzidine) reagent (Sigmafast DAB with metal enhancers) for 5 min or till appropriate staining color developed. Slices were washed in 0.1M PBS (twice, 10 min each), mounted onto subbed slides and, allowed to dry overnight. The following day, the slides were cleared by successive washes in 70%, 95% and 100% ethanol for 5 min each. Finally, the slides were rinsed in xylene for 3-5 min and coverslipped with permount.

Immunohistochemistry Image Analysis

Digital images were taken on an Olympus fluorescence microscope (equipped with a digital camera) using a 10X objective. Dorsal hippocampus was defined as Paxinos and Watson plate numbers 31 to 34, and three total images were taken from each slice, one of the CA1, one of the CA3, and one of the dentate gyrus subregion (Figure 8). Ventral hippocampus was defined as Paxinos and Watson plate numbers 39 to 43, and similar to the dorsal hippocampus, three total images were taken for each slice, one of the area CA1, area CA3, and dentate gyrus each. Medial prefrontal cortex was defined as Paxinos and Watson plate numbers 8 to 10 (Paxinos and Watson, 1998), and two total images were taken from each slice, one for the infralimbic cortex and one for the prelimbic cortex (Figure 3).

Following imaging of each structure, cells positively stained for Zif-268 were manually counted using Image Pro software. Cell counts were restricted to three of the five stained sections for each structure (i.e., 3 images per animal per structure were analyzed). For dorsal and ventral hippocampus, cell counts were restricted to an area containing the principal cell body layers. Cell counts were performed in the entire IL and PL sub-region of the mPFC. For each brain region, a percentage of control score was derived for each animal by dividing the mean cell count by the average adult cell count.

Data analysis and statistics

Statistical analyses were performed using SPSS. A one-way ANOVA was used to evaluate age effects, and Fisher's PLSD was used for *post hoc* comparisons. Planned comparisons were made using PLSD between adult and middle-aged and adult and aged

group as described during results. Averages were reported throughout as the mean ± standard error of the mean.

Results

Immediate early gene expression within the medial prefrontal cortex

Using western blots and immunohistochemistry, aging-related region-specific changes in immediate early gene expression (Zif-268 and Arc) within the mPFC were quantified.

Zif-268 expression reduces in the IL and PL subregion of the mPFC. Zif-268 expression was quantified within the IL and PL subregions of the mPFC. Western blots were used to detect any changes in overall protein expression. In addition, immunohistochemistry was used to confirm whether the possible changes in protein expression (as detected by western blots) result from a change in the number of cells expressing Zif-268 or the same number of cells expressing less protein.

Western blots revealed a non-significant reduction in Zif-268 expression within the mPFC during normal aging (Figure 2). A one-way ANOVA failed to detect significant differences in Zif-268 expression within the IL during normal aging [Figure 2A, $F(2,32) = 2.61$, $p = 0.09$]. However, a planned comparison revealed that Zif-268 expression is reduced in aged but not middle-aged rats relative to adults (post-hoc LSD, $p < 0.05$ and $p = 0.23$ respectively). Using immunohistochemistry, we quantified whether the number of cells expressing Zif-268 within the mPFC are altered during the course of normal aging. The number of Zif-268 expressing cells within the IL was reduced during normal aging (Figures 4A and 5A). An ANOVA revealed a significant effect of age on the number of Zif-268 expressing cells [$F(2,15) = 7.97$, $p < 0.01$]. Post hoc analysis confirmed that relative to adult rats, middle-aged and aged rats have fewer Zif-268 expressing cells within the IL ($p < 0.01$). No significant differences were found in the

number of IL cells expressing Zif-268 between middle-aged and aged rats (p = 0.99). Thus, aging results in fewer cells expressing Zif-268 within the IL, and these changes first become evident in middle age.

Within the PL, a one-way ANOVA revealed a trend for main effect of age on Zif-268 expression [Figure 2B, $F(2,38) = 2.91$, $p = 0.06$]. Post hoc tests indicated that Zif-268 expression is reduced in middle-aged rats ($p < 0.05$), and there is a trend towards reduced Zif-268 expression in aged animals ($p = 0.08$) relative to adults. In addition, immunohistochemistry also confirms an aging-related decrease in Zif-268 expression (Figures 4B and 5B). An ANOVA confirmed that within PL, aging significantly affected the number of Zif-268 expressing cells [$F(2,15) = 4.41$, $p < 0.05$]. Post hoc analysis revealed that Zif-268 expressing cells decrease in the PL of middle-aged and aged rats relative to adult rats ($p < 0.05$). Hence, the number of cells that express the immediate early gene, Zif-268, decreases in the IL and PL subregions of the mPFC, starting in middle age.

Arc expression is reduced within IL in aged rats. Within the IL subregion of the mPFC, an ANOVA revealed a trend towards reduced Arc expression [Figure 6A, $F(2,37) = 2.90$, $p = 0.68$]. A planned comparison revealed no change in Arc expression between middle-aged and young rats ($p = 0.48$), however Arc expression is significantly reduced in aged rats relative to young rats ($p < 0.05$). Within the PL, an ANOVA revealed no effect of age on Arc expression [Figure 6B, $F(2,37) = 0.75$, $p = 0.47$]. Thus, aging may cause region-specific changes in Arc expression within the mPFC, as evidenced by reduced Arc expression in the IL but not the PL subregion of aged rodents.

Immunohistochemistry was used to further analyze aging-related changes in Arc expression within mPFC. However, the expression of the IEG Arc was restricted to dendritic processes, and no somatic staining was observed within mPFC. Thus, the number of cells expressing Arc within mPFC subregions could not be analyzed.

To confirm that these aging-related changes in immediate early gene expression are not the result of a global decrease in protein levels during aging, we also quantified the expression of β-Actin during normal aging. β -Actin has been used extensively as a control for protein loading and expression (Vanguilder et al., 2012). These changes in protein expression during normal aging take place in the absence of any changes in the level of β-Actin in the IL [$F(2,39) = 0.77$, $p = 0.47$] as well as the PL subregions of the mPFC [$F(2,41) = 0.67$, $p = 0.51$]. Thus, aging-related changes in immediate early gene expression are both protein- and region-specific.

IEG expression within the hippocampus.

Expression of immediate early genes Zif-268 and Arc was quantified within the dorsal and ventral subregions of the hippocampus.

Zif-268 expression. Within the dorsal hippocampus, western blots revealed no change in Zif-268 expression as a function of age [Figure 7A, $F(2,40) = 0.68$, $p = 0.51$]. However, immunohistochemistry revealed region-specific changes in Zif-268 expression within the dorsal hippocampus subregions (Figure 9A and 10). The number of cells expressing Zif-268 within the principal cell layers (CA1, CA3 and dentate gyrus or DG) was counted. To account for any differences in the length of the cell layer imaged (or analyzed), the length of the principal cell layer was measured and the cell counts were

normalized to length of the cell layer. The normalized cell counts revealed aging-related changes in the CA1 and DG subregions of the dorsal hippocampus.

Within the CA1, aging reduces the number of Zif-268 positive cells [Figure 9A and 10A, $F(2,15) = 11.18$, $p < 0.01$]. Post hoc analysis indicated that Zif-268 positive cells are significantly reduced in the CA1 subregion of the middle-aged and aged dorsal hippocampus relative to that from adults ($p < 0.01$). Thus, Zif-268 immunoreactivity is reduced during normal aging within CA1 subregion of dorsal hippocampus.

In contrast to CA1, low basal expression Zif-268 was observed within the CA3 and DG subregions of hippocampus (see Figure 9). This is supported by previous findings demonstrating that basal expression of the IEGs, Zif-268 and Arc is extremely sparse in CA3 and DG subregions (Desjardins et al., 1997; Penner et al., 2010). Within the CA3 and DG, Zif-268 immunoreactivity is comprised of a few intensely labeled cells and an abundance of lightly stained cells. Within dorsal hippocampus, normal aging does not alter the number of intensely labeled cells in CA3 [Figure 9B, $F(2,15) = 1.37$, $p = 0.29$] nor does it alter the total number of labeled cells [sum of intense and light immunoreactivity; $F(2,15) = 1.97$, $p = 0.18$]. Hence, no effect of aging was observed in Zif-268 immunoreactivity within CA3 subregion of dorsal hippocampus.

Within the DG, the number of intensely labeled Zif-268 cells was reduced during aging [Figure 9C, $F(2,15) = 3.64$, $p = 0.06$]. Post hoc tests revealed a significant reduction in intensely labeled Zif-268 cells in the middle-aged as well as aged DG relative to adult ($p < 0.05$). However, the total number of Zif-268 labeled cells does not change as a function of age [$F(2,15) = 0.98$, $p = 0.4$]. This suggests that although a similar number of cells express Zif-268 across age groups, the level of Zif-268 expression

is reduced in DG. Hence, although western blots do not reveal aging-related changes in Zif-268 expression, immunohistochemistry indicates that the numbers of Zif-268 labeled cells are decreased within CA1 and are less intensely stained within the DG starting in middle age.

Within the ventral hippocampus, an ANOVA revealed no aging-related changes in Zif-268 expression [Figure 7B, $F(2,33) = 2.20$, $p = 0.12$]. However, planned comparisons revealed a trend towards reduced Zif-268 expression in middle-aged ($p = 0.05$) but not aged ($p = 0.1$) rats relative to adult rats. Similar to the dorsal hippocampus, immunohistochemistry revealed region-specific changes within the ventral hippocampus Zif-268 immunoreactivity (Figure 9B and 11). Specifically, the number of Zif-268-immunoreactive cells do not change within the CA1 [Figure 11A, $F(2,15) = 0.64$, $p = 0.55$] and the CA3 subregions [Figure 11B, $F(2,15) = 2.13$, $p = 0.16$ and $F(2,15) = 1.84$, $p = 0.2$ for intensely-labeled and total-labeled cells respectively]. However, there is a significant reduction in the number of Zif-268 intensely labeled cells within the DG subregion of the ventral hippocampus during aging [Figure 9C and 11C, $F(2,15) = 18.12$, $p < 0.001$]. Post hoc analysis confirmed that number of intensely-labeled Zif-268 cells is significantly reduced in the middle-aged and aged DG relative to adults ($p < 0.001$). These data indicate that reduction in Zif-268 expression within the ventral hippocampus is driven by a reduction in Zif-268 immunoreactivity within the DG subregion.

Arc expression. An ANOVA revealed that normal aging reduces Arc expression within the dorsal hippocampus [Figure 12A, $F(2,42) = 5.49$, $p < 0.01$]. Post hoc analysis confirmed that Arc expression is reduced in both middle-aged ($p < 0.05$) and aged animals ($p < 0.01$) relative to adults. However, no aging related change in Arc expression

was detected within the ventral hippocampus [Figure 12B, $F(2,41) = 0.69$, $p = 0.51$]. Hence, Arc expression is reduced within the dorsal but not ventral hippocampus starting in middle age.

Aging-related changes in Arc expression were also investigated using immunohistochemistry. Similar to Arc expression within the mPFC, Arc expression was mostly restricted to the dendritic regions in dorsal and ventral hippocampus and thus, cell counts for Arc immunoreactive neurons could not be obtained.

Furthermore, expression of the housekeeping protein β-Actin does not change in the hippocampus of middle-aged and aged rats relative to that from young rats [$F(2,41) = 0.16$, $p = 0.85$ and $F(2,38) = 0.73$, $p = 0.48$ for dorsal and ventral hippocampus respectively]. Hence, aging-related changes in protein expression within the dorsal and ventral hippocampus do not result from a general decrease of protein levels during aging.

Discussion

The present study investigated immediate early gene expression within mPFC and hippocampal subregions during normal aging. We found that aging-related changes in IEG expression are region-specific and first emerge during middle-age. Zif-268, a transcription factor, and Arc, an effector protein, were found to undergo distinct changes within the mPFC and hippocampus subregions indicating that aging affects different IEGs in a distinct manner. Finally, using western blots and immunohistochemistry we demonstrated that aging leads to a reduction in overall IEG levels as well as a reduction in the number of cells expressing IEGs.

Infralimbic cortex

Within the IL sub-region of the mPFC, western blots suggested a non-significant reduction in the IEG Zif-268 starting in middle age (Figure 2). In support of this, immunohistochemistry revealed a significant reduction in the number of Zif-268 immunoreactive cells starting middle age (Figure 4A and 5A). The level of Zif-268 protein or number of Zif-268 immunoreactive cells did not differ between middle-aged and aged rats. Aging also reduced levels of Arc, another IEG, in aged but not middle-aged IL relative to that from adults (Figure 6A). Thus, IEG expression (Zif-268 and Arc) is reduced in the IL from aged animals but only Zif-268 but not Arc is reduced in middle-aged IL.

Prelimbic cortex

Similar to the IL, western blots and immunohistochemistry revealed that basal Zif-268 expression is reduced with age in the PL neurons (Figure 2B). While western blots indicated a trend towards reduced expression of Zif-268 within the PL,

immunohistochemistry confirmed that the numbers of Zif-268 immunoreactive cells are reduced within the PL starting middle age (Figures 4B and 5B). On the contrary, aging does not lead to any significant changes in Arc expression within the PL (Figure 6B) indicating that not all IEG expression is sensitive to aging within the PL.

The aging-related changes in Zif-268 expression within IL and PL first become evident in middle age. This is in accordance with the extensive literature on the early onset of PFC dysfunction, which demonstrates executive functioning is first impaired beginning in middle age (Moore et al., 2006). Furthermore, within the mPFC starting in middle age intrinsic excitability of the IL and PL neurons is altered in opposite directions. While the excitability of the IL neurons is decreased, that of the PL neurons is increased (Kaczorowski et al., 2011). The implications of these data are two-fold. First, the direction of aging-related changes within the PL differ – while neuronal excitability is increased, Zif-268 expression is decreased. Second, within the mPFC, aging-related alterations start early on (i.e., during middle age). Although the precise nature of aging-related deficits and the relationship between neuronal excitability and IEG expression is as yet unknown, the current data demonstrates early onset of aging-related alterations within the mPFC.

Interestingly, the aging-related changes in the expression of Arc followed a distinct pattern than Zif-268 within the mPFC. Arc expression was only reduced in the IL of aged but not middle-aged animals indicating a difference between IEG expression in middle-aged and aged animals. In addition, no aging-related changes in Arc expression were detected within the PL. Thus, aging-related alterations within the mPFC are IEG-

specific, and expression of different IEGs is regulated differentially during the aging process.

Hippocampus

Within the hippocampus, basal Zif-268 and Arc expression as measured by western blot displays region-specific changes. While Zif-268 expression was unaltered within the dorsal hippocampus, there was a non-significant reduction of Zif-268 expression within ventral hippocampus during aging (Figure 7). Using immunohistochemistry, Zif-268 expression in different hippocampal principal cell layers was further analyzed. Number of Zif-268 immunoreactive cells was significantly reduced within the CA1 and DG cell layers of dorsal hippocampus starting in middle age (Figures 9 and 10). Within the ventral hippocampus, Zif-268 immunoreactivity is reduced within the DG but not CA1 subregion (Figures 9 and 11). No aging-related changes were observed in the CA3 Zif-268 immunoreactivity within the dorsal or ventral hippocampus (Figures 9, 10 and 11). Thus, basal Zif-268 expression declines in the CA1 and DG subregions of the dorsal and the DG subregion of the ventral hippocampus during normal aging with changes first evident in middle-aged animals.

Aging-related changes in basal Arc expression followed the opposite pattern. There was a significant reduction in Arc expression within the dorsal hippocampus starting in middle-age, but Arc expression within the ventral hippocampus remained unchanged (Figure 12). These findings are supported by data demonstrating that *arc* mRNA is downregulated in dorsal hippocampus CA1 neurons of aged rats with concomitant changes in DNA methylation in *arc* promoter and intragenic regions (Penner et al., 2010). Similarly, microarray analysis has also revealed reduced *arc* mRNA

expression in the CA1 subregion of the dorsal hippocampus (Blalock et al., 2003). Thus, aging leads to a reduction in basal *arc* mRNA as well as protein levels and these changes may begin in middle age.

Few studies have contrasted the aging-related changes in the dorsal and ventral hippocampus, with most studies either focusing on the dorsal hippocampus exclusively or failing to distinguish between the dorsal and ventral hippocampus. The lack of such studies makes it difficult to predict the specific contribution of the ventral hippocampus to aging-related cognitive decline. A study investigating the decline in adult neurogenesis in adult and middle-aged mice found that neurogenesis decreases faster in the ventral hippocampus than the dorsal hippocampus with age (Jinno, 2011). Moreover, the basal level of total CREB protein is decreased in the ventral but not the dorsal hippocampus of aged rats, with a significant reduction in number of pCREB immunoreactive cells in the DG subregion of the ventral hippocampus, but no change in the CA1 and CA3 subregions of the dorsal or the ventral hippocampus and the DG subregion of the dorsal hippocampus (Countryman and Gold, 2007). These findings indicate that ventral hippocampus may be more susceptible to the adverse effects of aging with the DG of ventral hippocampus being especially affected by these changes. The present study found similar results in the ventral hippocampus using immunohistochemistry, with Zif-268 immunoreactivity decreasing exclusively in the DG of the ventral hippocampus. Thus, aging results in distinct changes in the dorsal and ventral hippocampus. Basal IEG expression within the CA1 and DG subregions of the dorsal hippocampus and the DG subregion of the ventral hippocampus displaying reduced IEG expression during normal aging.

Conclusions

Frontal lobe-related cognitive decline begins in middle age, causing subtle deficits in executive functioning that is required for day-to-day activity. This occurs well before overt learning and memory deficits emerge (Moore et al., 2006). Although the IL and PL subregions of the mPFC regulate a wide variety of behaviors such as trace and context fear conditioning (Quinn et al., 2008), extinction (Quirk and Mueller, 2008) and goal-oriented behaviors (Killcross and Coutureau, 2003), data from our lab are the only published findings regarding the effect of aging on these structures (Kaczorowski et al., 2011). Little is known regarding the cellular and molecular mechanisms that underlie aging-related changes in the mPFC subregions and how these changes interact to influence behavioral deficits associated with aging. The present study demonstrates that changes in basal IEG expression may be evident as early as middle age and may contribute to cognitive decline. Determination of aging-related changes in basal IEG expression will further our understanding of the effect of normal aging on the IL and PL, and guide research on the other mPFC-dependent behaviors that may be affected during the normal aging process.

Although aging-related changes in the hippocampus have received much attention, these studies have primarily focused on changes in the dorsal hippocampus between young and aged animals. Many aging studies fail to include a middle-aged group (Penner et al., 2010, Buechel et al., 2011), which is necessary to differentiate the specific changes that contribute to the behavioral deficits from the non-specific alterations that accumulate during the aging process. The middle-aged group in the current research design allows for a determination of these early, behaviorally-relevant changes.

Relatively little is known regarding changes in the ventral hippocampus that accompany normal aging, making it difficult to predict the contribution of the ventral hippocampus dysfunction to aging-related cognitive decline. Here, we demonstrate that the ventral hippocampus, specifically the DG subregion, undergoes drastic reduction of Zif-268 immunoreactivity starting in middle age. The effect of these gene expression changes on hippocampus-dependent behaviors is yet to be determined.

Improved understanding of aging-related dysfunction in the hippocampus and the mPFC along with the age of onset of such dysfunction will be critical to establish how these structures interact during normal aging to bring about cognitive decline.

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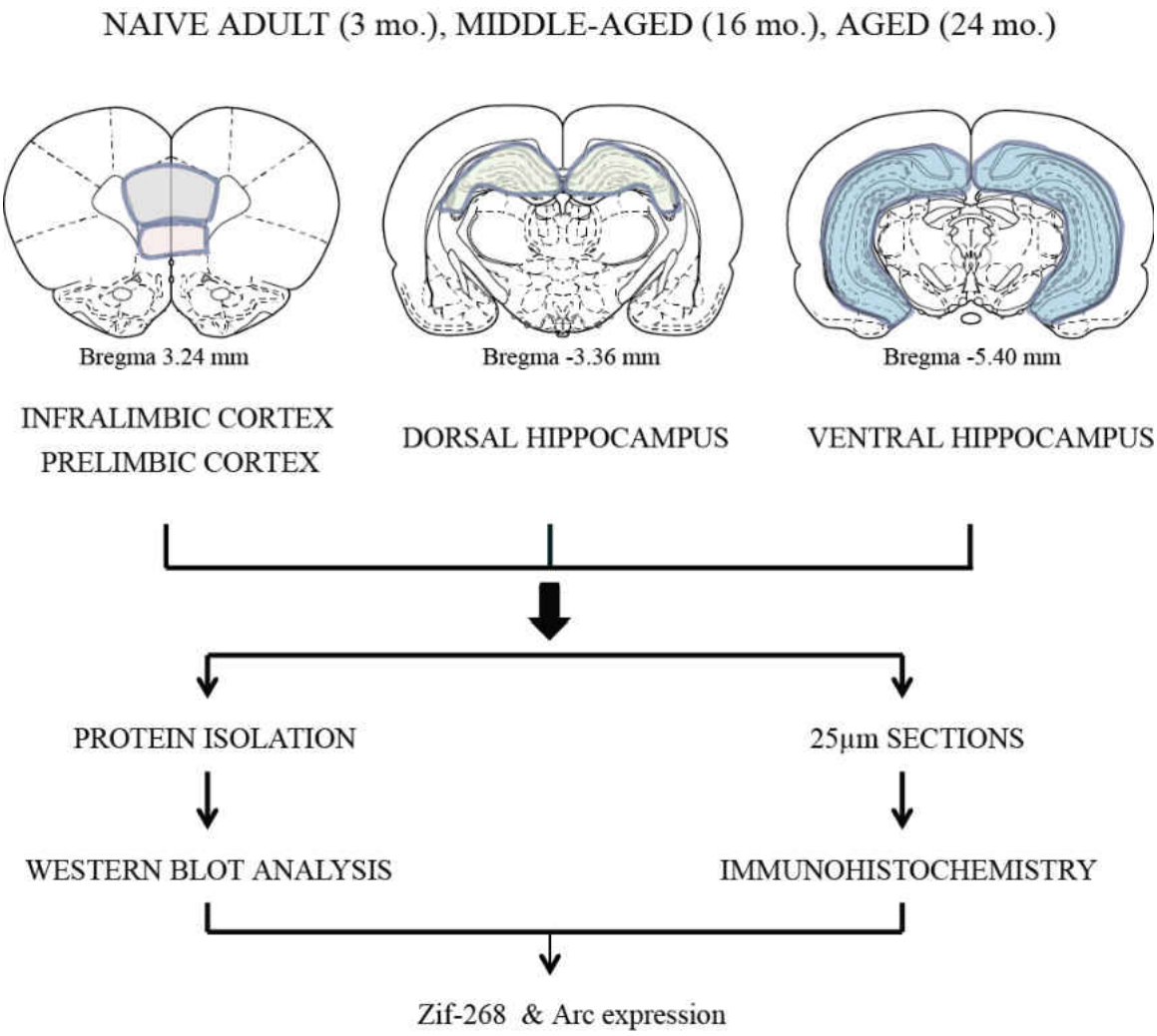


Figure 1. Experimental setup. Experimentally naive adult, middle-aged and aged animals were anaesthetized, perfused intra-cardially with 0.9 % saline, brains removed and hemisected. One hemisphere was processed for western blot analysis and the other for immunohistochemistry in a counterbalanced manner. For a subset of brains, the entire brain was processed for western blot analysis. Basal expression of IEGs Zif-268 and Arc was quantified in medial prefrontal cortex (IL and PL) and hippocampus (dorsal and ventral) as described in the Methods.

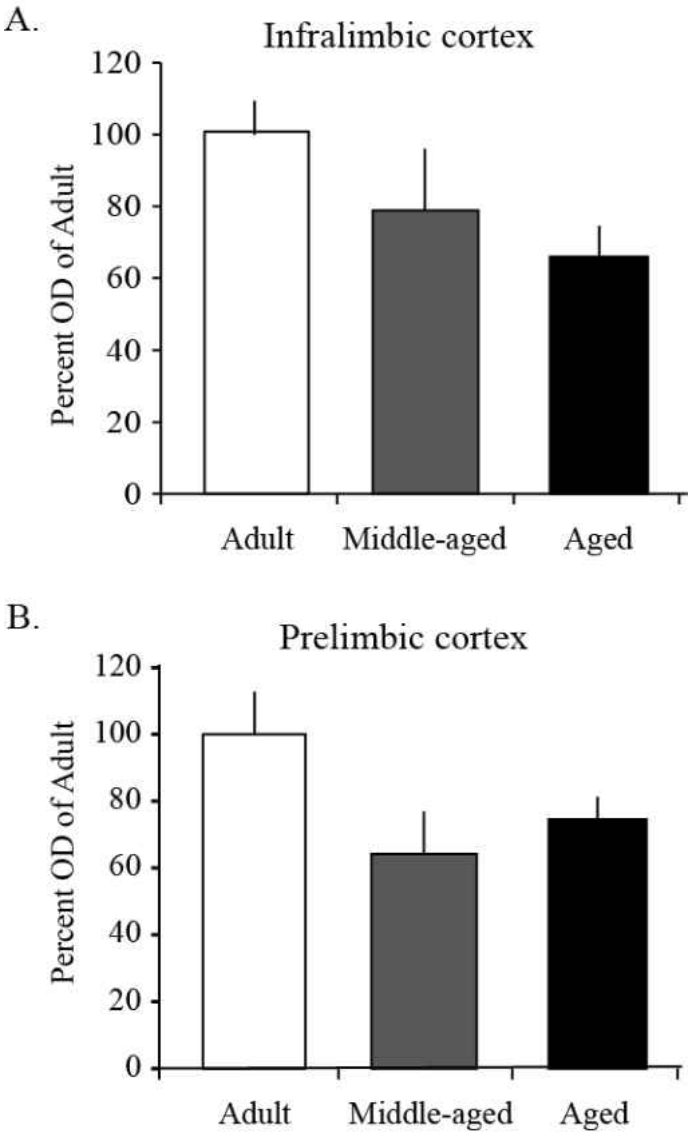
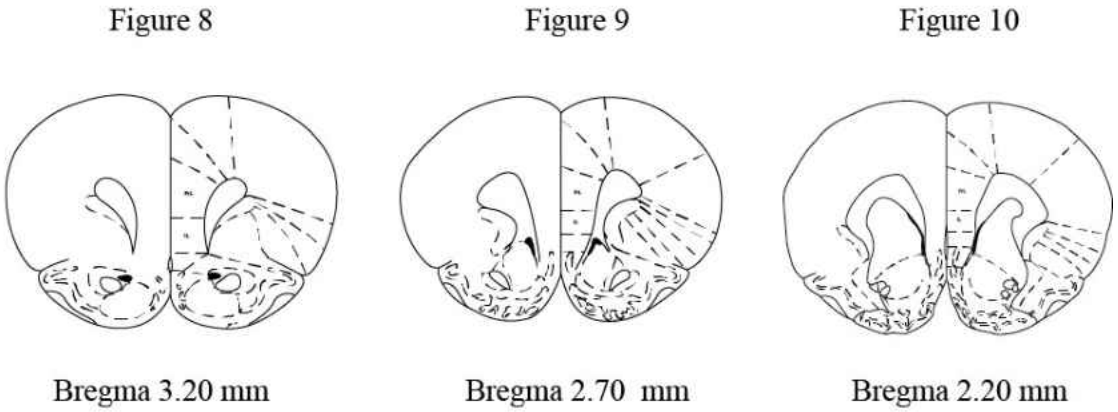


Figure 2. Aging-related changes in mPFC Zif-268 protein expression. A. Within the IL subregion of mPFC, planned comparisons indicate that Zif-268 expression is significantly reduced for aged ($p < 0.05$) but not middle-aged rats ($p = 0.23$) relative to adults. No significant differences were found in Zif-268 expression between middle-aged and aged rats ($p = 0.43$). **B.** Zif-268 expression within PL subregion of mPFC is also reduced during normal aging. Planned comparisons confirm that Zif-268 expression is significantly reduced within PL in aged rats ($p < 0.05$) and show a trend towards reduced expression within middle-aged rats ($p < 0.08$) relative to adults.

A.



B.

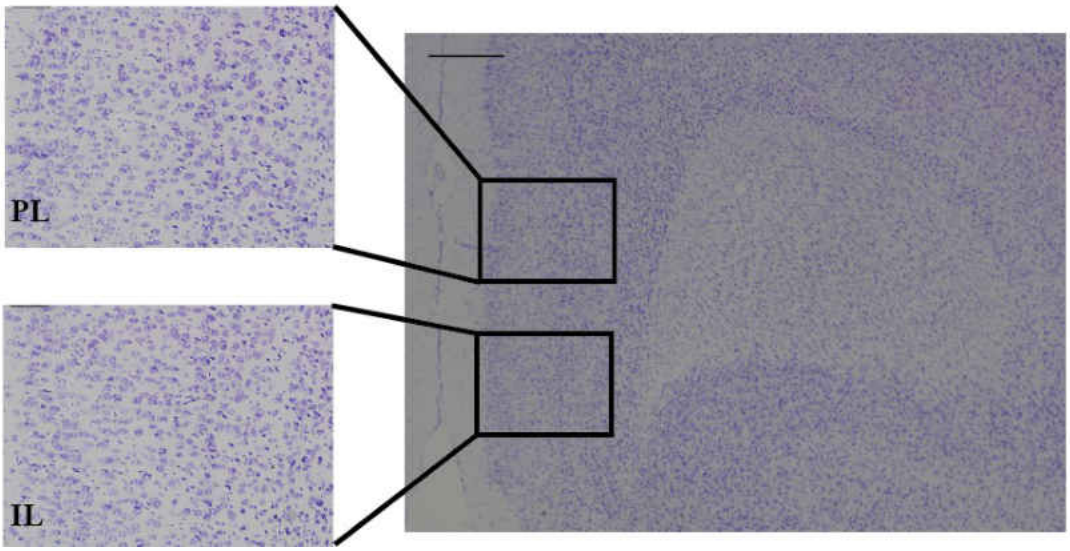
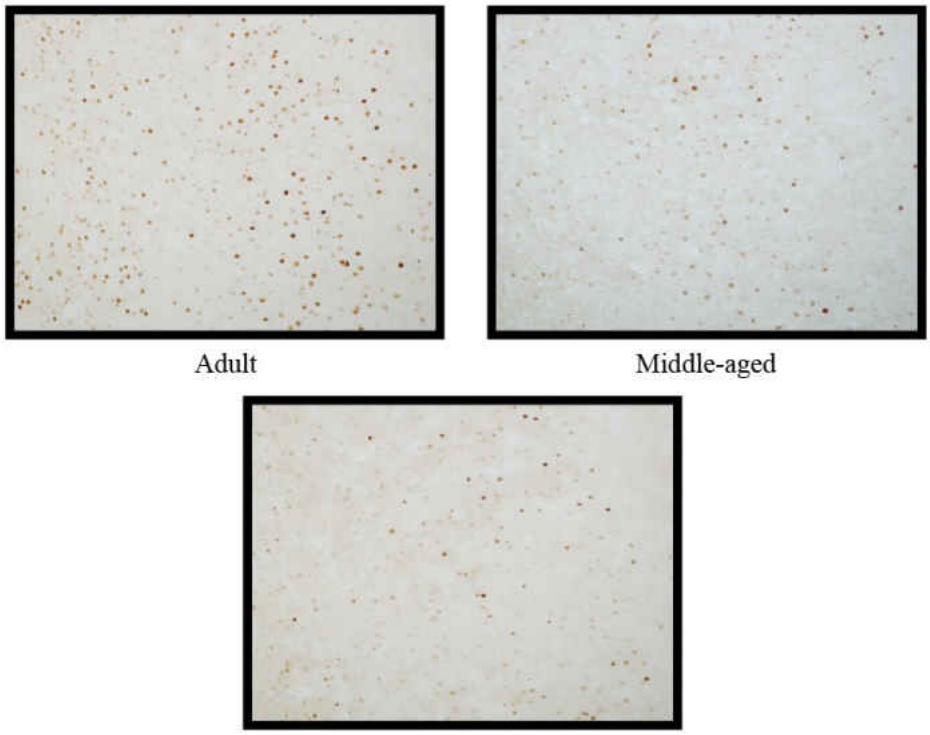


Figure 3. Cresyl ciolet stained sections illustrating the location of image analysis within mPFC. For analysis of immunohistochemistry, Paxinos and Watson (1998) plates 8 – 10 were imaged. Cresyl violet stained sections show representative areas imaged for infralimbic (IL) and prelimbic (PL) cortex. On the right is a low power (2X), cresyl stained section of mPFC (scale 1 mm). Insets on the left depict where IL (bottom) and PL (top) images were taken using a 10X objective (scale 200 µm).

Figure 4. Zif-268 immunoreactivity within mPFC. A. Representative immunohistochemistry images showing that aging reduces the number Zif-268 positive cells within IL of middle-aged and aged rats relative to adults. **B.** Representative immunohistochemistry images showing that aging reduces the number Zif-268 positive cells within PL of middle-aged and aged rats relative to adults. For A and B, left-right is medial-lateral orientation and top-botton is dorsal-ventral orientaion. (Figure on the next page)

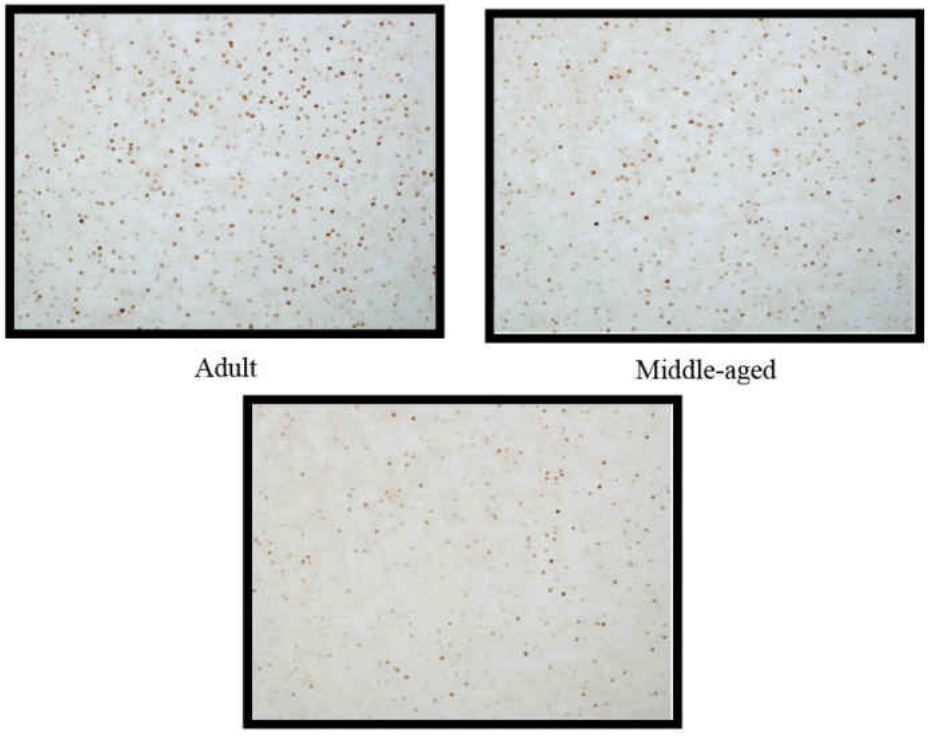
A. Infralimbic cortex



Adult Middle-aged

Aged

B. Prelimbic cortex



Adult Middle-aged

Aged

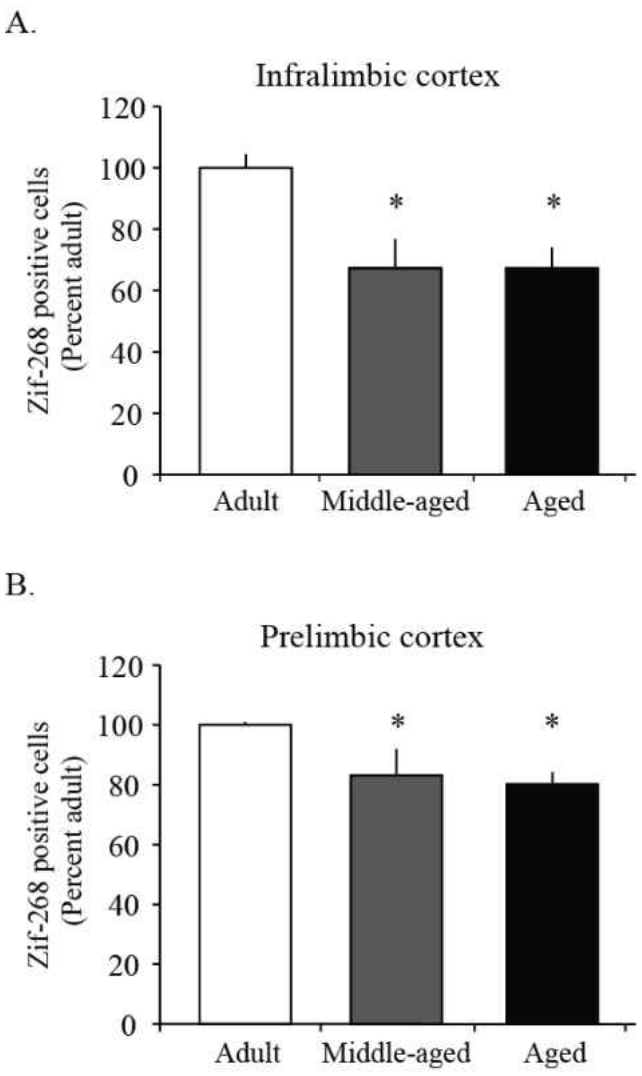


Figure 5. Normal aging reduces Zif-268 immunoreactive cells within medial prefrontal cortex. A. The number of Zif-268-immunoreactive cells decreases in middle-aged and aged IL relative to IL of adult rodents ($p < 0.01$) indicating that reduced Zif-268 expression results from fewer cells expressing Zif-268. **B.** Similar to IL, there are fewer Zif-268 immunoreactive cells in middle-aged and aged rat IL relative to that from adult rats ($p < 0.05$).

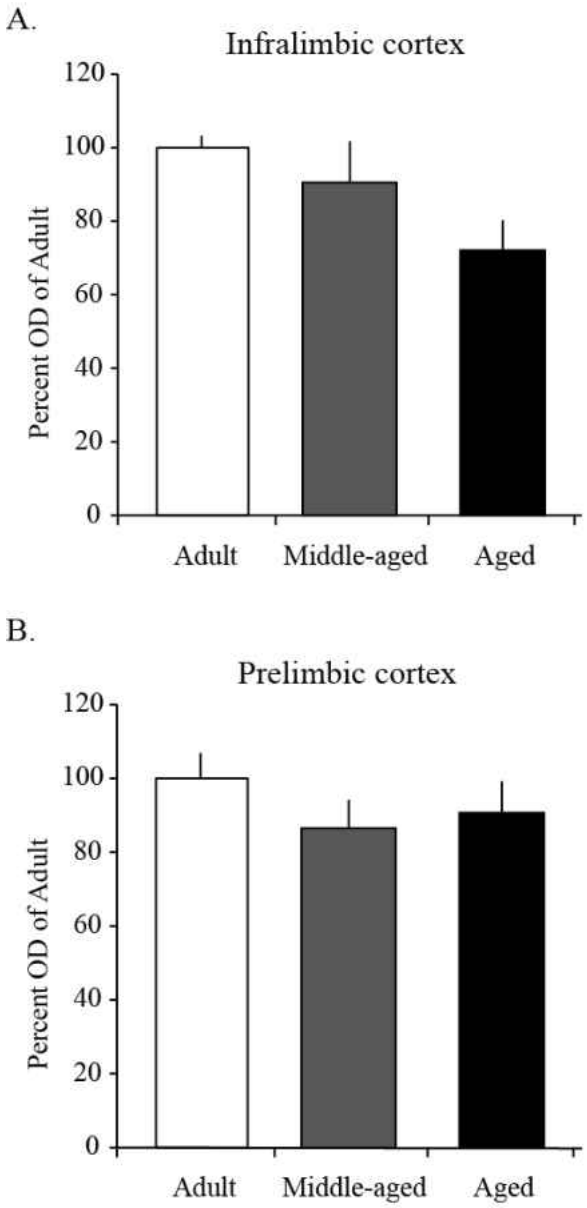


Figure 6. Region-specifc changes in mPFC Arc expression during normal aging. A. Expression of Arc may reduce during normal aging within IL subregion of mPFC ($p < 0.07$). Post hoc tests revealed that Arc expression within IL from aged but not middle-aged rats is reduced realtive to adults ($p < 0.05$ and $p = 0.49$ respectively). **B.** Normal aging does not lead to any change in Arc expression within PL subregion of mPFC ($p = 0.48$).

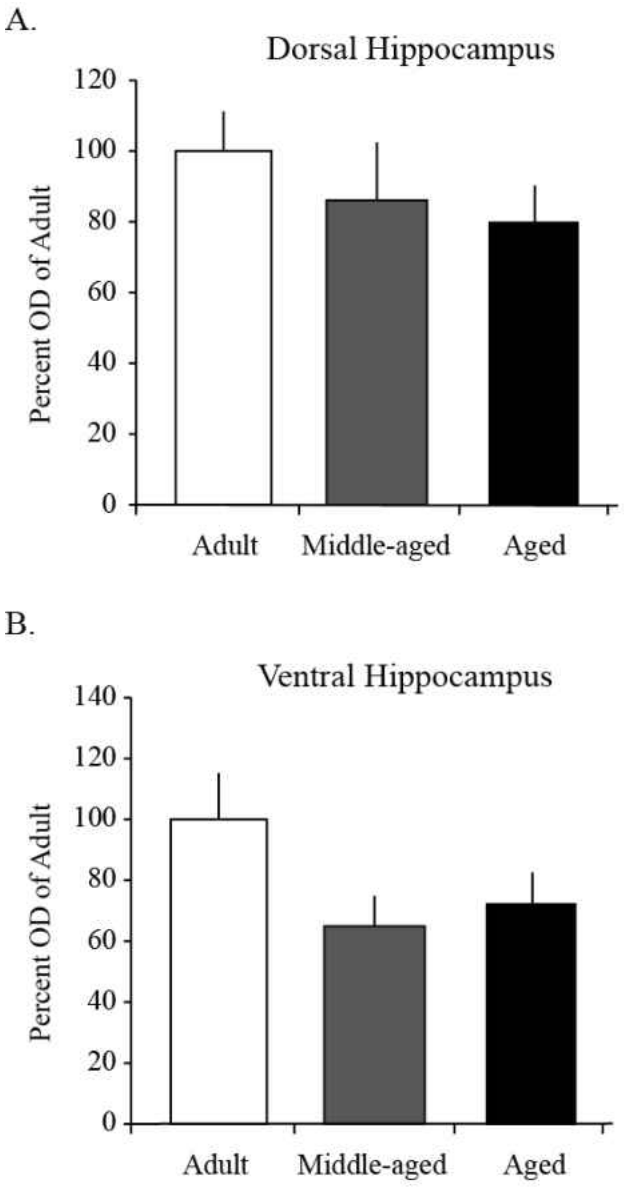


Figure 7. Hippocampal Zif-268 protein expression during normal aging. A. Within dorsal hippocampus, western blots indicate no effect of aging on Zif-268 epxression (p = 0.51). **B.** Zif-268 expression within ventral hippocampus undergoes statistically non-significant reduction (p = 0.13). Planned comparisons confirm a trend towards reduced Zif-268 expression in middle-aged but not aged rat ventral hippocampus.

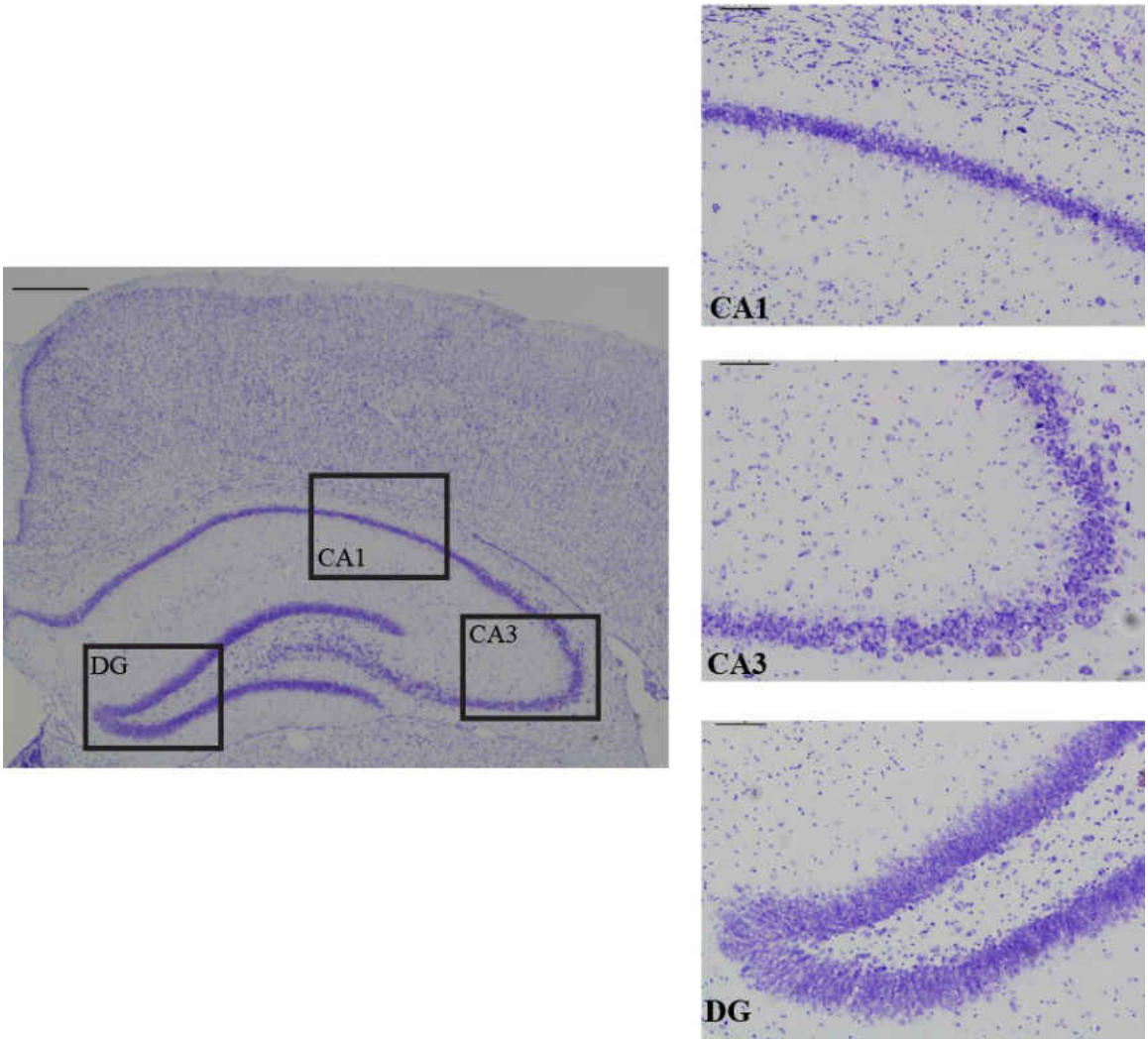
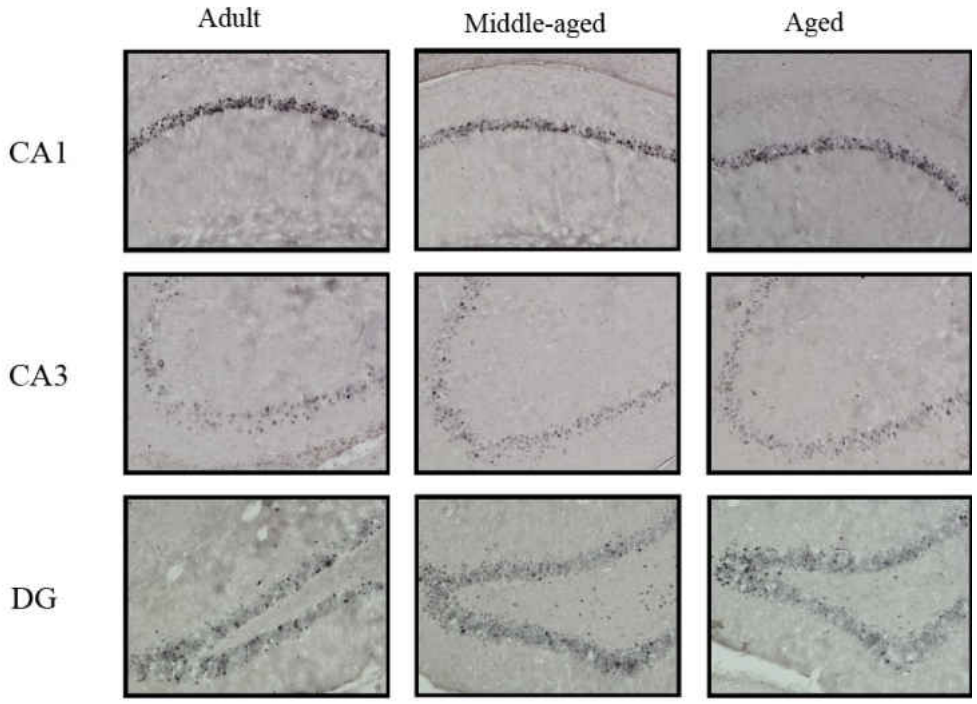


Figure 8. Cresyl violet stained sections illustrating the location of image analysis within hippocampus. For analysis of immunohistochemistry, Paxinos and Watson (1998) plates 31 – 34 were imaged for dorsal hippocampus and plates 39 – 43 for ventral hippocampus. Cresyl violet stained sections show representative dorsal hippocampus section imaged for CA1, CA3, and dentate gyrus (DG) subregions. On the left is a low power (2X), cresyl violet stained section of dorsal hippocampus (scale 1 mm). Insets on the right depict where CA1, CA3 and DG images were taken using a 10X objective (scale 200 µm).

Figure 9. Zif-268 immunoreactivity within hippocampus during normal aging. A. Representative immunohistochemistry images showing that aging reduces the number Zif-268 positive cells within CA1 and DG subregions of dorsal hippocampus within middle-aged and aged rats relative to adults. **B.** Representative immunohistochemistry images showing that aging reduces the number Zif-268 positive cells within DG subregion of ventral hippocampus within middle-aged and aged rats relative to adults. (Figure on the next page)

A. Dorsal hippocampus



B.Ventral hippocampus

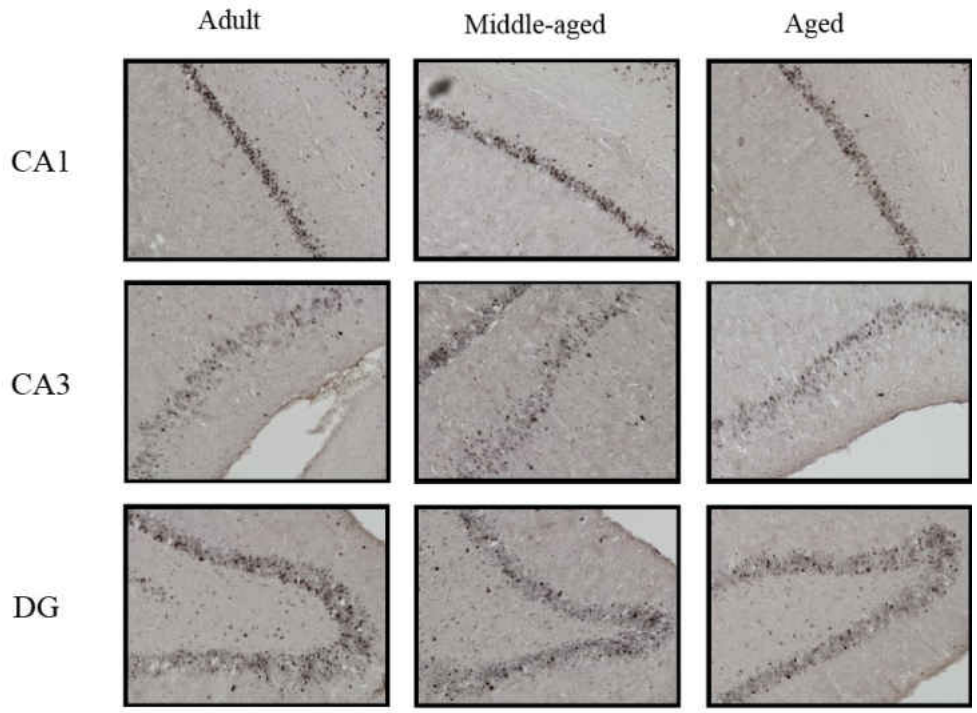


Figure 10. Number of Zif-268 immunoreactive cells decreases within CA1 and DG subregions of the dorsal hippocampus. A. Fewer cells in the CA1 subregion of the dorsal hippocampus express Zif-268 in middle-aged and aged rats (all values, $p < 0.01$). **B.** Zif-268 immunoreactivity within CA3 subregion of the dorsal hippocampus does not change during normal aging. **C.** Within the DG subregion of the dorsal hippocampus, there is a trend towards fewer Zif-268 positive cells with age (#, $p < 0.55$). Post hoc tests confirmed that the number of intensely labelled Zif-268 cells is significantly reduced in middle-aged and aged rats relative to adults. (Figure on the next page)

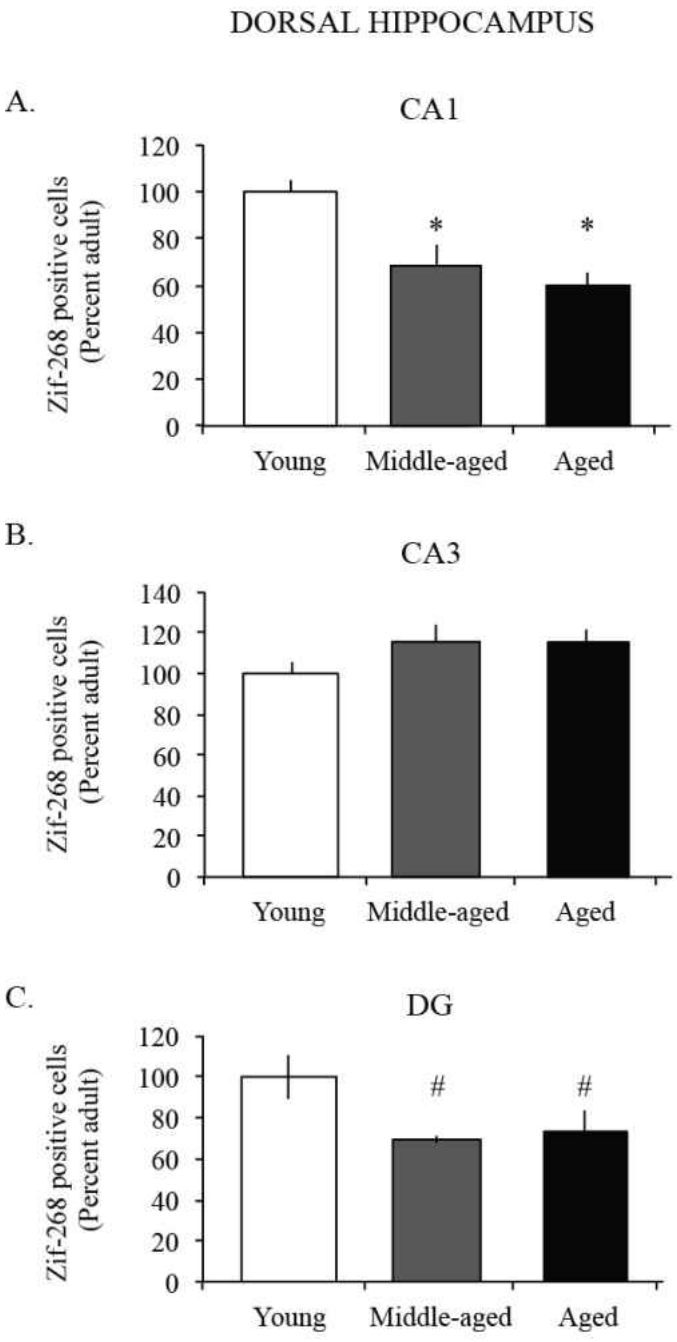
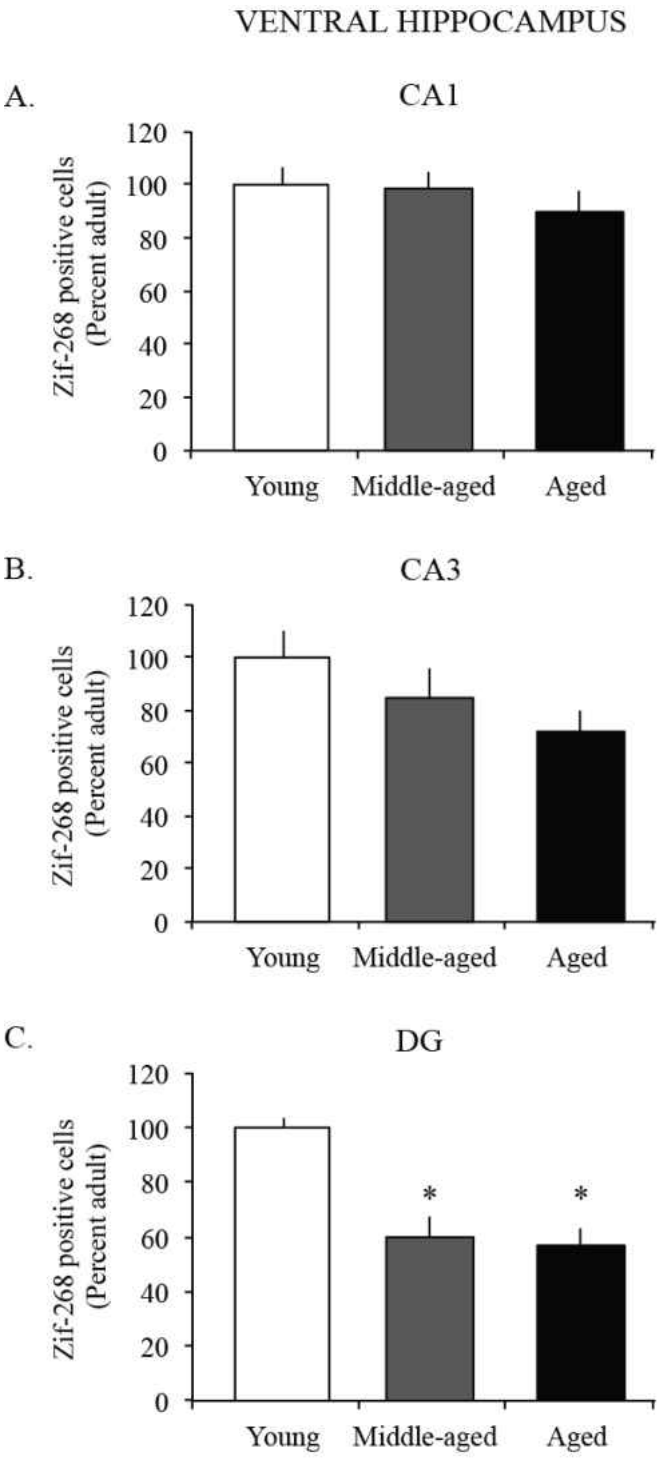

DORSAL HIPPOCAMPUS
A.
CA1
Zif-268 positive cells (Percent adult)
120
100
80
60
40
20
0
*
*
Young
Middle-aged
Aged
B.
CA3
Zif-268 positive cells (Percent adult)
140
120
100
80
60
40
20
0
Young
Middle-aged
Aged
C.
DG
Zif-268 positive cells (Percent adult)
120
100
80
60
40
20
0
#
#
Young
Middle-aged
Aged

Figure 11. Number of Zif-268 immunoreactive cells decreases within DG subregion of ventral hippocampus. A. In contrast to Zif-268 immunoreactivity within CA1 subregion of dorsal hippocampus (Figure 10A), the number of Zif-268 positive cells within CA1 subregion of the ventral hippocampus does not change with age. **B.** Zif-268 immunoreactivity within CA3 subregion of the ventral hippocampus does not change during normal aging. **C.** There is a drastic reduction in the number of intensely labelled Zif-268 immunoreactive cells within the DG sub-region of ventral hippocampus (all values, $p < 0.001$). (Figure on the next page)


VENTRAL HIPPOCAMPUS
A.
CA1
Zif-268 positive cells (Percent adult)
120
100
80
60
40
20
0
Young
Middle-aged
Aged
B.
CA3
Zif-268 positive cells (Percent adult)
120
100
80
60
40
20
0
Young
Middle-aged
Aged
C.
DG
Zif-268 positive cells (Percent adult)
120
100
80
60
40
20
0
*
*
Young
Middle-aged
Aged

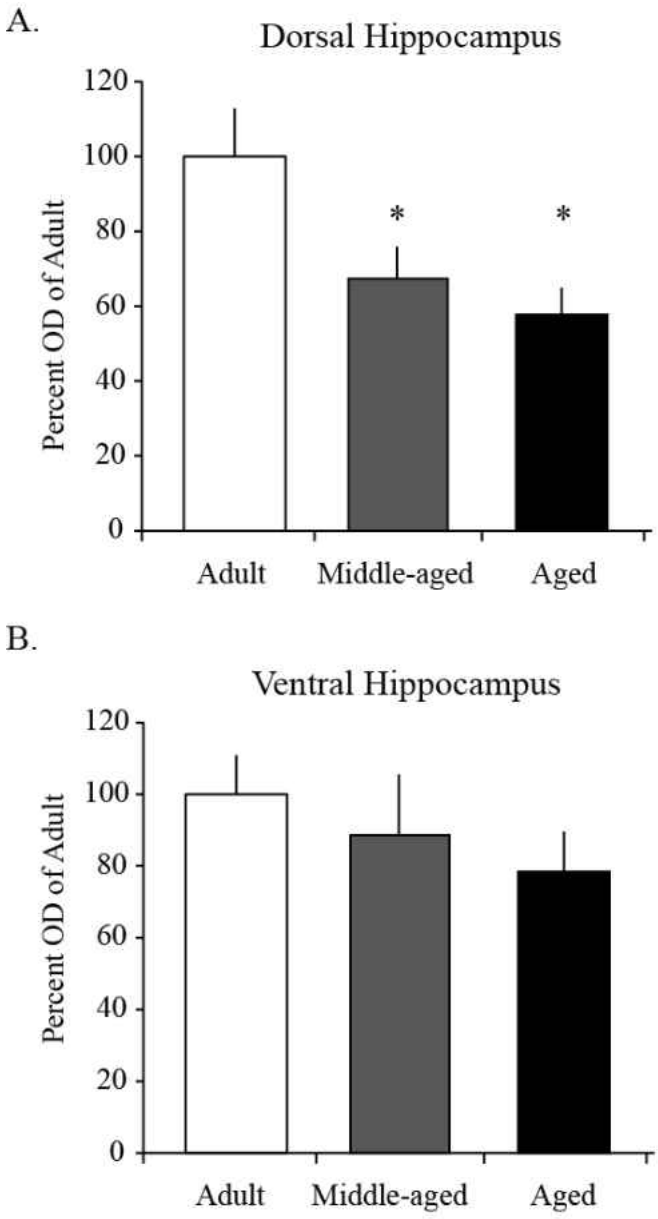


Figure 12. Region-specific changes in hippocampal Arc expression first emerge in middle age. A. Expression of Arc significantly decreases within the dorsal hippocampus of middle-aged and aged rats relative to that of adults ($p < 0.05$ and $p < 0.01$ for middle-aged and aged rats respectively). **B.** Aging does not alter Arc expression within the ventral hippocampus ($p = 0.51$).