NEURAL CORRELATES OF VISION LOSS

LINKING VISUAL DEFICITS WITH NEUROBIOLOGICAL CHANGES IN VISUAL CORTEX

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ABSTRACT

During postnatal development, visual experience initiates synaptic plasticity mechanisms that guide the refinement and maturation of visual cortex necessary to support the emergence of visual functions. Lack of normal visual experience during development can lead to vision loss, a condition called amblyopia. Additionally, even if our vision developed properly early in life, our vision naturally declines as we age. The mechanisms underlying vision loss associated with amblyopia and aging are not fully understood, and the studies in this thesis were designed to increase our understanding of the neural basis of vision loss through the linkage of synaptic protein expression to changes in vision.

In the first part of this thesis, I examined the impact of monocular deprivation on synaptic proteins in visual cortex, and on vision. Using Western blot analysis I showed that monocular deprivation causes a rapid, and sustained loss of AMPAR proteins in the region of cat visual cortex representing the center of vision. Because AMPARs play a key role mediating visual processing, I extended these findings by using behavioural measurements to show that the sustained loss of AMPARs in the central region is correlated with long-lasting binocular acuity deficits that are most severe in the center of vision. These findings showed that disrupting binocular vision early in development leads to experience-dependent changes that are greatest in the center of vision.

In the second part of this thesis, I examined age-related changes in the expression of a group of synaptic proteins associated with glutamatergic and GABAergic synapses. I found an age-related decline in the expression of Ube3A, a protein necessary for ocular dominance

plasticity, across sensory and non-sensory regions of cat, macaque, and human cortex. However, there was a selective loss of Ube3A relative to other synaptic proteins that occurred only in human cortex. Finally, I found a substantial age-related decline in expression of both glutamatergic and GABAergic synaptic proteins across cat visual cortex, suggesting fewer synapses in aging. Together, the findings from this thesis provide new insight into the neural basis of vision loss, and provide a foundation for the development of therapeutic interventions for cortical vision loss.

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LIST OF ABBREVIATIONS

AMPA (R)	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (receptor)
DE	deprived eye
EPSC	excitatory post synaptic current
GABA (R)	gamma amino butyric acid (receptor)
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IPSC	inhibitory post synaptic current
LGN	lateral geniculate nucleus
LTD	long term depression
LTP	long term potentiation
MD	monocular deprivation
NMDA (R)	N-methyl-D-aspartate (receptor)
OD	ocular dominance
PCA	principal component analysis
PSD-95	post-synaptic density 95
RGC	retinal ganglion cell
RO	reverse occlusion
SVD	singular value decomposition
UPS	ubiquitin proteasome system
V1	primary visual cortex
VGAT	vesicular GABA transporter
VGLUT1	vesicular glutamate transporter 1

DECLARATION OF ACADEMIC ACHIEVEMENT

Chapter 2 is a manuscript that has been submitted to Vision Research. This chapter was a collaboration between myself, Justin Balsor, Simon Beshara, Dr. Brett Beston, Dr. David Jones, and Dr. Kathryn Murphy. I was the lead on designing and performing the experiments, analyzing the data, and I wrote the manuscript with Dr. Kathryn Murphy.

Chapter 3 is a paper that was published in Frontiers in Aging Neuroscience. This chapter was a collaboration between myself, David Irwin, Dr. David Jones, and Dr. Kathryn Murphy. I was the lead on designing and performing the experiments, analyzing the data, and I wrote the paper with Dr. Kathryn Murphy.

Chapter 4 is being prepared for submission to Neurobiology of Aging. This chapter was a collaboration between myself, Dr. David Jones, and Dr. Kathryn Murphy. I was the lead on designing and performing the experiments, analyzing the data, and I wrote the manuscript.

Chapter 3 Citation

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Chapter 1. General Introduction

1.1 Development & Organization of Visual Cortex

Humans and other animals have the remarkable ability to translate light into a pattern of neural activity that results in our perception of the world around us. Vision alerts us to danger and guides our movements to ensure survival. Importantly, vision is a means of communication. As such, loss of vision has a great impact on our quality of life.

Normal visual experience during development is critical for the proper maturation of the central visual pathway. Visual experience supports maturation by initiating synaptic plasticity mechanisms which mediate the refinement of neural connections within visual cortex. However, if abnormal visual experience occurs during development, refinement of neural connections is not properly guided, and leads to poor vision. In humans, this condition is known as amblyopia. Similarly, as we age we experience a normal decline in visual perception that developed early in life, also leading to poor vision. Because amblyopia and aging share some features of vision loss, the mechanisms underlying the neural basis of both poor vision during amblyopia and aging may also share some features, but these processes are far from being fully understood. In this introduction I will discuss the organization and development of the visual cortex, and experience-dependent synaptic plasticity mechanisms in visual cortex. Then, I will discuss visual deficits associated with amblyopia and aging. This will build the foundation for the studies described in this thesis, which were designed to increase our understanding of the neural basis of vision loss in amblyopia and aging through the linkage of synaptic protein expression to changes in vision.

Visual Pathway

Vision begins with light falling on the retina, where photoreceptors (rods and cones) transduce incoming light energy into electrical activity. This activity feeds onto bipolar cells, which in turn feed onto retinal ganglion cells (RGC). In addition to these feedforward connections, there are also lateral connections made between the photoreceptors, bipolar, and RGCs. Specifically, horizontal cells form connections between photoreceptors and bipolar cells, and amacrine cells form connections between bipolar cells and RGCs. The convergence of visual information transduced by photoreceptors onto RGCs forms the receptive field of RGCs, which refers to the area on the retina (and of visual space) where a stimulus can induce a response in the neuron. Additionally, the receptive field of RGCs are arranged so there is an ON region (activated by light) and an OFF region (activated by no light). The receptive field of a RGC is circular and arranged with either an ON-center (OFF surround) or OFF-center (ON surround) organization. This organization allows RGCs to transit information about contrast. The degree of convergence between photoreceptors and RGCs determines the size of the receptive field of the RGC. The degree of convergence is important because the size of receptive fields of RGCs determines their spatial resolution, which ultimately limits visual acuity (Cleland et al., 1979). Importantly, convergence is not uniform across the retina. In the fovea, the region of the retina where the center of gaze is focused, typically there are ~ 3-4 RGCs for every cone, whereas just outside the fovea, there is ~ 1 RGC for every cone, and in the peripheral retina there are more cones than RGCs (humans: Sjostrand et al., 1999; primates: Wassle et al., 1990). Thus, the receptive fields of RGCs found in the peripheral retina are much larger, and this explains why acuity is so much worse in the periphery, where convergence is high. While the fovea is specific

to humans and other species (including non-human primates, birds and reptiles), cats have a similar area of specialization within their retina called the area centralis. The area centralis is characterized by the greatest number of cones (Steinberg et al., 1973), highest density of RGCs (Stone, 1978), and smallest receptive fields (Cleland et al., 1979), and thus is the area that ultimately determines visual acuity in the cat.

The spatial relationship of information received by the retina is preserved throughout the visual pathway to primary visual cortex. This topographic arrangement of the visual field onto the retina is called retinotopy. From the retina, axons of RGCs bundle together and exit the eye as the optic nerve eventually terminating in a layer-specific manner in the lateral geniculate nucleus (LGN) of the thalamus. Importantly, retino-geniculate afferents from each eye remain segregated and are organized topographically according to their receptive field location on the retina. Neurons in the LGN then project to the primary visual cortex (V1) where they synapse in layer IVc. Importantly, inputs from each eye are still segregated at this point. From layer IVc, neurons project to and form synapses in layer IVb, which is the first cortical location in the primary visual pathway where information from both eyes is combined. From layer IV, neurons project to other layers within V1, and eventually other visual areas in the cortical hierarchy of the visual system. The convergence of inputs beyond layer IV gives rise to increasingly more complex receptive fields, which underlies more complex visual processing. For example, receptive fields of complex cells are not defined by fixed ON and OFF regions. The result is that complex cells don't have a strong preference for an orientation, but are selective for the *direction* of motion of a stimulus. Parallel to these feedforward connections are feedback connections that project from later visual areas back to earlier areas. Together, feedforward and feedback

projections drive and modulate visual activity necessary for visual processing (Lamme et al., 1998).

Organization of Visual Cortex

The greatest insight into the organization and function of V1 came from the early work of Hubel & Wiesel who showed that receptive fields of neurons in cat V1 are selective for specific properties, and are organized into a "functional architecture" based on these properties (Hubel & Wiesel, 1959, 1962). In particular, neurons in V1 are selective for stimulus orientation and ocular dominance: orientation selectivity of V1 neurons refers to the preferential response of a neuron to bars of light of a particular orientation (0-180 degrees), which is dependent on the shape of the neuron's receptive field, and ocular dominance (OD) of a neuron refers to the preferential response to input from a specific eye. Hubel & Wiesel quantified OD using extracellular single unit recordings to compare the response of a neuron following presentation of a stimulus to either eye (Hubel & Wiesel, 1962), and found that neurons in V1 are organized based on their OD preference. Specifically, they found that neurons with similar OD are organized into columns oriented perpendicularly to the surface of V1. Additionally, by injecting radio-labeled proline into one eye, Hubel & Wiesel were able to anatomically demonstrate the columnar arrangement of OD within macaque V1 (Wiesel et al., 1974), and this arrangement was later shown in cat V1 (Levay et al., 1978). These OD columns are well defined in layer IVc, where neurons receive only monocular input from thalamocortical afferents, and as such there is an abrupt shift between neurons that exclusively respond to the left or right eye. Above and below layer IVc, where neurons are binocular, there is a gradual shift in ocular dominance as one moves across the surface of V1, ranging from cells that respond exclusively to the contralateral

eye, to both eyes equally, or exclusively to the ipsilateral eye. Overall, in cat V1 ~80% of neurons are binocular, and most are found in the central visual field representation (Hubel & Wiesel, 1962).

As mentioned, a hallmark organizing principle in V1 is retinotopy. The retinotopic map of primary visual cortex refers to the spatial organization of neurons: neurons with receptive fields that are adjacent in visual space are located next to each other in V1. The retinotopic organization of cat V1 has been extensively mapped using physiological and anatomical measures (Tusa et al., 1978). This mapping technique revealed that a large portion of V1 (~50%) is devoted to neurons representing the central 10 degrees of visual field. This "cortical magnification" reflects the specialization that was initially set up in the retina, as neurons in the central visual field representation are characterized by the smallest receptive fields (Hubel & Wiesel, 1962; Tusa et al., 1978) and process the highest spatial frequencies (Tootell et al., 1981). Just as in the retina, visual functions are limited by the receptive field properties of neurons in the central visual field representation of V1.

Experience-Dependent Development of Visual Cortex

Visual functions are not mature at birth. but rather arise following substantial postnatal development of the eye, retina, and visual cortex. For example, the size of the eye and lens of newborn kittens is ~50% smaller than adults, and vision remains largely occluded by a dense vascular network (the tunica vasculosa lentis) that only recedes after 3 weeks of age (Thorn et al., 1976). Furthermore, the size of receptive fields of RGCs (Rusoff & Dubin, 1977), and photoreceptor spacing continues to mature postnatally (Tucker et al., 1979). Finally, V1 undergoes substantial physiological and anatomical refinement postnatally that underlies the

emergence of visual abilities. This refinement, and ultimately the development and maturation of V1 relies on a combination of intrinsic mechanisms and postnatal visual experience. In cats, neurons in V1 are selective for orientation within 3 weeks of age (Hubel & Wiesel, 1963; Sherk & Stryker, 1976; Crair et al., 1998) and ocular dominance columns are formed within 2 weeks of age (Crair et al., 2001). Furthermore, if kittens are reared without visual experience (dark rearing or binocular lid suture), they still develop orientation selectivity (Blakemore & Van Sluyters, 1975) and ocular dominance columns (Crair et al., 1998), although orientation selectivity is slightly weaker (Sherk & Stryker, 1976) and ocular dominance is largely dominated by contralateral input. Beyond 3 weeks of age, however, lack of binocular visual experience leads to deterioration in orientation selectivity, and ocular dominance remains largely biased towards contralateral input (Crair et al., 1998). Visual experience beyond 3 weeks of age is necessary to sharpen and maintain orientation selectivity and strengthen ipsilateral input, so that V1 shifts to a binocular distribution (Crair et al., 1998). It is clear that even though experience-independent mechanisms support the initial development of receptive field properties, visual experience is critical for refining and maintaining these properties.

A dramatic example which makes obvious that normal visual experience is required for proper development of V1 is the effect of monocular deprivation (MD) on binocularity and function of V1. In cats, MD early in development reduces the number of binocular neurons in V1: neurons in V1 initially shift their responsiveness away from the deprived eye (DE), and then slowly increase responsiveness to the open eye (Wiesel & Hubel, 1965; Hubel & Wiesel, 1970). These physiological changes in responsiveness are also accompanied by rapid morphological changes at the level of dendritic spines (Oray et al., 2004; Mataga et al., 2004). Ultimately, these

rapid changes are hardwired by slower anatomical changes including rearrangement of thalamocortical afferents such that afferents serving the DE shrink, and those serving the open eye expand (Hubel et al., 1977; Shatz & Stryker, 1978; LeVay et al., 1980; Antonini & Stryker, 1993). However, if the deprivation is reversed by suturing closed the eye that was initially open and opening the eye that was initially closed, a procedure called reverse occlusion (RO), there can be complete reversal of the physiological (Blakemore & Van Sluyters, 1974; Movshon, 1976; Van Sluyters, 1978) and anatomical changes (Swindale et al., 1981; Crewther et al., 1983). Furthermore, simply re-opening the deprived eye can restore responsiveness to that eye (Blasdel & Pettigrew, 1978). The ability to shift back and forth following MD and RO reveals a high degree of plasticity in V1. This plasticity, however, is restricted to a critical period early in development. In cats, the critical period for OD plasticity begins around 4 weeks of age, peaks between 4-6 weeks of age, then gradually begins to decline (Olson & Freeman, 1980). During the critical period, as little as one day of MD can cause a shift in OD (Movshon & Dursteler, 1977; Olson & Freeman, 1980; Mioche & Singer, 1989) and prolonging MD beyond the critical period can cause a permanent shift in which almost all neurons respond exclusively to the open eye (Hubel & Wiesel, 1970). Importantly, the loss of responsiveness is not simply due to disuse, because kittens that receive binocular deprivation retain binocular neurons (Wiesel & Hubel, 1965; Blakemore & Van Sluyters, 1974). Additionally, if kittens are reared with strabismus, where the *amount* of activity through each is the same, but the image each eye receives is different, the number of binocular cells in V1 is reduced (Hubel & Wiesel, 1965; Van Sluyters & Levitt, 1980). Together, these findings suggest that the *quality* of visual experience, and not just the quantity, is critical for development of V1 to support normal vision. The consequence of MD

on the development of vision is severe. In kittens, MD causes severe acuity deficits in the DE (Dews & Wiesel, 1970; Giffin & Mitchell, 1978) and loss of binocular functions such as stereopsis (Timney, 1983). As such, the monocular deprivation paradigm introduced by Hubel & Wiesel (1963) has become a premier model for studying the human developmental disorder, amblyopia.

1.2 Synaptic Plasticity Mechanisms

The studies by Hubel & Wiesel laid the foundation for our understanding of the development and organization of V1, and were crucial for guiding subsequent research devoted to understanding the neurobiological mechanisms that underlie plasticity in developing V1. The critical period for OD plasticity has since been characterized across a number of species including macaques (Horton & Hocking, 1997), rats (Fagiolini et al., 1994), ferrets (Issa et al., 1999), and mice (Gordon & Stryker, 1996). OD plasticity has been used as a powerful paradigm to link molecular mechanisms of experience-dependent plasticity to anatomical, physiological, and behavioural changes. Due to the competitive nature of OD shifts, Hebbian forms of synaptic plasticity such as long term potentiation (LTP) and long term depression (LTD) were initially thought to be the main contributors to OD shifts. It has become increasingly apparent, however, that non-Hebbian, homeostatic plasticity mechanisms also contribute to OD plasticity.

OD shifts are composed of two phases. In the first phase, there is a rapid reduction of neural responsiveness to the deprived eye; in the second phase, there is a potentiation of neural responsiveness to the open eye, but also a slight potentiation to the deprived eye (Frenkel & Bear, 2004; Mrsic-Flogel et al., 2007). The initial loss of responsiveness is believed to be mediated by LTD (Heynen et al., 2003; Yoon et al., 2009), whereas the delayed potentiation is due to a combination of LTP and homeostatic plasticity, such as synaptic scaling (Smith et al., 2009; Mrsic-Flogel et al., 2007). These forms of plasticity often proceed cooperatively, but through distinct cellular and molecular mechanisms including activation of glutamate and GABA (gamma amino butyric acid) receptors. This section will discuss the role of glutamatergic and GABAergic synaptic function in experience-dependent plasticity in V1.

Role of AMPARs and NMDARs in experience dependent plasticity in V1

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) and NMDA (N-methyl-D-aspartate) receptors are the two major types of ligand-gated ionotropic glutamate receptors in V1 (McLennan, 1983; Huntley et al., 1994). Both types of receptors are present on the post-synaptic membrane of excitatory synapses, where a network of proteins, such as PSD-95, anchor and stabilize them (Kennedy, 1997; El-Husseini et al., 2000). Additionally, both NMDARs (Rodriguez-Moreno et al., 2010; Larsen et al., 2014) and AMPARs are also found on pre-synaptic membranes (Engelman & MacDermott, 2004; Rusakov et al., 2005; Takago et al., 2005). Both AMPAR and NMDARs have specific properties that mediate different components of excitatory post synaptic currents (EPSC), and these properties depend on the subunit composition of the receptor.

AMPARs are tetrameric structures that mediate the majority of fast excitatory transmission (<10ms) in the cortex (Kleppe & Robinson, 1999)**.** There are four different subunits (GluA1-4) that can be included within an AMPAR, and different combinations of these subunits give the AMPAR distinct properties. For example, AMPARs containing the GluA2 subunit are impermeable to Ca++ (Jonas et al., 1994; Cull-Candy et al., 2006). Changing either the number or subunit composition of AMPARs in the postsynaptic membrane can alter synaptic strength, and thus the trafficking of AMPARs in and out of synapses is a major mechanism for the regulation and expression of synaptic plasticity (for review see Anggono & Huganir, 2012). This has been demonstrated in V1, where AMPAR trafficking has been linked with experience-dependent plasticity mechanisms underlying OD shifts. In rodents, MD induces a rapid internalization of GluA2-containing AMPARs, showing an LTD mechanism underlying the

initial loss of responsiveness to the deprived eye (Yoon et al., 2009; Lambo & Turrigiano, 2013). The role of AMPAR trafficking extends beyond the initial Hebbian type plasticity that is believed to underlie the initial loss of responsiveness following MD. Because MD induces a delayed potentiation not only to the non-deprived eye, but also to the *deprived* eye, homeostatic plasticity mechanisms are thought to play a role in OD plasticity (Mrsic-Flogel et al., 2007). Homeostatic plasticity mechanisms can work to keep activity of a single cell, neural circuit, or the entire brain within an optimal range, in the face of prolonged changes in activity (Turrigiano & Nelson, 2004). For example, prolonged inactivity induced by tetrodotoxin causes insertion of GluA2-containing AMPARs that mediates a homeostatic increase in neuronal activity (Gainey et al., 2009). Furthermore, blocking insertion of GluA2-containing AMPARs prevents the subsequent restoration of activity to V1 after MD, demonstrating a clear role of AMPARs in experience-dependent plasticity in V1 (Lambo & Turrigiano, 2013). The rate and direction of AMPAR insertion and internalization are dependent on interactions between AMPARs and intracellular proteins called AMPAR-interacting proteins. For example, the AMPAR-interacting proteins GRIP and PICK-1 modulate AMPAR trafficking by regulating the surface accumulation and internalization of AMPARs. PICK-1 stabilizes intracellular AMPAR stores (Gardner et al., 2005), removes surface AMPARs (Terashima et al., 2004; Perez et al., 2001), and is required for some forms of synaptic scaling (Anggono et al., 2011), LTP, and LTD (Terashima et al., 2008).

Like AMPARs, NMDARs are tetrameric structures. However, rather than mediating the fast component of an EPSC, NMDARs mediate a very slow component (several hundred milliseconds) (Lester et al., 1990). There are 3 families of NMDAR subunits, with 7 total subunits identified (GluN1, GluN2A-D, GluN3A-B) which combine in specific combinations to

form functional NMDARs (Monyer et al., 1992). Two GluN1 subunits are obligatory for receptor function, and the other 2 subunits can be a combination of GluN2 or GluN3 subunits. Within the GluN2 and GluN3 subunit families, different subtypes confer distinct functional properties to the receptor (for review see McBain & Mayer, 1994). For example, GluN2A containing receptors typically have a faster decay time, and lower binding affinity to glutamate, compared to GluN2B-containing receptors (Flint et al., 1997). Furthermore, GluN2A and GluN2B are developmentally regulated across the central nervous system: the GluN2B subunit is typically more predominant early in postnatal development, whereas the GluN2A subunit is typically more predominant in the mature central nervous system (Monyer et al., 1994; Sheng et al., 1994; Wang et al., 1995; Portera-Cailliau et al., 1996; Shi et al., 1997). Several other properties of NMDARs make them quite different from AMPARs. First, in order for NMDARs to be activated, both glutamate and glycine must bind to the receptor (Kleckner & Dingledine, 1988). Second, the channel of NMDARs is blocked by a magnesium ion at resting membrane potential, and this ion is only removed when the membrane is depolarized (Nowak et al., 1984). Third, all NMDARs pass calcium ions, which most AMPARs do not (Yuste & Katz, 1991). Because NMDARs require both glutamate binding and membrane depolarization to be activated they act as a coincidence detector, meaning they activate only during simultaneous pre- and post-synaptic activity. This property, and their ability to pass calcium, has made NMDARs important for various types of plasticity, including LTP and LTD (for review see Hunt & Castillo, 2012). In V1, NMDAR- mediated LTD and LTP are thought to underlie the initial reduction in response to the deprived eye, and the potentiation to the open eye, respectively (Smith et al., 2009). In support of this idea, blocking NMDARs reduces OD plasticity in kittens (Kleinschmidt et al.,

1987, Gu et al., 1989; Bear et al., 1990), and prevents the internalization of AMPARs that underlies the depression of deprived eye responses (Yoon et al., 2009). Furthermore, the developmental regulation of GluN2A/2B is important for critical period plasticity. Reducing the GluN2A/2B ratio permits potentiation of the non-deprived eye inputs, presumably by lowering the threshold for LTP (Cho et al., 2009).

Role of Ube3A in experience-dependent plasticity in V1

Tied to the regulation of glutamate receptors, and AMPARs in particular, is Ube3A, which is an E3 ubiquitin ligase that is part of the ubiquitin-proteasome system (UPS). Ube3A catalyzes the attachment of ubiquitin molecules to specific proteins marking them for degradation by the UPS. In humans, maternal deletion or mutation of the gene encoding Ube3A results in the developmental disorder Angelman's Syndrome, which is characterized by a halt in cognitive development after ~1 year of age, resulting in severe mental retardation (Kishino et al., 1997). Because Angelman's syndrome children develop fairly normally, and then suddenly stop, the possibility exists that Ube3A plays a role in experience-dependent plasticity mechanisms across the cortex. Indeed, mouse models of Angelman's Syndrome (Ube3A KOs) have shown that Ube3A is required for experience-dependent OD plasticity in V1 (Yashiro et al., 2009; Sato & Stryker, 2010). OD plasticity is severely impaired in developing V1 of Ube3A KO mice and MD in juvenile Ube3A KO mice does not induce an OD shift (Yashiro et al., 2009, Sato & Stryker, 2010). Additionally, Ube3A KO mice have severe LTP and LTD deficits (Jiang et al., 1998; Mardirossian et al., 2009; Yashiro et al., 2009), reduced spine density (Dindot et al., 2008; Sato & Stryker, 2010) and reduced mEPSC frequency (Yashiro et al., 2009). However, if Ube3A KO mice are dark-reared they retain ocular dominance plasticity until exposed to light (Yashiro et al.,

2009*)*. Research into the substrates of Ube3A indicates that the plasticity impairments in Ube3A KOs may be rooted in deficits of AMPAR-mediated plasticity. An identified substrate of Ube3A, Arc, (Greer et al., 2010) regulates AMPAR trafficking by increasing AMPAR internalization, which decreases AMPAR surface expression (Chowdhury et al., 2006; Shepherd et al., 2006). Normally, Ube3A tags Arc with ubiquitin, and regulates its degradation. However, when Ube3A expression is reduced Arc levels accumulate, and AMPARs become over-internalized (Greer et al., 2010), leading to a selective decrease in AMPAR surface expression, and reduction of the AMPA/NMDA ratio.

Role of $GABA_A$Rs in experience-dependent plasticity in V1

$GABA_A$ receptors are pentameric ionotropic receptors that mediate the majority of fast inhibitory synaptic transmission in the brain (Semyanov et al., 2004). Nineteen $GABA_A$ receptor subunits have been identified (α1-6, β1-3, γ1-3, δ, ρ1-3, ε, π, and θ), and like the glutamate receptors discussed above, different combinations of these subunits form $GABA_A$ receptors with different properties (Luscher & Keller, 2004). For example, early in development $GABA_A$ receptors are composed mainly of α3 subunits, which have a lower binding affinity for GABA (Bohme et al., 2004), and slower inhibitory post synaptic current (IPSC) decay time (Bosman et al., 2002). As maturation continues, however, $GABA_A$ receptors switch to being composed mainly of α1 subunits (Hendrickson et al., 1994; Fritschy et al., 1994; Chen et al., 2001; Murphy et al., 2005) which have higher binding affinity, and faster IPSC decay time (Bosman et al., 2002).

Much recent work supports the hypothesis that the balance between excitation and inhibition (the E:I balance) determines critical period timing in V1, and small fluctuations in the

balance can dramatically alter cortical plasticity (Hensch et al., 1998; Fagiolini & Hensch, 2000; Huang et al., 1999; Hanover et al., 1999; He et al., 2006; Iwai et al., 2003). Early in development, the balance favours excitation, because inhibitory circuitry has not matured (Luhmann & Prince, 1991; Benevento et al., 1992; Guo et al., 1997; Murphy et al., 2005). An emerging view is that there is a range in the strength of inhibition in which plasticity can occur, and that going above or below this range may limit the chance for plasticity (Jiang et al., 2005). The initial maturation of GABAergic inhibition crosses the lower limit of the range, opening the window for plasticity, and as inhibitory circuitry continues to mature GABAergic inhibition crosses the upper limit of the range, attenuating plasticity. In this model, the maturation of GABAergic inhibition refers to the proliferation of GABAergic synaptic contacts onto pyramidal cells in V1 (Blue & Parnavelas, 1983; Huang et al., 1999; Chattopadhyaya et al., 2004)

The onset of inhibitory circuit maturation coincides with the beginning of the critical period for ocular dominance plasticity. In support of the hypothesis that an inhibitory threshold must be reached in order to trigger the critical period, mice lacking the GABA synthesizing enzyme, glutamic acid decarboxylase isoform 65 (GAD65), do not display the typical shift in OD that follows MD during the normal critical period, suggesting that the critical period cannot be started in these mice (Hensch et al., 1998). However, OD plasticity can be rescued at any age in the GAD65 knockout mice by infusion of benzodiazepines, which increase GABAergic transmission. Additionally, studies have demonstrated that injecting benzodiazepines into a normal animal induces a precocious critical period that is dependent on activation of $GABA_A$ receptors containing the α1 subunit (Fagiolini et al., 2004). In mice treated with benzodiazepines the critical period still only occurs once, which suggests that maturation of inhibition starts an

irreversible cascade of events that ultimately closes the critical period (Fagiolini & Hensch, 2000). Several other factors can contribute to the maturation of inhibition, and therefore the timing of the critical period. For example, over-expression of the neurotrophin BDNF accelerates maturation of inhibitory connections, initiating an early critical period (Huang et al., 1999; Hanover et al., 1999). Conversely, dark rearing an animal reduces BDNF expression, which prevents the increase in GABAergic input onto pyramidal cells (Morales et al., 2002; Kreczko et al., 2009), and delays the onset of critical period plasticity (Fagiolini et al., 1994).

Glutamatergic and GABAergic Mechanisms for Visual Processing

It is clear that together, AMPA, NMDA, and $GABA_A$ receptors play a key role mediating synaptic plasticity in developing V1. Furthermore, these receptors play a crucial role in the physiology underlying vision. Both excitatory and inhibitory synaptic mechanisms play a key role shaping receptive field properties of neurons in V1. Hubel & Wiesel were the first to propose a feedforward model for receptive field formation for simple cells in V1. In a feedforward model of receptive field formation, the excitatory drive from thalamocortical afferents shapes the receptive fields of V1 neurons (Hubel & Wiesel, 1962). In support of this model, even if all inhibitory inputs to a neuron are blocked, the excitatory inputs are sufficient to maintain orientation selectivity of neurons in V1 (Nelson et al., 1994). Additionally, NMDARs have been shown to be necessary for development of orientation selectivity in V1 (Ramoa et al., 2001; Fagiolini et al., 2003). Finally, AMPARs mediate the majority of feedforward visual processing, and blocking AMPAR activity in V1 reduces the main stimulus driven neuronal response (Self et al., 2012). In contrast, NMDARs mediate the majority of feedback visual

processing, and blocking NMDAR activity prevents modulatory activity within V1 that is necessary for complex visual functioning (Self et al., 2012).

Although a feedforward model can account for the orientation tuning of neurons in V1, there are certain properties, like contrast invariance, that can't be explained by summing the input of excitatory drive onto V1 neurons. An alternative model suggests these properties emerge due to an interaction between excitatory feedforward drive and intracortical inhibitory inputs (Blakemore & Tobin, 1972). Support for this theory was initially driven by several studies that showed that blocking cortical GABA-mediated inhibition with the $GABA_A$ antagonist, bicuculline, reduces orientation selectivity of neurons in cat V1 (Sillito, 1975; Tsumoto et al., 1979; Eysel et al.,1998). However, subsequent studies showed that *direct* inhibition is not required for orientation selectivity because suppressing cortical input to simple cells in V1, by either cooling (Ferster et al., 2006) or electrical stimulation (Chung & Ferster, 2006), does not have a great impact on orientation selectivity. Thus, input from the LGN is sufficient to maintain orientation selectivity.

These studies support the well-accepted model that orientation selectivity of simple cells in V1 does *not* require inhibition, but can be solely determined by excitatory input from the LGN, and that properties such as contrast invariance can be accounted for by the non-linearity of V1 neurons (Priebe & Ferster, 2008; 2012). Though inhibition is not required for orientation selectivity to exist, it seems likely that inhibition plays an important role *modulating* orientation selectivity, probably by regulating inputs beyond the receptive field formed by feedforward drive from the LGN. The role of inhibition, therefore, likely plays an important role in visual perception, which reflects the response of a population of neurons to a stimuli, rather than the

response of a single neuron to a stimuli presented to its receptive field. In support of this, activation of inhibitory parvalbumin positive (PV+) interneurons can sharpen orientation and direction selectivity of neurons in mouse V1, which leads to better visual perception as seen by improvement on an orientation discrimination task (Lee et al., 2012).

Though further research will be needed to tease apart the specific contributions of AMPA, NMDA, and GABA receptors to receptive field formation in V1, it is clear that that together, excitatory and inhibitory mechanisms cooperate to shape the receptive field properties in V1 that ultimately underlie visual perception.

1.3 Amblyopia

Amblyopia is a condition caused by abnormal binocular vision early in development. The most common causes of amblyopia are anisometropia (unequal refractive power in the two eyes), deprivation amblyopia (ex. cataract), or strabismus (misaligned eyes). The best chance at recovery of vision is correction of refractive error, or removal of the optical obstruction early enough in development so that vision can develop normally. However, many children still experience poor vision following optical correction. Amblyopia is characterized by a wide range of vision loss in the amblyopic eye including poor spatial vision and contrast sensitivity (Bradley & Freeman, 1981; Levi & Klein, 1985; Mckee et al., 2003; Levi et al., 2006; Levi et al., 2011). Furthermore, a number of binocular functions are impaired, such as stereopsis and eye movements (for review see Birch, 2013). The standard treatment for amblyopia is patching therapy, a treatment approach based on the competitive nature of plasticity in V1, and the assumption that patching the "good" eye will force the amblyopic eye to compete and become stronger. Unfortunately, this treatment does not guarantee recovery of normal vision. Despite correcting the optical deficits, and beginning patching treatment early in postnatal life, 15-50% of amblyopic children continue to suffer from residual, or recurrent visual deficits (Birch, 2013). The failure of patching treatment highlights an incomplete understanding of amblyopia.

Neural Basis of Amblyopia

Non-human animal models of amblyopia have been key for understanding the neural basis of amblyopia. Macaque monkeys reared with abnormal visual experience (anisometropia or strabismus) end up with similar visual deficits as human amblyopes, including poor spatial vision (Harwerth et al., 1981; Kiorpes et al., 1987; Kiorpes, 1992) and reduced contrast sensitivity

(Kiorpes et al.,1987, 1998) in the amblyopic eye. Similarly, cats that are monocularly deprived during the critical period for OD plasticity have reduced acuity (Dews & Wiesel, 1970; Giffin & Mitchell, 1978; Murphy & Mitchell, 1986, 1987) and contrast sensitivity in their DE (Kratz & Lehmkuhle, 1983). Furthermore, the severity of visual deficits is related to the length and onset of the period of deprivation, with longer deprivations resulting in worse acuity (Dews & Wiesel, 1970). Importantly, although reverse occlusion (RO) can reverse many of the anatomical and physiological changes caused by MD, acuity cannot be restored so simply. In fact, RO only promotes a transient improvement of acuity in the previously deprived eye, an improvement that is quickly lost upon restoration of binocular vision (Mitchell et al., 1984). Additionally, RO can actually impair vision in the non-deprived eye, resulting in a bilateral amblyopia (Murphy & Mitchell, 1986; 1987; Harwerth et al., 1989). These studies have given great insight into the risks and limitations of patching therapy in humans.

Physiological studies have shown that in addition to a loss of binocular neurons, abnormal visual experience alters the spatial properties of neurons in V1, including reduced optimal spatial frequency and spatial resolution (Eggers & Blakemore, 1978; Movshon et al., 1987; Kiorpes et al., 1998). Interestingly, these effects are largely restricted to the region of V1 that represents the central visual field (Kiorpes et al., 1998). Furthermore, monocularly depriving kittens causes dramatic changes in the expression of synaptic proteins, including a loss of glutamate receptor subunits in the central region of V1 (Murphy et al., 2004; Beston et al., 2010). Importantly, loss of the NMDA receptor subunit GluN1 occurs in OD columns for *both* eyes within the central region. These regional effects raise the possibility that monocular deprivation may affect the non-deprived eye. Amblyopia, however, is typically thought of as a monocular disorder, and

vision in the non-deprived eye (or fellow eye in humans) is assumed to be normal. While many clinical studies have reported visual deficits in the fellow eye of amblyopes, this is confounded by the fact that the patients had all received patching therapy at some point, making it difficult to determine whether the visual deficits are purely the result of early deprivation (Lewis et al., 1992; Varadharajan & Hussaindeen, 2012).

Amblyopia: A binocular disorder

While patching remains the primary treatment in the clinic, much amblyopia research over the past decade has explored alternative therapies, including perceptual learning. Perceptual learning is a procedure of prolonged training on a visual task designed to improve vision in the amblyopic eye (Li et al., 2005; Polat et al., 2004; Zhou et al., 2006). Typically perceptual learning in amblyopia has been done monocularly, but more recent approaches have achieved good visual recovery using *binocular* perceptual learning paradigms (Hess et al., 2010; Ooi et al., 2013). This fits with an emerging view that although amblyopia initially develops due to a monocular insult, the fundamental cause is due to abnormal binocular visual experience (Birch, 2013). By that rationale, if correlated binocular vision is required for development of vision, then restoring correlated binocular vision is likely a requirement for good, permanent recovery. Unfortunately patching therapy continues to *decorrelate* binocular input. Therefore, therapies that use correlated binocular vision should be optimal for recovery (Mitchell & Duffy, 2014). In support of this hypothesis, animal models have shown that recovery of vision is faster if both eyes are opened following monocular deprivation (compared to reverse occlusion; Mitchell et al., 2001). Furthermore, correlated binocular vision following MD leads to faster and better recovery of physiological responses in V1 and visual acuity (Kind et al., 2002). Additionally,

interrupting MD with short periods of binocular vision can prevent development of amblyopia in cats (Mitchell et al., 2003, 2006). This growing body of evidence for binocular vision being essential for recovery has fueled new treatment paradigms that use a binocular approach to promote recovery from amblyopia. For example, paradigms that reduce suppression of the deprived eye by *balancing* input to both eyes during perceptual learning tasks have shown to elicit good recovery (Hess et al., 2010; Knox et al., 2012; Ooi et al., 2013). However, most of these novel paradigms have not yet reached the clinic, where patching paradigms still prevail. It is clear that a complete understanding of the visual deficits associated with amblyopia and the neural basis of vision loss will be crucial to guide the development of new treatments that promote good, permanent recovery of vision.

1.4 Aging

Age-related changes in visual function

In some respects, the effects of aging on vision are similar to those associated with amblyopia. In humans, these age-related deficits may include reduced visual acuity (Wood & Bullimore, 1995; Li et al., 2000), contrast sensitivity (Owsley et al., 1983; Wood & Bullimore, 1995; Del Viva & Agostini, 2007) motion perception (Trick & Silverman, 1991; Bennett et al., 2007), and poor performance on complex visual integration tasks (Kennedy et al., 2009) (for a review of the general effects on aging on vision see Faubert, 2002; Owsley, 2011; Andersen, 2012). However, not all visual functions decline during aging (Betts et al., 2005; Billino et al., 2008), and the range of deficits can vary across individuals (Johnson & Choy, 1987). Interestingly, most deficits that are experienced cannot be accounted for by ocular, retinal, or subcortical changes, and are therefore likely due to cortical deterioration (Spear, 1993). However, unlike the extensive understanding of the changes that occur during development of V1, very little is known about the changes that occur during normal aging of V1. Initially, a generalized loss of neurons throughout V1 was thought to underlie age-related visual deficits. However, detailed anatomical studies across a range of species have since shown there is not a significant loss of neurons in V1 throughout the lifespan (Leuba & Garey, 1987; Vincent et al., 1989; Peters et al., 1997; Hua et al., 2008). Furthermore, the selective loss of visual deficits supports an emerging view that age-related visual deficits are due to much more selective and subtle changes in the visual system (Billino et al., 2008). For example, a selective reduction of contrast sensitivity to medium and high spatial frequency stimuli occurs in aging, but not to low frequency stimuli (Crassini et al., 1988). Additionally, complex visual functions, such as

biological motion detection, appear to be more susceptible to deficits during aging (Habak & Faubert, 2000; Billino et al., 2008), which may reflect region- or cell-specific vulnerability to the effects of aging.

Macaques and cats have been important animal models for studying the neural basis of age-related vision loss. The average lifespan of a cat is 15 years of age, while that of a macaque monkey is 25 years of age. A series of studies from Audie Leventhal's group compared the electrophysiological properties of V1 neurons in young cats (2-4 years old) and macaques (4-9 years old) with old cats (11-14 years old) and macaques (23-31 years old). Using extracellular single-unit recordings in V1, it was shown that there is a degradation of physiological properties of neurons in old cats and macaques including reduced orientation and direction selectivity (Schmolesky et al., 2000; Hua et al., 2006), spatial resolution (Zhang et al., 2008; Hua et al., 2011), contrast sensitivity (Zhou et al., 2011) and signal timing (Wang et al., 2005). The predominant view is that a loss of GABAergic inhibition underlies these physiological deficits in V1. In support of this, application of a GABA agonist can restore orientation and direction selectivity of V1 neurons in old cats to levels observed in young cats (Leventhal et al., 2003). Furthermore, Hua et al (2008) have shown that there is a loss of GABAergic neurons in V1 of old cats, and studies of post mortem human V1 have shown losses in GABAergic synaptic proteins in aging (Pinto et al., 2010). It seems unlikely, however, that GABAergic inhibition is the only mechanism affected during aging. More recent studies of human V1 have shown a selective loss of dendritic spines on spiny stellate cells in layer IV of V1 during normal, healthy aging (Mavroudis et al., 2012). Because spiny stellate cells receive a large proportion of excitatory feedforward activity from the LGN, this raises the possibility that aging also affects

glutamatergic transmission. Age-related losses in glutamatergic synaptic components have been shown across other areas of cortex , such as loss of NMDARs (Magnusson et al., 2005; Bai et al., 2004; Liu et al., 2008; Zhao et al., 2009) and AMPARs in hippocampus and prefrontal cortex (Liu et al., 2008; Yu et al., 2011). However it remains unknown if there are similar losses in V1. Understanding how *both* glutamatergic and GABAergic synaptic components change during the lifespan will be crucial for linking age-related deficits in visual function with neurobiological changes in V1.

Age-related reduction in experience-dependent plasticity

In addition to the changes that disrupt visual function, it seems likely that age-related changes in V1 may affect mechanisms that underlie plasticity of V1. Though it is well accepted that adult V1 does retain some plasticity, it is limited compared to the plasticity observed during postnatal development. For example, MD can induce an OD shift in adult mice; however when compared to juvenile mice, there are several distinct differences. First, OD shifts in adults only occur following nearly double the length of MD, second, the shift is smaller in magnitude, and third, it is primarily driven by potentiation to the non-deprived eye inputs, rather than depression of the deprived-eye inputs (Sato & Stryker, 2008).

Much of our understanding of the factors which decrease the potential for plasticity come from adult animal models in which ocular dominance plasticity has been reinstated (for review see Bavelier et al., 2010). These factors which act as "brakes" on plasticity generally fall into one of two categories: functional or structural. A major functional brake on plasticity is alterations in the E:I balance. This claim is supported by the observation that reducing inhibition in adult V1 by blocking $GABA_A$ receptors with picrotoxin or by reducing GABA synthesis

restores ocular dominance plasticity (Harauzov et al., 2010). Moreover, plasticity- inducing paradigms, such as environmental enrichment (Sale et al., 2007), dark exposure (He et al., 2007), caloric restriction (Spolidoro et al., 2011), and fluoxetine administration (Maya Vetencourt et al., 2008) cause a reduction in GABA and GAD levels. Additionally, infusion of benzodiazepines prevents plasticity under these paradigms. Although this result may seem paradoxical to the *increase* in inhibition that initiates plasticity in development, it is clear that an inhibitory threshold must be reached to trigger critical period plasticity in development, and that maturation of inhibition ultimately restricts plasticity, closing the critical period. In support of this theory, when inhibitory neuron precursors are transplanted into the adult mouse brain, ocular dominance plasticity can be induced (Southwell et al., 2010). In contrast to functional brakes, structural brakes are those that impose a physical barrier to plasticity. For example, the expression of myelin-related proteins and chondroitin sulphate proteoglycans in the extracellular matrix can restrict axon outgrowth, which ultimately restricts synaptic rearrangement. By removing these physical barriers, ocular dominance plasticity can be re-instated in adult V1 (Pizzorusso et al., 2002; McGee et al., 2005; Pizzorusso et al., 2006; Carulli et al., 2010). Importantly, many of these paradigms promote successful recovery of *vision* in amblyopic animals. Further characterization of the age-related changes in V1 will be critical for guiding interventions that can re-instate plasticity, and will be important for designing treatment paradigms to improve visual deficits associated with amblyopia and aging.

The research presented in chapters 2-4 was performed to gain insight into the neural basis of vision loss during amblyopia and aging. More specifically this thesis asked 1) How does monocular deprivation impact regional expression of synaptic proteins in V1? and can the pattern

of regional changes be linked with changes in vision? and 2) how does the expression of synaptic proteins in V1 that play a key role mediating plasticity and the physiology underlying vision change across the lifespan? These questions were examined in three separate projects and are described in the following chapters.

Preamble for Chapter 2

Previous work from our lab had shown that the impact of long term monocular deprivation (MD) on the expression of AMPA and NMDA receptors was greatest in the region of cat V1 representing the center of vision (Beston et al., 2010). Furthermore, anatomical studies revealed a loss of NMDAR expression in the columns representing both eyes within the center of vision (Murphy et al., 2004). In contrast, the region of V1 representing the peripheral visual field was relatively spared (Murphy et al., 2004; Beston et al., 2010). Because glutamate receptors play a key role mediating feedforward and feedback activity in V1 (Self et al., 2012), these studies suggested that 1) the impact of MD on vision is greatest in the center of vision and 2) MD affects vision in both eyes.

In general, studies that use MD in cats to induce amblyopia typically describe acuity in the non-deprived eye, or binocular acuity, as normal (Dews & Wiesel, 1970; Giffin & Mitchell, 1978). However, recent studies in rodents have shown that the initial impact of MD is on cortical activity driven by both eyes (Kuhlman et al., 2013), but this effect is transient (Kuhlman et al., 2013; Hengen et al., 2013). Furthermore, brief MD in rodents causes rapid internalization of AMPARs, but with longer MD, receptors are inserted back into synapses (Heynen et al., 2003; Lambo & Turrigiano, 2013).

Taken together, these findings formed the foundation for the studies in chapter 2 which aimed to correlate regional changes in AMPAR expression with changes in vision. Specifically, we asked: 1) How quickly does the loss of AMPARs occur after MD? 2) Does MD have an impact on binocular vision?

By rearing cats with varying lengths of MD, we found that MD induces a rapid, but transient loss in the expression of AMPAR proteins in the regions of visual cortex representing the peripheral and monocular visual field, but a sustained loss in the region representing the center of vision. To relate the regional findings in AMPAR proteins with vision, we measured acuity in the center of vision and found that MD caused long-lasting binocular acuity deficits that were most severe in the center of vision. Thus, there was a clear correlation between the sustained loss of AMPAR proteins in the central region of V1 and the long-lasting acuity deficits in the center of vision.

Importantly, the rapid, but transient changes in the the regions of visual cortex representing the peripheral and monocular visual field are similar to the time course of experience-dependent changes that occur in rodent V1 (Kuhlman et al., 2013; Hengen et al., 2013; Lambo & Turrigiano, 2013). Furthermore, the impact of MD on vision mirrors similar features of human amblyopes, including severe vision loss in the fovea, and binocular vision loss. Thus, our animal model for amblyopia bridges findings in rodents, and humans.

Preamble for Chapter 3

Just as vision loss can occur during development, it can also occur during aging. Similar to the visual deficits associated with amblyopia, age-related loss of vision is cortical in nature, because often vision loss occurs despite having normal optics and a healthy retina. However, despite the number of studies that have characterized age-related changes in vision, there is very little known about the neurobiological changes in V1 that occur during aging.

A key difference between young and old V1 is the capacity for synaptic plasticity. The mechanisms that mediate ocular dominance plasticity during development are not readily available in adults. Ube3A is an enzyme that tags other proteins with ubiquitin, thus marking them for degradation. Importantly, Ube3A is critical for ocular dominance plasticity during development (Yashiro et al., 2009; Sato & Stryker, 2010) and knocking out Ube3A during development leads to rigidity of ocular dominance plasticity that is very similar to the reduced plasticity seen in older animals. This led us to ask whether aging V1 is characterized by a loss of Ube3A expression.

We quantified Ube3A expression across a range of species (cats, macaques, humans) and cortical areas (V1, V3, V4, frontal, auditory) and found that for all species, and across all cortical areas, there was a loss of Ube3A throughout the lifespan. However, when the loss of Ube3A was compared to a group of other synaptic proteins, we found a selective loss of Ube3A that occurred only in human cortex. This selective loss of Ube3A highlights a species-specific vulnerability to aging and the implications of this are important for translating findings between species.

Recent therapies designed to restore age-related vision loss include perceptual learning paradigms, and a reduced capacity for plasticity in aging is likely to have an impact on the

effectiveness of these paradigms. It is clear that a complete understanding of the limitations on plasticity in aging V1 will be crucial for optimizing therapies to improve age-related vision loss.

Preamble for Chapter 4

The studies from chapter 3 (Williams et al., 2010) showed a loss of Ube3A expression in aging V1. Just before we published the paper, a new study by Greer et al. (2010) revealed that one of the substrates for Ube3A is the protein, Arc. Greer et al., (2010) showed that Ube3A selectively tags Arc with ubiquitin, thus marking it for degradation. Because Arc contributes to the internalization of AMPARs, when Ube3A expression was knocked down, Arc levels accumulated, and as a consequence, AMPARs became over-internalized (Greer et al., 2010). This led to a decrease in the number of AMPARs expressed at synapses, and reduced excitatory synaptic transmission.

We therefore predicted that aging might impact the expression of AMPARs. However, the prevailing view to date is that a loss of inhibition in aging drives age-related vision loss (Leventhal et al., 2003), although it is likely that a loss of inhibition isn't the only mechanism driving age-related changes because restoring inhibitory activity in V1 does not completely rescue age-related degradation of neuronal properties (Leventhal et al., 2003).

Interestingly, glutamate receptors mediate specific types of visual processing. AMPARs mediate the main feedforward, stimulus driven response, while NMDARs mediate modulatory feedback activity, driving more complex visual processing (Self et al., 2012). Blocking these receptors in macaques selectively blocks the neuronal responses that underlie both feedforward and feedback activity in a figure-ground segregation task. Thus, glutamate receptors play a key role mediating visual processing in V1, and a loss of glutamate receptors during aging may impact visual processing mechanisms in V1 that underlie visual perception.

Taken together, we asked 1) Does expression of proteins associated with glutamatergic synapses changes across the lifespan? and 2) What components of the GABAergic system change across the lifespan? We quantified a conserved set of pre-and post-synaptic proteins found at glutamatergic and GABAergic synapses and found a widespread loss of all glutamatergic and most GABAergic synaptic proteins throughout the lifespan. Furthermore, the losses occurred across all regions of V1. This study lays the foundation for subsequent studies to investigate the nature of the loss in order to determine the impact on the circuitry in V1 that underlies vision.

Chapter 2. Experience-dependent central vision deficits: neurobiology and visual acuity

Abstract

Abnormal visual experience during childhood often leads to binocular dysfunction, including acuity deficits that affect both eyes, especially in central vision. In animal models using monocular deprivation, the non-deprived eye is often considered normal and therefore not studied intensively. This leaves open the question whether monocular deprivation induces the binocular dysfunction typically found in amblyopia. In previous studies of monocularly deprived cats, we found a loss of excitatory receptors restricted to the central visual field representation in visual cortex (V1), including both eyes' columns. This led us to ask two questions about the effects of monocular deprivation on central vision: how quickly are the receptors lost? and is there an impact on the vision of both eyes? We found that just a few hours of monocular deprivation in cats caused a rapid loss of AMPA receptor proteins across all of V1, but after slightly longer deprivation, expression recovered in the region representing the visual periphery, leaving only the center of vision with a long-term loss. When we studied the visual acuity of cats with early deprivation, followed by many months of binocular vision, we uncovered a persistent acuity deficit in the center of vision that affected both eyes. Together, these findings show that monocular deprivation can reduce central visual acuity of both eyes and suggests that the role of AMPA receptors in feedforward processing is an important mechanism underlying those binocular acuity deficits.

2.1 Introduction

Early visual experience shapes the maturation of circuits in visual cortex (V1) and development of visual perception. Abnormal visual experience during the critical period by depriving one eye of vision (monocular deprivation, MD) causes a loss of responsiveness in V1 to the deprived eye, poor acuity through that eye, and binocular dysfunction. These changes are known as ocular dominance plasticity (Hubel et al., 1977) and just a few weeks of MD early in life can have a long-term impact on vision (Dews & Wiesel, 1970). Often the visual deficits are restricted to the deprived eye; however, several studies of children with amblyopia have found deficits in both eyes (Leguire et al., 1990; Chatzistefanou et al., 2005; Simons, 2005; Birch, 2013), especially for vision in the fovea (Agervi et al., 2010).

Animal studies using MD typically describe changes in V1 affecting the deprived eye (Dews & Wiesel, 1970; Giffin & Mitchell, 1978). But two recent studies have shown that the initial impact of MD on V1 is a loss of responsiveness in binocular excitatory neurons (Kuhlman et al., 2013; Hengen et al., 2013) followed by transient reduction in activation of inhibitory interneurons (parvalbumin-positive, PV+) that affects responsiveness of both the open and deprived eye (Kuhlman et al., 2013). The input to PV+ neurons is dominated by AMPA receptors containing GluA2/3 subunits (Kooijmans et al., 2014). Furthermore, AMPARs and their trafficking proteins are involved in both rapid and long term physiological changes caused by MD (Heynen et al., 2003; Yoon et al., 2009; Lambo & Turrigiano, 2013) as well as mediating the main feedforward stimulus driven response of neurons in V1 (Self et al., 2012). In deprived animals there is a loss of feedforward input to V1 neurons from the deprived eye (Ma et al., 2013) and in normal animals feedforward responses set up the spatial tuning properties of V1

neurons (Lamme et al., 1998). Thus, AMPARs sit at a nexus connecting ocular dominance plasticity and feedforward tuning of V1 neurons that ultimately underlies visual acuity.

Previously, we studied the effect of long-term MD in cats on expression of a large collection of synaptic receptors in V1 and found a loss of AMPARs restricted to the part of V1 representing the center of vision (Beston et al., 2010). Furthermore, anatomical investigation of expression of another glutamate receptor (NMDAR) showed that the central loss includes both eyes' ocular dominance columns (Murphy et al., 2004). Those studies, however, did not address how quickly receptors are lost in the center of vision, or if the loss has an impact on the vision of both eyes. Typically, the vision of the non-deprived eye is described as normal (Dews & Wiesel, 1970), but the acuity of both eyes in the center of vision has not been probed after MD. This led us to ask two questions: how quickly are AMPARs lost in the central region of V1? Does MD affect the acuity of both eyes? We addressed these questions by studying the effect of various lengths of MD on expression of AMPAR proteins in V1 and on long-term changes in the visual acuity of both eyes.

2.2 Methods

Animals and Rearing Conditions.

A total of 22 kittens, born and raised in a closed colony, were used in this study. To determine the effect of brief monocular deprivation (MD 6 hours - 7 days) on AMPAR expression in V1 we used 5 animals, and to study changes in visual acuity after early monocular deprivation we used 17 animals. All experimental procedures were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision research and approved by the McMaster University Animal Research Ethics Board. The animals used to examine rapid changes in AMPAR protein expression in V1 were studied at the peak of the critical period for ocular dominance plasticity in the cat (5 weeks of age, Olson & Freeman, 1980) and received either no deprivation, 6 hours, 1 day, 4 days, or 7 days of monocular deprivation. Acuity measurements were made on animals reared with either normal vision (n=3), or monocular deprivation for different durations during the critical period to create mild (deprivation from 4-6 weeks of age, n=8), moderate (deprivation from eye opening to 5 weeks of age, n=3), or more severe vision loss (deprivation from eye opening to 6 weeks of age, n=3). After deprivation these animals were given about 3 months of binocular visual experience during which visual acuity was measured daily. All animals were born in house and

Monocular deprivation was done by suturing the eyelids closed with 5-0 vicryl using aseptic surgical techniques, gaseous anesthetic (isoflurane, 1.5%-5%, in oxygen) for induction and maintenance of anesthesia, and following procedures that have been described previously (Murphy & Mitchell, 1987). The sutured eyes were checked daily to ensure that the lid margins

remained closed. At the end of deprivation the closed eye was re-opened by carefully parting the fused lid margins using aseptic surgical techniques.

Measurement & analysis of AMPAR subunit and trafficking proteins

Tissue collection

Animals were euthanized with Euthanol (165mg/kg), and perfused transcardially with cold 0.1M phosphate buffered saline (PBS) (4˚C; 80-100 ml/min). The brain was removed from the skull and immersed in ice cold PBS. A series of tissue samples (approx. 2mm X 2mm) were taken from V1 as described previously (Beston et al., 2010). For each cat, we used samples from V1 representing the center of vision (<5˚, n=2)), the visual periphery (~10˚-~50˚, n=2-3), and the monocular field of vision (>60˚, n=1) (Tusa et al., 1978). Each cortical tissue sample was rapidly frozen on dry ice and stored at -80˚C. All tissue samples were taken from visual cortex contralateral to the deprived eye.

Tissue sample preparation

The tissue sample was suspended in cold homogenization buffer (1 ml buffer: 50 mg tissue, 0.5m*M* DTT, 1m*M* EDTA, 2 m*M* EGTA, 10 m*M* HEPES, 10mg/L leupeptin, 100n*M* microcystin, 0.1 m*M* PMSF, 50 mg/L soybean trypsin inhibitor) and homogenized using a glass-glass Dounce tissue homogenizer. Homogenized samples were suspended in 10% SDS, heated for 10 minutes, and then stored at -80 ℃. Protein concentrations were determined using bicinchoninic acid (BCA) assay guidelines (Pierce, Rockford, IL) and samples were diluted to a standard concentration of 1μg protein/ml.

Immunoblotting

The samples (25 μg) were separated on polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF-FL) (Millipore, Billerica, MA). Each sample was run three times. Blots were pre-incubated in blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) for 1 hour (Li-cor Biosciences; Lincoln, NE), then incubated in primary antibody overnight at 4°C using the following concentrations: GluA2, 1:2000 (Invitrogen, Carlsbad, CA), Ube3A (E6AP), 1:2000 (Bethyl Laboratories, Montgomery, TX), PICK-1, 1:200 (NeuroMab, Davis, CA), β-Tubulin; 1:4000 (Imgenex, San Diego, CA). The blots were washed with PBS containing 0.05% Tween (Sigma, St. Louis, MO) (PBS-T) (3 x 10 min), incubated (1 hour, room temperature) with the appropriate IRDye labeled secondary antibody (Anti-Mouse, 1:8000, Anti-Rabbit, 1:10,000) (Li-cor Biosciences; Lincoln, NE), and washed in PBS-T (3 x 10 min). The bands were visualized using the Odyssey scanner (Li-cor Biosciences; Lincoln, NE) then the blots were stripped and reprobed so that each blot was probed with each of the antibodies (Blot Restore Membrane Rejuvenation kit, Chemicon International, Temecula, CA).

Band Analysis

To analyze the bands we scanned the blots (Odyssey Infrared Scanner) and quantified the bands using densitometry (Licor Odyssey Software version 3.0; Li-cor Biosciences; Lincoln, NE). Density profiles were determined by performing a subtraction of the background, integrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in band size. β-tubulin normalization was used as the loading control, and for each sample, expression of the synaptic proteins was divided by β-tubulin expression. We verified that β-tubulin expression did not vary across conditions (all p values were n.s.) and thus was an appropriate loading control (Fig. 1). A control sample, made

by combining a small amount from each sample, was run on each gel so the density of each sample was quantified relative to the control (sample density/control density).

Statistical Analysis

We made scatterplots showing all 3 runs (grey symbols) and average expression level for each animal (black symbols). All results were plotted as a percentage of the average expression for a normal 5 week old animal. We used a bootstrapping test to evaluate whether monocular deprivation caused a significant change in protein expression. Bootstrapping is a common statistical method for small sample sizes when parametric statistical methods are not appropriate. We used Matlab (The MathWorks, Inc, Natick, MA) to simulate a data set with the same mean and standard deviation as the experimental results from the normal animal. To determine if monocular deprivation caused a significant decrease in protein expression, we compared the simulated data set of the normal animal with average protein expression for each length of monocular deprivation (6 hrs, 1 day, 4 days, or 7 days). We ran Monte Carlo analyses that randomly sampled from the simulated normal distribution N times, where N was the number of measurements made for each length of deprivation, region of V1, and antibody (N ranged from 2 to 9). This random sampling was repeated 1,000,000 times to create a distribution of average expression levels expected for those sample sizes (N). The statistical significance for a particular condition was determined using a one-sided comparison with the simulated distribution. Deprivation conditions were identified as significantly less than normal ($p<0.05$) when their average expression was observed less than 5% of the time in the simulated averages. Finally, those simulations were used to calculate confidence intervals (CI) around the normal data and included those on each scatterplot.

Curve-fitting

To describe the time course of changes in protein expression as a function of the length of monocular deprivation we used a model fitting approach (Christopoulos & Lew, 2000). Single- or double-exponential decay functions [(y= yo+ a*exp(-t*b)+c*exp(-t*d); where t=length of monocular deprivation in days] were fit using the results from all samples and the on-line curve-fitting tool zunzun.com. The best fitting curve was found by least squares analysis, and the goodness-of-fit (R) and statistical significance of the fit (p) was calculated. The time constants (τ) for each phase of the decay function were calculated and 3τ was used to describe the length of time for loss or recovery of protein expression.

Principal component analysis

To assess the factors that effect the pattern of changes in AMPAR proteins we ran a multivariate analysis using principal component analysis (PCA). Protein expression was compiled into an mxn matrix, where the rows (m=3) represent the AMPAR proteins (GluA2, PICK-1, Ube3A), and the columns (n=26) represent all of the samples from the 5 animals (central=10, peripheral=11, monocular=5). The data were centered by subtracting the mean column vector, and then singular value decomposition (SVD) was applied to calculate the principal components in Matlab (The MathWorks, Inc, Natick, MA). The SVD represents expression of all proteins for each sample as a vector in high dimensional space. The PCA identifies the directions in "protein expression space" that represents the all the variance in the data.

Visual Acuity Measurement and Analysis

Measurement of Visual Acuity

Daily measurements of binocular visual acuity were started at 6 weeks of age after all deprived animals had finished their period of monocular deprivation (Giffin & Mitchell, 1978; Murphy & Mitchell,1987; 1991). The target stimulus was a high contrast (Michelson contrast 0.86) sine-wave grating displayed on a computer monitor (Sony GDMFW900, 24", 0.23mm dot pitch, 2304 x 1440 pixels, 8-bit gamma correction, 80 Hz frame rate) at a luminance of ~40cd/ m2. For each trial, 2 stimuli were displayed on either side of a center divider, the target sine-wave grating and a scrambled version of the grating. We chose to use the scrambled grating instead of a uniform grey field to control for any slight variations in luminance between the grating (target) and the alternative (negative) stimulus. Cats viewed the stimuli from the jumping stage above the landing platform and jumped down to the right or left side to indicate their selection. The viewing distance was varied from 50-85cm. Both the grating and scrambled stimuli were presented in a circular aperture that was either 4º, 8º, or 16º in diameter. The largest size aperture (16º) was used to measure development of binocular grating acuity until mature levels were reached at about 110 days of age. Acuity measurements with the smaller stimuli were only made after grating acuity had reached mature levels.

A two-alternative forced-choice paradigm and modified staircase procedure were used to measure acuity thresholds. Training began between 5 to 6 weeks of age and animals learned the task by first viewing an easy target stimulus (0.1 cycles/deg sine-wave grating) and were trained to jump to the target stimulus. Correct jumps were rewarded with food (chicken liver) and petting, while incorrect jumps resulted in withholding the rewards and immediately repeating the trial. The animals learned to jump toward the target stimulus and met criteria of 10 consecutive correct jumps to the easy stimulus within a few sessions (3-5 sessions). After this brief learning

period, binocular visual acuity thresholds were measured (5 sessions/week) for 10-12 weeks until the animals were about 110 days of age. Sessions were typically 45-60 minutes long and ~45 trials were needed to obtain a grating acuity threshold.

Each testing session began with an easy target and the position of the 2 stimuli were interchanged from left to right in a pseudo-random order. Thresholds were determined using a modified staircase procedure where the spatial frequency of the grating was increased in small steps, 16 steps per octave. Using these small steps, performance was nearly error free until the spatial frequency approached the threshold level. Typically, performance fell from flawless to chance in just 2 steps providing a reliable measure of acuity thresholds. Animals usually received only 1 trial for easy stimuli. The minimum number of trials was increased to 3 for stimuli close to threshold. When an error was made, additional trials were given until the animal made either 5 consecutive correct responses, or 7 correct responses in a maximum of 10 trials. Sessions ended when the animal no longer achieved criterion within 10 trials. For each session the last spatial frequency passed was taken as the acuity threshold.

After binocular acuity thresholds stabilized at adult levels (~110 days of age) we measured thresholds separately for each eye by placing an opaque contact lens (EyeTech Optics, Coquitlam BC) in the other eye. We also measured grating acuity in the center of vision using smaller diameter stimuli (4º and 8º) and the same procedures described above. Both binocular and monocular acuities for the smaller stimuli were determined by measuring thresholds in 3 to 5 separate sessions.

Quantitative Analysis of Acuity Measurements

The development of binocular grating acuity measured with the 16º stimulus was quantified by fitting a sigmoid function to the thresholds from each animal [y= a+((b-c)/(1+(t/c) ^d))] where t=age (days). We used the age when the function reached 90% of asymptotic level as the time when adult acuity was attained. The average age and SEM were calculated for each group. There was a lot of overlap for individual functions within the normal and 2 weeks deprived groups so we also fit separate sigmoid functions to all of the data from normal animals and animals deprived for 2 weeks. The final binocular grating acuity, standard deviation, and 95% confidence interval (95% CI) for each animal were calculated using the last 10 acuity thresholds. Average binocular and deprived eye (monocular for normals) grating acuities and SEMs were determined for each of the 3 different stimulus sizes (4º, 8º, or 16º in diameter).

Statistical Analyses

We used the same bootstrapping test described above to evaluate the significance of final acuities for each deprived animal compared to normals. A normal distribution of visual acuities was simulated with the same mean and standard deviation as the last 10 acuity measurements from the normal animals. We ran a Monte Carlo analysis that randomly sampled 10 times from the simulated normal acuity distribution then repeated that 1,000,000 times to create a normal distribution acuities for a sample size of 10 thresholds. We calculate the probability that the acuity of deprived animals was from that normal distribution and the z-score for the magnitude of the difference in deprived acuity away from the average acuity for normal animals.

To determine if there were differences among the 4 groups in the day-to-day variability in acuity we ran a repeated measures ANOVA using the last 10 binocular acuity thresholds measured with the 16º stimulus. Separate repeated measures ANOVAs were run to determine the

effect of stimulus size on visual acuity thresholds. Post-hoc analyses were done using Tukey HSD. In addition, the effect of stimulus size was quantified by fitting linear functions using least squares to binocular and deprived eye (monocular for normal animals) acuities, then the slopes were compared using an ANCOVA.

2.3 Results

Western Blot Analysis

We used β-tubulin as the loading control and verified that it was not affected by monocular deprivation (MD). Expression of β-tubulin was not different from normal after any length of deprivation (6hrs, 1 day, 4 days, 7 days MD) or within any region of V1 (central, peripheral, monocular) (Fig. 1A, B, C; all p values n.s.).

Monocular deprivation causes a transient loss of AMPAR proteins in the peripheral and monocular regions of V1, but sustained loss in the central region

To examine if the loss of AMPAR proteins in V1 is restricted to the central region we quantified expression of the AMPAR subunit, GluA2, and two AMPAR trafficking proteins, PICK-1 and Ube3A, after different lengths of MD (6 hrs, 1 day, 4 days, 7 days) and in 3 regions of V1 representing the center of vision (< 5˚, central), visual periphery (~10˚-~50˚, peripheral), and monocular crescent (>60˚, monocular) (Fig. 2). We included the AMPAR trafficking proteins, PICK-1 and Ube3A, because they are involved in dynamic movement of AMPARs in and out of the postsynaptic membrane, especially GluA2 subunit containing receptors, and are important mechanisms regulating experience-dependent synaptic plasticity (Terashima et al., 2004; Yashiro et al., 2009; Greer et al., 2010; Sato & Stryker, 2010; Lee & Kirkwood 2011; Shepherd & Bear 2011). Thus, quantifying these AMPAR-associated proteins provides additional information about receptor dynamics that may differ from experience-dependent changes in expression of the AMPAR subunit, GluA2.

We found a rapid loss of all 3 AMPAR proteins after 6 hrs of MD that was similar in all regions of V1. With longer deprivation there was recovery of expression, but only in the

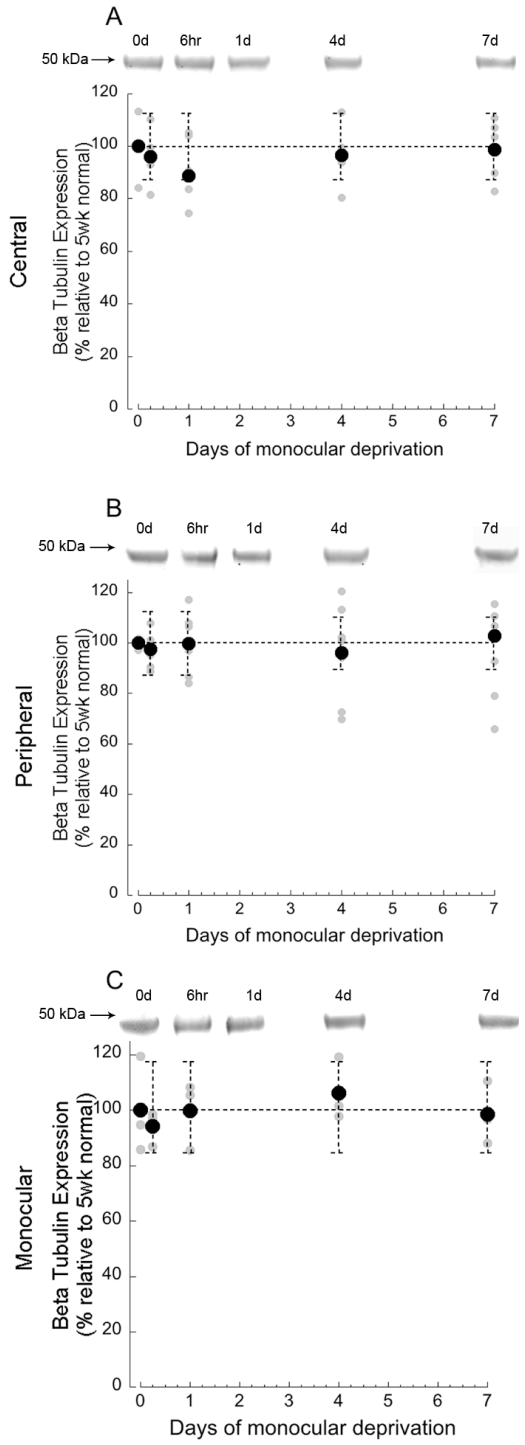

Figure 1. Effect of monocular deprivation on β-tubulin expression in the central (A), peripheral (B), and monocular (C) regions of V1. Expression levels are shown as a percentage of a 5 week old animal that did not receive monocular deprivation (dotted line). Grey dots are results from all runs, and black dots are the average for each animal. Example bands are shown above the graphs. 95% confidence intervals are displayed around the normal distribution. Monocular deprivation had no significant effect on β-tubulin expression in central (A), peripheral (B), and monocular (C) regions of V1 (all p values n.s).

peripheral and monocular regions (Fig. 2D-I). The changes in expression of the 3 AMPAR proteins were well fit by single- or double-exponential decay functions from which we calculated the amount of change from normal levels of expression and time constants to quantify the rate of rapid loss (3τloss) and subsequent recovery (3τrecovery) of protein expression.

In the central region in V1, just 6 hrs of MD caused a rapid loss of the 3 AMPAR associated proteins which dropped ~30-40% below normal levels (GluA2 40% $p<0.001$, PICK-1 30% $p<0.01$, Ube3A 40% $p<0.001$) (Fig 2A-C). That loss persisted in the central region with longer MD and remained ~30-40% below normal levels after 7 days of MD (GluA2, PICK-1, Ube3A, all $p<0.001$). The time constants for the curve fits to each protein showed that the loss happened very quickly, within 5-7 hours of MD (Fig. 2A, GluA2 curve-fit $R= 0.64$, $p= 0.0001$, 3τloss=7 hrs; Fig. 2B, PICK-1, curve-fit $R=0.58$, $p=0.008$, 3τloss=7.1 hrs;Fig. 2C, Ube3A, curve-fit $R= 0.61$, $p=0.0003$, 3τloss= 5 hrs).

In the peripheral region, 6 hrs of MD also caused a rapid loss of the AMPAR proteins which fell to ~40-50% below normal (GluA2 50% $p<0.001$, PICK-1 50% $p<0.001$, Ube3A 40% $p<0.001$) (Fig 2D- F). In contrast to the central region, the loss in the periphery was transient and after longer MD there was recovery of AMPAR protein expression (Fig 2D-F). The extent of recovery was variable and after 7 days MD some samples had normal or above normal levels, while other samples remained below normal. On average, however, GluA2 recovered to levels that were not different from normal (n.s.), while PICK-1 ($p<0.05$) and Ube3A ($p<0.01$) remained ~15-20% below normal levels. Curve fits to those data showed a pattern of rapid loss (range for 3τloss 6.6-9.5 hrs of MD), similar to that found for the central region, followed by slow recovery (range for 3τrecovery 10.7-27.7 days of MD) after longer MD (Fig. 2D, GluA2, curve-fit R=

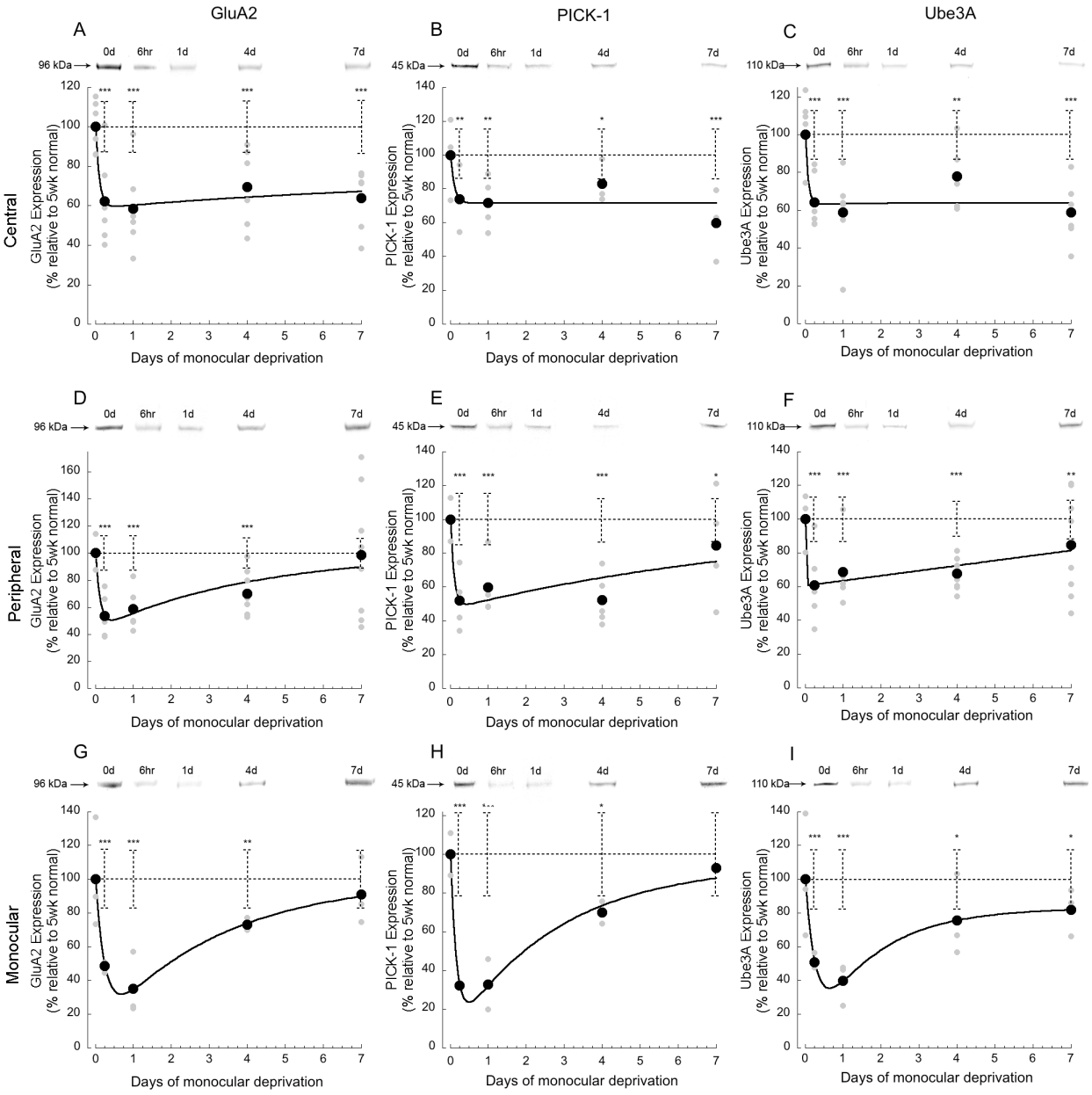

Figure 2. Effect of monocular deprivation on GluA2, PICK-1, Ube3A expression across the central (A,B,C), peripheral (D,E,F), and monocular (G,H,I) regions of V1. Expression levels are shown as a percentage of a 5 week old kitten that did not receive monocular deprivation (dotted line). Grey dots are results from all runs, and black dots are the average for each kitten. Example bands are shown above the graphs. Single or double exponential decay functions were fit to describe changes in expression. 95% confidence intervals are displayed around the normal distribution. In the central region, GluA2, PICK-1, and Ube3A expression fell rapidly (30-40%) after 6 hours of MD, and persisted for 7 days MD (all p values <0.001) (A,B,C). In the peripheral region, GluA2, PICK-1, and Ube3A expression fell rapidly (40-50%) after 6 hours MD, and either completely recovered (GluA2, p=0.4) or partially recovered (PICK-1, p<0.05, Ube3A, p<0.01) after 7 days MD (D,E,F). In the monocular region, GluA2, PICK-1, and Ube3A expression fell rapidly (50-70%) after 6 hours of MD, and either completely recovered (GluA2, p= 0.2, PICK-1, p=0.3) or partially recovered (Ube3A, p<0.05) (G, H, I). Levels of significance are indicated by asterisks: * = p<0.05, ** = p<0.01. ***= p<0.001

0.56, p=0.0006, 3τloss=8.6 hrs, 3τrecovery= 12.2 days; Fig. 2E, PICK-1, curve-fit R=0.6, p=0.003, 3τloss=6.6 hrs, 3τrecovery= 27.7 days; Fig. 2F, Ube3A, curve-fit R=0.5, p=0.004, 3τloss= 9.5 hrs, 3τrecovery= 10.7 days). The timing of the transient loss and subsequent recovery was similar to the physiological changes found in rodent V1 (Kuhlman et al., 2013).

The monocular region also had a transient loss of the 3 AMPAR proteins after 6 hrs and 1 day of MD (Fig. 2G-I). The transient loss in the monocular region was greater than in central or peripheral regions, and fell by ~65-75% of normal levels (GluA2 70% $p<0.001$, PICK-1 75% $p<0.001$, Ube3A 65% $p<0.001$). With longer MD (4 or 7 days) there was substantial recovery so that after 7 days of MD the levels for the 3 AMPAR proteins were either not different from normal or slightly below normal levels (GluA2 10% n.s., PICK-1 10% n.s., Ube3A 20% $p<0.05$). The curve fits showed that the monocular region had a slightly more prolonged period of protein loss (range for 3τloss 11.4-19.6 hrs of MD) than the central or peripheral regions, but faster recovery (range for 3τrecovery 4.9-9.6 days of MD) for all 3 AMPAR proteins (Fig. 2G, GluA2, curve-fit R= 0.84, $p<0.0001$, 3τloss= 17.7 hrs, 3τrecovery= 9.6 days; 2H, PICK-1, curve-fit R= 0.96, $p<0.0001$, 3τloss= 11.4 hrs, 3τrecovery= 8.3 days; 2I, Ube3A, curve-fit R= 0.78, p=0.0006, 3τloss= 19.6 hrs, 3τrecovery= 4.9 days).

Principal component analysis shows that MD has regional effects on AMPAR protein expression

The effect of different lengths of MD on expression of GluA2, PICK-1, and Ube3A was very similar within each region, but different across regions in V1. That observation suggested that MD has different effects on plasticity for the center of vision, where there was sustained loss, versus the visual periphery or monocular regions, where the loss was transient. Since we measured multiple AMPAR proteins and regions in V1 we used a multidimensional analysis to

determine what factors accounted for the variance in the data. We took a data driven approach, and analyzed the expression of GluA2, PICK-1 and Ube3A in the different regions of V1 using principal components analysis (PCA). First, a 3x26 matrix of protein expression was made where the 3 rows were the proteins (GluA2, PICK-1, Ube3A) and the 26 columns were the samples from all regions and animals. The data were centered by subtracting the mean column vector, and singular value decomposition (SVD) was applied to calculate the principal components. The first principal component accounts for the largest variation in the data and for this dataset the eigenvalue for PCA1 explained 88.4% of the variance, PCA2 explained 7.3%, and PCA3 explained 4.3% (Fig. 3A). Only PCA1 met the Guttman-Kaiser criterion (an eigenvalue > 1) to be used for further analysis (Jackson, 1991).

The next step was to determine potential biological factors for PCA1. We plotted the basis vector for PCA1 (Fig. 3B) and found that the weights for GluA2, PICK-1 and Ube3A were positive and had similar amplitudes. Those weights suggested that the main factor underlying PCA1 was the variance in total protein expression, which was supported by a very strong correlation between PCA1 and the sum of GluA2, PICK-1, and Ube3A, that we called total protein ($R= 0.99$, $p<0.0001$). We plotted total protein expression as a function of the different lengths of MD for the 3 regions in V1 (Fig. 3C). Total AMPAR protein levels dropped in the 3 regions within 6 hrs and in the monocular region it continued to fall reaching a minimum after about 1 day of MD. Interestingly, the levels of total protein expression in the 3 regions were very similar after 4 days of MD, but just a few more days of MD showed recovery in peripheral and monocular regions. This analysis clearly illustrates the rapid transient loss of AMPAR proteins in all of V1 with subsequent recovery in visual periphery and monocular regions, and

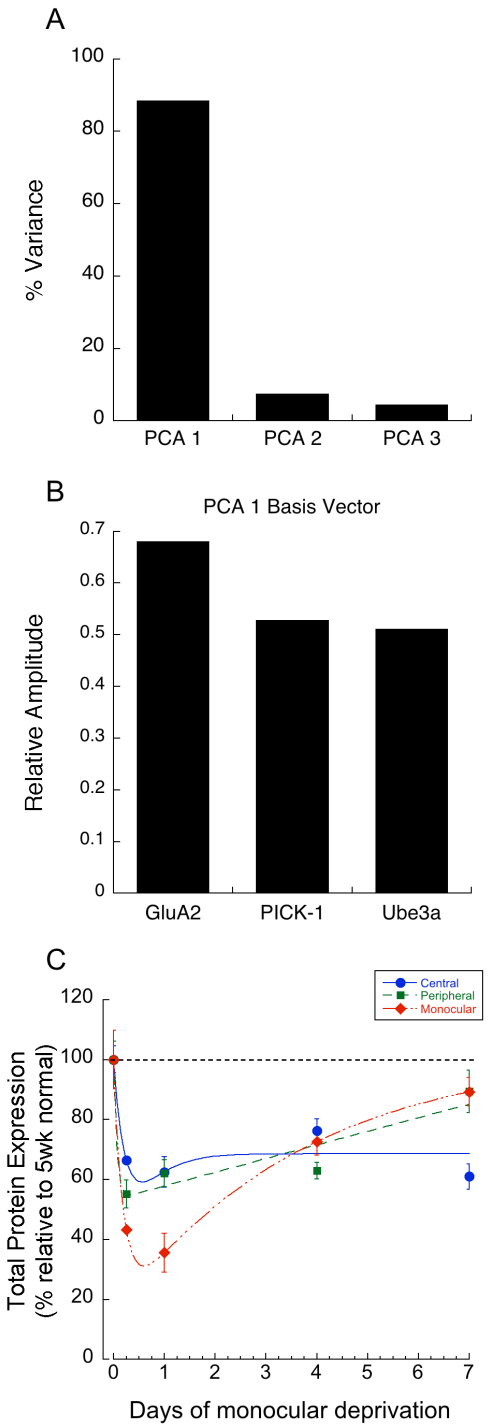

Figure 3. Principal component analysis. The percent variance captured by each component of the SVD analysis of protein expression in central, peripheral, and monocular regions of V1 (A). The first principal component represents the only significant portion (88.4%) of the SVD. The influence of each protein on the first principal component was reflected by the relative amplitude in the basis vectors (B). Total protein expression as a function of the length of monocular deprivation in the central (blue), peripheral (green), and monocular (red) regions of V1 (C).

only the center of vision is left with sustained loss. The sustained loss after 1week MD was similar to the results from our previous study with longer MD (Beston et al., 2010) and led us to ask if this pattern of experience-dependent changes in AMPAR proteins in V1 is reflected in the impact of MD on visual acuity.

Visual Acuity

To assess whether changes caused by early MD have long term impact on the development of vision in both eyes we made daily measurements of binocular grating acuity in normal and monocularly deprived animals. The animals were MDed for either 2 wks (from 4-6 wks of age), 5wks (eye opening to 5wks of age), or 6 wks (eye opening to 6wks of age) to create a range of vision deficits from mild to more severe. Then binocular grating acuity measurements were started once the deprived eye was opened and continued until ~4 months of age. We used a large diameter (16º) grating to measure the development of binocular grating acuity because it would stimulate a large region of V1 including both the center of vision and visual periphery. The thresholds were measured binocularly so neither eye received any patching that could give the other eye a competitive advantage.

Development of binocular acuity is impaired after long, but not short-term MD

Binocular acuity of normal animals (n=3) (open black symbols) and 2wk MD animals (n=8) (filled red symbols) developed together similarly (Fig. 4A). There was a lot of overlap among the animals in each group, so we plotted sigmoid functions fit to all the thresholds from normal ($y= 11.2 + ((0.3-11.2)/(1+(t/74.8)^{12.6}))$, $R=0.96$, $p<0.0001$) or 2wks MD animals ($y= 11.4 + ((0.3-11.4)/(1+(t/75.1)^{9.4}))$, $R=0.96$, $p<0.0001$). Those curves almost completely overlapped,

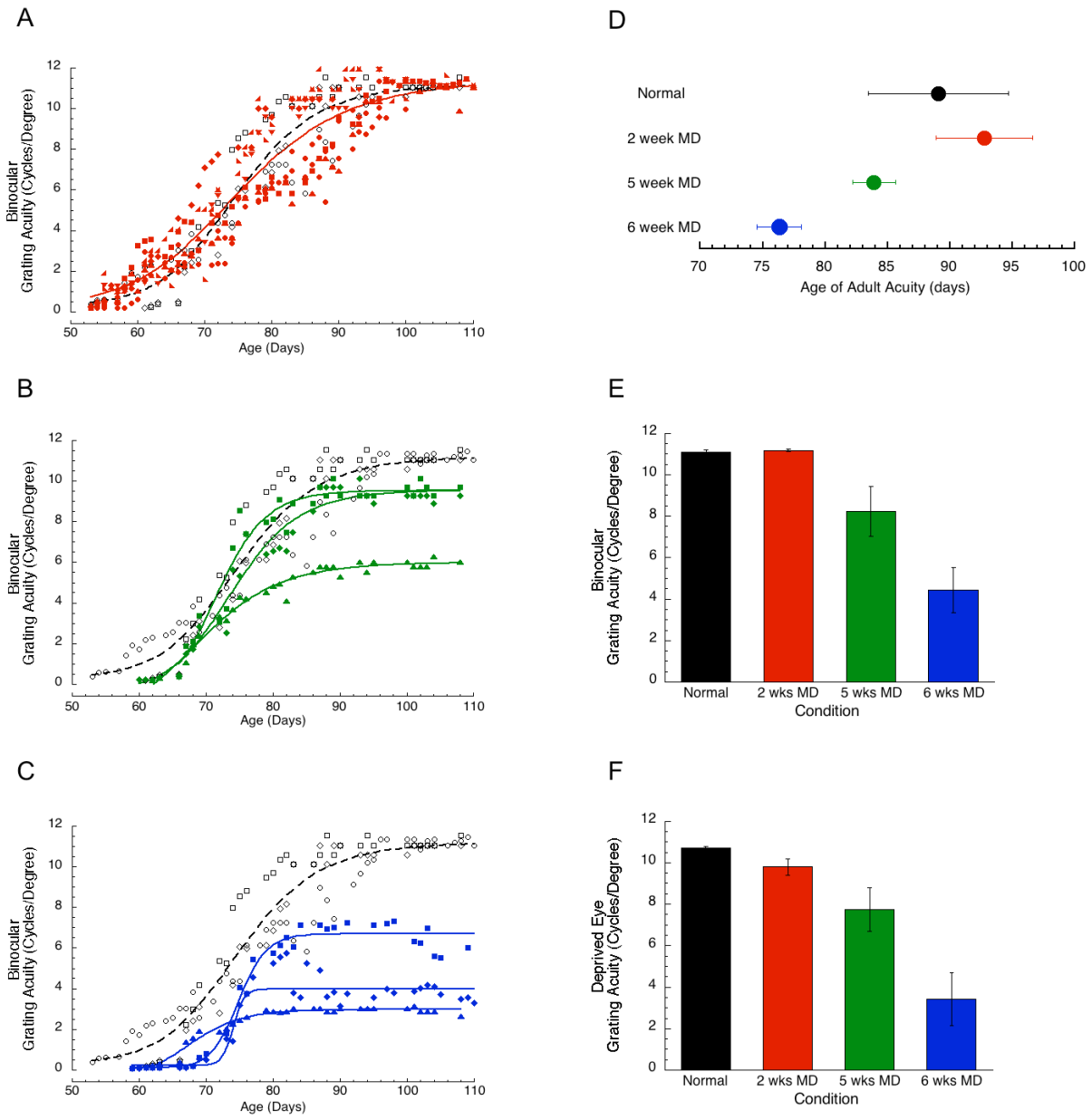

Figure 4. Effect of monocular deprivation on recovery of binocular and deprived eye grating acuity. Binocular grating acuity developed similarly for normal animals (open circles, n=3) and animals monocularly deprived for 2 weeks during the critical period (red symbols, n=8) (A). Animals monocularly deprived from eye opening until 5 (green symbols, n=3) (B) or 6 weeks of age (blue symbols, n=3) initially developed like normals, but stopped earlier, and developed poor binocular acuity (C). The age at which normals and each deprivation condition reached 90% of their final asymptote (D). Comparison of final average binocular (E) and deprived eye (F) grating acuity for the group of normal animals and each deprivation condition.

and we used the equations to calculate when adult acuity was reached (90% of asymptotic level) and found that normal animals reached adult binocular acuity at 89 days of age (SEM 5.6 days) and 2wk MD animals attained mature binocular acuity at 92 days of age (SEM 3.9 days) (Fig. 4D).

The comparison between normal and 2wk MD animals found similar development (Fig. 4A) and adult binocular acuities (normals, average = 11.1 cycles/deg, SEM 0.1 cycles/deg; 2wk MDs, average = 11.2 cycles/deg, SEM 0.1 cycles/deg.) (Fig. 4E), but quite a different pattern of acuity development emerged for animals deprived for longer periods. Initially, development of binocular acuity for 5wk and 6wk MDs followed the normal trajectory but then stopped earlier. For 5wk MDs, binocular acuity reached mature levels at 84 days of age (SEM 2 days) and for 6wk MDs it was even earlier at 76 days of age (SEM 2 days) (Fig. 4D). Both of those ages were earlier than normal (89 day SEM 4) or 2wk MD (92 days SEM 4) animals, suggesting that longer deprivation shortens the period for visual development. Furthermore, adult binocular acuities were 8.2 cycles/deg. (SEM 1.2 cycles/deg.) for 5wk MDs and only 4.4 cycles/deg. (SEM 1.1 cycles/deg.) for 6wk MDs, both of which were well below normal binocular acuities thresholds (Fig. 4B, C, E).

The last 10 thresholds were used to calculate the average binocular acuity and variability for each animal and we ran a repeated measures ANOVA with those measures to verify that each group had similar day-to-day variability in acuity ($p=0.7$). Then, we used a bootstrapping analysis to calculate the probability that the binocular acuity for each deprived animal was drawn from the distribution of normal acuities. The probabilities were converted into z-scores and two-tail p values were calculated to identify deprived animals with binocular acuity different from

normal. None of the 2wk MD animals were different from normal (all p values > 0.05) but all of the animals deprived for longer (5wks or 6 wks MD) had binocular acuity that significantly worse than normal (5wks MD $p<0.00001$; 6wks MD $p<0.00001$) (Fig. 4E).

Deprived eye acuity is worse after short and long-term MD

After mature binocular acuity was reached we measured acuity of the deprived eye and monocular acuity for normal animals. We used the bootstrap analysis to compare deprived eye acuities with normal monocular acuity and found that even the 2wk MD group had acuity less than normal (Fig. 4F). Deprived eye acuity of the 2wk MDs was ~1 cycle/deg. worse than normal animals (normal monocular acuity, 10.7 cycles/deg., SEM 0.1 cycles/deg.; deprived eye acuity 2wks MD, 9.8 cycles/deg., SEM 0.4 cycles/deg., $p<0.0001$) (Fig. 4F). Longer deprivation caused greater acuity deficits for the deprived eye compared with normals (deprived eye acuity 5 wks MD, 7.7 cycles/deg., SEM 1.1 cycles/deg., $p<0.00001$; 6 wks MD, 3.4 cycles/deg., SEM 1.3 cycles/deg., $p<0.00001$) (Fig. 4F). Thus, all of the MD conditions led to acuity deficits in the deprived eye that persisted even though the eye was opened during the critical period and there had been months of binocular vision with daily testing of grating acuity.

Finally, we assessed if any of the animals had a binocular advantage or disadvantage. Many visual functions have an improvement in performance when viewed with both eyes compared to one eye (binocular advantage) and in some circumstances when the vision of the 2 eyes is different there can be binocular loss. We used the bootstrap analysis to compare binocular and deprived eye (or monocular) acuities for every animal to determine if any of the groups had a binocular advantage or disadvantage. Normal animals had a small but not significant binocular advantage (average 11.1 cycles/deg) over monocular (10.7 cycles/deg.)

($p>0.05$) suggesting that if normal cats have a binocular advantage for high contrast grating acuity then the gain is very small. There were no significant differences between binocular and deprived eye acuities for animals that had 5 or 6 wks MD ($p>0.05$). Only 2 wk MDs had better binocular (average = 11.2 cycles/deg, SEM 0.1 cycles/deg.) than deprived eye acuity (9.8 cycles/deg., SEM 0.4 cycles/deg.)($p<0.01$). Importantly, none of the MDed animals had binocular acuity that was worse than the deprived eye acuity so we did not find any evidence that MD caused a binocular disadvantage for grating acuity.

Measuring acuity in the center of vision reveals acuity deficits after both short and longer MD

The lack of binocular acuity deficit in the animals MDed for 2 weeks was puzzling because a short period of MD caused a loss of AMPAR proteins in the central region of V1 (Fig 2), similar to the loss after longer periods of MD (Beston et al., 2010). We considered two possibilities: first, that 2 weeks of MD only affects vision of the deprived eye; and second, that measuring binocular acuity with the large (16º) stimulus was masking a deficit by stimulating a large region of V1 beyond the center of vision. To address these possibilities we reassessed deprived eye and binocular acuity using a small (4º), medium (8º), or large diameter grating (16º) where the small stimulus diameter was within the cat's center of vision.

For normal animals, monocular and binocular acuity were not affected by measuring thresholds with smaller diameter stimuli (repeated-measures ANOVA $p=0.3$)(Fig. 5). In contrast, all of the MDed animals had significantly worse deprived eye and binocular acuities when measured with the smaller stimuli, and the deficits grew linearly with the size of the stimulus (repeated-measures ANOVA for 2wk, 5wk, 6wk MDs all p values < 0.05). Even animals

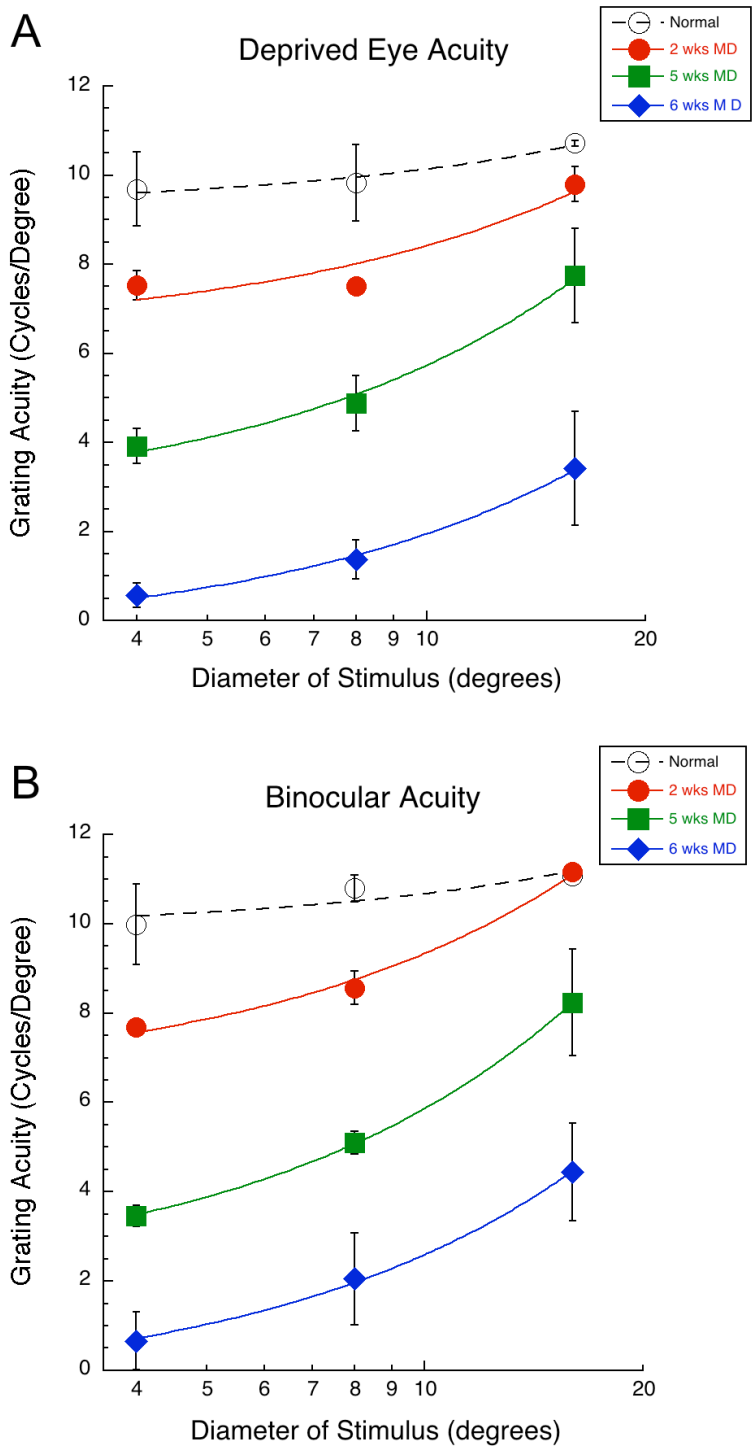

Figure 5. Effect of monocular deprivation on acuity in the center of vision. Deprived eye acuity increases linearly as a function of stimulus size for all deprived animals (repeated measures ANOVA; 2wk p=0.04; 5wk p=0.02; 6wk p=0.05), but monocular acuity remained the same for normals (p=0.3) (A). Binocular acuity also increases linearly as a function of stimulus size for all deprived animals (repeated measures ANOVA; 2wk p=0.02; 5wk p=0.03; 6wk p=0.001) but remained the same for normal animals (p=0.3) (B).

deprived for 2 wks had significantly worse grating acuity, more than 2 cycles/deg. in the center of vision (binocular and deprived eye) for the small (4º) and medium (8º) sized stimuli compared with the large stimulus (Fig. 5). Interestingly, the slopes of the linear functions fit to the binocular and deprived eye acuities were not different among the 3 groups of MDed animals (ANCOVA p=0.5). Thus, using smaller stimuli we found that both binocular and deprived eye acuity deficits scaled linearly as the stimulus size was reduced down to the center of vision. Furthermore, the entire acuity vs size function dropped down with longer periods of MD to the point where animals deprived to 6 wks were left with rudimentary vision in the center of vision. These results suggest that the impact of MD on visual acuity has two phases: first, a loss in the center of vision; then after longer MD a widespread loss of acuity affecting the center and visual periphery.

2.4 Discussion

In this study we asked whether the monocular deprivation (MD) induced loss of AMPAR proteins in V1 was reflected in changes in visual acuity. We studied expression of AMPAR proteins because they are tied to visual experience-dependent plasticity and feedforward processing necessary for spatial receptive field properties in V1 neurons. To relate changes in AMPAR proteins with visual acuity we studied the effects of MD in two groups: one to examine how quickly expression of AMPAR proteins changed in different regions of V1 and the second to determine the long-term consequences of MD on acuity in the center of vision. We found that just a few hours of MD caused a rapid loss of AMPAR proteins across all of V1. But after slightly longer MD (a few days) expression recovered in the visual periphery leaving only the center of vision in V1 with a long-term loss of AMPAR proteins. When we examined the long-term impact of MD on acuity we found a persistent loss of acuity in the center of vision even in animals with relatively short MD and many months of binocular vision. Furthermore, the visual acuity of both eyes was effected. Our results suggest that an experience-dependent loss of AMPAR proteins in the central region of V1 contributes to amblyopic vision loss by effecting the processing of visual information for both eyes.

We used cats in this study because they have a well formed area centralis in the retina that supports a central visual field with high resolution acuity. They also have a much larger V1 than rats (Duffy et al., 1998) making it is possible to analyze multiple tissue samples from different regions of V1. In addition, the jumping stand technique allowed us to make accurate and reliable estimates of acuity thresholds, and the large number of cats (n=17), provided confidence about the differences between normal (n=3) and deprived animals (n=14). Previous studies have

reported lower acuities for normal cats (range 4-8 cycles/deg.), but the spacing and size of receptive fields in the area centralis predict grating acuity as high as 20 cycles/deg. (Campbell et al., 1973; Cleland et al., 1979; 1980; Jacobson et al., 1976; Robson & Enroth-Cugell, 1978; Steinberg et al., 1973). In this study, normal cats achieved acuity thresholds of about 12 cycles/deg., consistent with a recent study that measured similarly high grating acuity thresholds in cats (Clark & Clark, 2013). This increased sensitivity for measuring acuity, especially of normal cats, no doubt contributed to finding acuity deficits in all of the deprived animals.

We used the same techniques as the previous study that found a loss of AMPAR proteins in the central region of V1 after MD (Beston et al., 2010). The Western blot technique provide good quantification of the available pool of these AMPAR associated proteins. It does not, however, give an indication of cell types, layers, or synapse locations where MD causes changes in the expression of these AMPAR proteins. The rapid and transient time course of MD induced changes in AMPAR proteins identify important stages in this plasticity that are useful in designing future studies using detailed anatomical preparations to map changes in the connectome.

How might the loss of AMPAR proteins contribute to amblyopic vision deficits? AMPARs mediate much of the feedforward processing that shapes the spatial properties of V1 neurons (Lamme et al., 1998; Self et al., 2012), and blocking AMPAR activity reduces the main stimulus driven response in V1 (Self et al., 2012). The spatial properties of V1 neurons limit visual acuity (Parker & Hawken, 1985) and because AMPARs mediate the feedforward drive that shapes those receptive field properties it is reasonable to propose that a loss of AMPARs in the center of vision would have an adverse impact on high resolution acuity. The most direct way to test that

relationship is by manipulating AMPARs either by selectively reducing AMPARs in the central region of V1 in normal cats, or by prevent the loss of AMPARs caused by MD. A challenge for those studies is that the powerful techniques for genetic manipulations of AMPARs are currently only available in rodent models, but the rodent lacks a well developed center of vision with high resolution acuity.

The relationship between the losses of AMPAR proteins and visual acuity must involve changes in V1 circuitry that probably includes parvalbumin-positive (PV+) interneurons. First, the dominant excitatory input to PV+ neurons is mediated by AMPAR rich synapses containing the GluA2 subunit, and MD reduces excitatory drive onto binocular PV+ neurons (Kuhlman et al., 2013). Second, activation of PV+ neurons shapes spatial receptive field properties of V1 neurons that underlie visual perception (Lee et al., 2012). Together, these make PV+ neurons a likely component of V1 circuitry linking AMPAR changes with visual acuity. There is a growing consensus that PV+ circuitry is pivotal for experience-dependent plasticity (Hensch, 2014) and determining the functional impact of AMPAR loss on PV+ neurons will be important for determining the neural basis of acuity loss in the center of vision. For example, PV+ neurons control gamma rhythms (Cardin et al., 2009; Sohal et al., 2009) and the amplitude of gamma rhythms increases as the stimulus size expands from within to outside the fovea (Ray & Maunsell, 2011).

What neural mechanisms might drive experience-dependent changes in the center of vision? Previous studies have found differences in experience-dependent plasticity mechanisms between binocular and monocular regions of rodent V1 during development (Nataraj & Turrigiano, 2011; Lambo & Turrigiano, 2013) and in adults (Kuo & Dringenberg, 2012). Our lab

extended those regional findings when we identified MD induced changes in synaptic proteins in the portion of cat V1 that represents the center of vision (Beston et al., 2010; Murphy et al., 2004). Those changes would contribute to reduced plasticity, a shift in the excitatory:inhibitory balance, and an acceleration of receptor maturation in V1. A difference between cats and rodents is that, like primates, cats have a peak retinal cone density in the center of vision (Steinberg et al., 1973). It is not clear, however, why a peak in cone density might lead to greater sensitivity to abnormal visual experience. Another mechanisms that dominates the center of vision are OFF responses. These are more common and stronger than ON responses in the center of vision and the OFF dominance follows through to at least V1 (Ahmad et al., 2003; Jin et al., 2007; Kremkow et al., 2014). Disrupting the ON/OFF balance in the retina causes a loss of contrast sensitivity (Schiller et al., 1986) and reduced orientation selectivity of V1 neurons (Sarnaik et al., 2014). The maturation of ON/OFF circuitry in the retina is activity-dependent (Tian & Copenhagen, 2003) and visual deprivation in cats leads to permanent changes in that circuitry which could induce a shift favouring OFF responses (Burnat et al., 2012). A recent study has shown that OFF responses dominate low spatial frequencies in cat V1 (Kremkow et al., 2014) and thus an experience-dependent shift in favour of OFF responses is likely to have the greatest impact on high spatial resolution in the center of vision.

What unanswered questions about amblyopia might be addressed using this animal model? In this study we found a number of acuity changes in MDed cats that are similar to those reported for human amblyopes. These include: greater deficits in the center of vision, acuity deficits in both eyes, and long lasting effects of early abnormal visual experience. Thus, the cat provides a good model to identify neural mechanisms in the center of vision that are vulnerable

to abnormal visual experience and then develop neuroplasticity based therapies that provide long-term recovery of high resolution spatial vision in both eyes.

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Chapter 3. Dramatic Loss of Ube3A Expression During Aging of the Mammalian Cortex

Publication Reference

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Abstract

Neurobiological studies of aging are beginning to link functional changes with a loss of experience-dependent plasticity. In the visual system, age-related functional changes include decreases in visual acuity, orientation selectivity, motion perception, and ocular dominance plasticity. A recent paper has shown that Ube3A, an E3 ubiquitin ligase that is absent in Angelman's Syndrome, is required for experience-dependent plasticity during development of the visual cortex. Knocking out Ube3A during development leads to rigidity of ocular dominance plasticity that is strikingly similar to the reduced plasticity seen in older animals. Furthermore, ubiquitin ligases have been linked with age-related neurodegenerative disorders and longevity. Ubiquitin ligases selectively mark proteins for degradation, and a balance between synaptic proteins and their degradation is important for neural transmission and plasticity. This led us to ask whether normal aging is characterized by a loss of Ube3A in the cortex. We used Western blot analysis in order to quantify Ube3A expression across the life span of humans, macaque monkeys, and cats. We found that Ube3A expression declines across the lifespan in human, monkey, and cat cortex. The losses were substantial (50-80%) in all areas studied which includes V1, V3, V4, frontal, and auditory cortex. In addition, when compared with other synaptic proteins there was a selective loss of Ube3A in human cortex. The progressive loss of Ube3A expression during cortical aging is an important new finding. Furthermore, the selective loss of Ube3A in human cortex highlights a specific vulnerability in human brain aging that may signify a dramatic shift in cortical function and plasticity.

3.1 Introduction

A common way of describing the behavior of older adults is that they are more rigid and set in their ways. The wide range in sensory and cognitive functional changes that accompany normal, healthy aging are suggestive of widespread cortical dysfunction. Neurobiological studies are beginning to link these functional changes and common descriptions with a loss of experience-dependent plasticity that reflects age-related changes in synaptic plasticity mechanisms (Burke & Barnes, 2006). In the visual system, age-related changes include perceptual changes in luminance, colour, motion, and texture (Faubert, 2002), and neurophysiological loss of orientation tuning (Schmolesky et al., 2000) and ocular dominance plasticity (Sato & Stryker, 2008). The common explanation for these changes is a loss of GABA mediated inhibition in the aging visual cortex because application of GABA to visual cortical neurons reinstates normal receptive field tuning properties (Leventhal et al., 2003). But many changes occur at the aging synapse (Wong et al., 2000; Bai et al., 2004), and it is likely that more than just one synaptic mechanism contributes to visual deficits in aging. Recent studies have highlighted the important role of ubiquitin-mediated degradation of proteins at the synapse as a potent mechanism for changing synaptic structure and function (DiAntonio & Hicke, 2004), and the loss of ubiquitin ligases in neurodegeneration (Ardley & Robinson, 2004). Yet, little has been known about changes in the expression of ubiquitination machinery in human cortex across the lifespan into normal, healthy aging.

Ube3A, an E3 ubiquitin ligase, is absent in the neurodevelopmental disorder, Angelman Syndrome, leading to mental retardation early in life (Kishino et al., 1997). A recent study has shown that Ube3A is necessary for experience-dependent ocular dominance plasticity during

development (Yashiro et al., 2009). Ube3A knockout mice are resistant to the synaptic plasticity that underlies monocular deprivation driven changes in ocular dominance. This rigidity of synapses is strikingly similar to the reduced experience-dependent plasticity seen in older animals, who also show no shift in ocular dominance patterns when challenged with monocular deprivation (Sato & Stryker, 2008).

Neuronal activity interacts with ubiquitination pathways to regulate synaptic structure and function in response to changes in synaptic activity; for example, by facilitating the functional reorganization of the synapse by remodeling the postsynaptic density composition (Ehlers, 2003). Furthermore, disruptions in ubiquitination pathways are linked with specific neurodegenerative diseases including Alzheimers and Parkinson's disease (Ardley & Robinson, 2004). Finally, a recent study has shown that an E3 ligase homologous to Ube3A is necessary for caloric restriction to extend longevity (Carrano et al., 2009). It is clear that an appropriate balance between synaptic proteins determines synaptic efficacy and function, and thus a controlled regulation between their synthesis and degradation is important for maintaining normal synaptic transmission and plasticity (Cline, 2003). Taken together, these results led us to ask whether normal aging in visual cortex is characterized by a loss of Ube3A at the synapse.

To better understand age-related changes in Ube3A expression in the visual cortex we quantified expression levels in tissue samples from macaque monkey, cat, and human visual cortex. In addition, we compared changes in primary visual cortex with extrastriate areas, auditory, and frontal cortex. We found a progressive loss of Ube3A expression in visual cortex during aging that was similar for the 3 species and cortical areas. Furthermore, our findings

reveal a selective age-related loss of Ube3A in human cortex that becomes apparent at the transition into old age and may signify a loss of cortical plasticity.

3.2 Methods

Tissue Collection

Human cortical tissue samples were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). Tissue samples were obtained within 24 hours post-mortem. The occipital lobe was dissected according to gyral and sulcal landmarks. The samples were from close to the posterior pole of the left hemisphere of human primary visual cortex, including both superior and inferior portions of the calcarine fissure. At the Brain and Tissue Bank the left hemisphere was sectioned coronally, rinsed with water, blotted dry, placed in a quick-freeze bath (dry ice and isopentane), and stored frozen (-70°C). Samples were from 28 individuals ranging in age from 20 days to 79 years of age (Table 1). The individuals had no history of neurological or mental health disorders.

Macaque monkey tissue samples were obtained from the Alzheimer's Research Center at the Medical College of Georgia (Augusta, GA). Samples were from 8 macaque monkeys, ranging in age from 4 to 33 years old (Table 2). The right hemisphere was removed, cut into thick coronal sections, fresh frozen, and stored at -80°C. The sections used were from the posterior pole of the hemisphere including primary visual cortex (V1) and extrastriate areas V3a and V4v.

Cat tissue samples were obtained from 7 animals, ranging in age from 2.5 to 11 years old (Table 3). Samples were collected from primary visual cortex (V1), frontal cortex, and auditory cortex (A1). Cats were euthanized with Euthanol (165mg/kg), and transcardially perfused with cold 0.1M PBS (4˚C; 80-100 ml/min) until the circulating fluid was cleared. The brain was quickly removed from the skull and immersed in cold PBS. To facilitate quantification of

Table 1. Human tissue samples. The age and postmortem interval for each of the human cortical tissue samples. The star (★) indicates that a sample was obtained from that visual cortical area.

Human Age	Postmortem Interval (hours)	Sex	Cause of death	V1	V3	V4
20 days	14	F	pneumonia	★		
86 days	23	F	not known	★		
96 days	12	M	bronchopneumonia	★		
98 days	16	M	cardiovascular disorder	★		
119 days	22	M	bronchopneumonia	★		
120 days	23	M	pneumonia	★		
133 days	16	M	accidental	★		
136 days	11	F	pneumonia	★		
273 days	10	M	sudden infant death syndrome	★		
1.34 years	21	M	dehydration	★		
2.16 years	21	F	cardiovascular disorder	★		
3.34 years	11	F	accidental	★		
4.56 years	15	M	accidental	★		
5.39 years	17	M	accidental	★		
8.14 years	20	F	asphyxia	★	★	★
8.59 years	20	F	surgical complications	★	★	★
9.13 years	20	F	asthma	★	★	★
12.45 years	22	M	cardiovascular disorder	★	★	★
13.27 years	5	M	asphyxia	★	★	★
15.22 years	16	M	multiple injuries	★	★	★
19.21 years	16	F	multiple injuries	★	★	★
22.98 years	4	M	multiple injuries	★	★	★
32.61 years	13	M	cardiovascular disorder	★	★	★
50.43 years	8	M	cardiovascular disorder	★		★
53.90 years	5	F	cardiovascular disorder	★	★	★
69.30 years	12	M	cardiovascular disorder	★	★	★
71.91 years	9	F	multiple medical disorders	★	★	★
79.50 years	14	F	drug overdose	★	★	★

Table 2. Monkey Tissue Samples. The age and cortical area of the tissue samples. The star (★) indicates that a sample was obtained from that visual cortical area.

Monkey **Age**	**V1**	**V3a**	**V4v**
4	★	★	★
13	★		★
20	★	★	★
25	★	★	★
25	★	★	★
27.5	★	★	★
30	★	★	★
33	★	★	★

Table 3. Cat Tissue Samples. The age and cortical area of the tissue samples. The star (★) indicates that a sample was obtained from that cortical area.

Cat **Age**	**V1**	**A1**	**Frontal**
2.5	★	★	★
2.5	★	★	★
7.3	★	★	★
9	★	★	★
10	★	★	★
11	★	★	★
11	★	★	★

regional differences within V1, the area was divided into a series of small tissue samples (approx. 2 X 2mm) covering the central, peripheral, and monocular visual field representations (Tusa et al., 1978). For each cat, 2 samples of both central and peripheral and 1 from the monocular visual field representation were used. Each cortical tissue sample was rapidly frozen on dry ice and stored at -80˚C. All experimental procedures were approved by the McMaster University Animal Research Ethics Board and the McMaster Human Research Ethics Board.

Tissue Sample Preparation

Pieces of tissue (50-100 mg) were cut from the coronal sections of human and macaque monkey cortex using the gyral and sulcal pattern to identify primary visual cortex (V1), and presumptive areas V3a and V4v. For the cat the whole 2X2mm pieces of tissue samples were used to prepare the samples for Western blotting. Samples were suspended in cold homogenization buffer (1 ml buffer:50 mg tissue, 0.5m*M* DTT, 1m*M* EDTA, 2 m*M* EGTA, 10 m*M* HEPES, 10mg/L leupeptin, 100n*M* microcystin, 0.1 m*M* PMSF, 50 mg/L soybean trypsin inhibitor) and homogenized using a glass-glass Dounce tissue homogenizer. A portion of the homogenate was removed, the remainder was then subjected to a subcellular fractionation procedure in order to enrich for synaptic proteins. Remaining homogenate was taken up with a 3ml syringe, where it was passed through a 5μm pore hydrophillic filter (Millipore, Billerica, MA). The filtered product was then centrifuged for 10 minutes at x1000g. Supernatant was removed, leaving a pellet enriched for synaptic proteins, the synaptoneurosome. The pellet, homogenate, and supernatant were then re-suspended in 100μl of 1% boiling SDS and then stored at -20 degrees C. Protein concentrations were determined using bicinchoninic acid

(BCA) assay guidelines (Pierce, Rockford, IL). Samples from cat were synaptoneurosome preparations, while human and macaque samples were homogenate preparations.

Immunoblotting

Samples (25 μg) were separated on polyacrylamide (SDS-PAGE) mini-gels (Precise Protein Gels) (Pierce Biotechnology Inc, Rockford, IL) and transferred to polyvinylidene difluoride (PVDF-FL) (Millipore, Billerica, MA) membranes. Each sample was run twice. Blots were pre-incubated in blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) for 1 hour (Li-cor Biosciences; Lincoln, NE), after which blots were incubated in primary antibody overnight at 4°C using the following concentrations: Ube3A (E6AP), 1:1000; (Bethyl Laboratories, Montgomery, TX), Synapsin; 1:8000 (Invitrogen, Carlsbad, CA), Synaptophysin; 1:2000 (Sigma, St.Louis, MO), PSD95; 1:32,000 (Chemicon International, Temecula, CA), Gephyrin; 1:2000 (Imgenex, San Diego, CA), GAPDH; 1:4000 (Imgenex, San Diego, CA). The blots were washed with phosphate buffer saline containing 0.05% Tween (Sigma, St. Louis, MO) (PBS-T) (3 x 10 min), incubated (1 hour, room temperature) with the appropriate IRDye labeled secondary antibody, (Anti-Mouse, 1:8000, Anti-Rabbit, 1:10,000) (Li-cor Biosciences; Lincoln, NE), and washed in PBS-T (3 x 10 min). The blots were visualized using the Odyssey scanner (Li-cor Biosciences; Lincoln, NE). The IRDye secondary antibodies and Odyssey scanner system have a wide linear dynamic range so that strong and weak bands could be accurately quantified on the same blot. The blots were stripped and prepared to be reprobed with additional antibodies (Blot Restore Membrane Rejuvenation kit, Chemicon International, Temecula, CA).

Analysis

To analyze the expression levels we scanned the blots (Odyssey Infrared Scanner) and quantified the bands using densitometry (Licor Odyssey Software version 3.0; Li-cor

Biosciences; Lincoln, NE). Density profiles were determined by performing a subtraction of the background, integrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in band size. GAPDH normalization was used as the loading control and for each sample expression of the synaptic proteins was divided by GAPDH expression. We verified that GAPDH expression was not correlated with age for any of the groups (all p-values were > 0.15) and thus was an appropriate loading control for this study. A control sample (a mixture of all the samples) was run on all of the gels and the density of each sample was measured relative to that control (sample density / control density).

We plotted scattergrams of Ube3A expression that included both the average expression level for each case and every point from all runs. To facilitate comparing the pattern of changes the results were normalized to the average for young adults. For humans, the young adult average was calculated from the 19, 22, and 32 year old samples. For cats, the 2.5 year old samples, and for macaque monkeys, the 4 year old samples. To help describe the pattern of change in expression across the lifespan a weighted average curve was fit to Ube3A expression using the locally weighted least squares method (dotted lines). In order to quantify changes in Ube3A expression relative to a collection of other synaptic proteins, we calculated an index of Ube3A to the average of 4 other synaptic proteins ((Ube3A-Other)/(Ube3A+Other)). The other synaptic proteins were synapsin, synaptophysin, PSD-95, and gephyrin. Statistical comparisons of differences in Ube3A expression levels between young vs old adults (human: young -- 19-32 years, old -- 50-79 years; macaque monkey: young -- 4-13 years, old -- 20-33 years; cat: young -- 2.5 years, old -- 7-11 years) were calculated using Wilcoxon Mann Whitney and Kruskal–Wallis nonparametric analysis of variance.

3.3 Results

In this study, we characterized the expression of E3 ubiquitin ligase, Ube3A, in 3 different species: humans, macaque monkeys, and cats. These species are commonly used to study perceptual and neurobiological changes in the aging visual system. Using Western blot analysis, we quantified the developmental trajectory of Ube3A expression across the lifespan. We measured expression in primary visual cortex (V1), because it is has been shown that Ube3A is required for experience-dependent plasticity in visual cortex (Yashiro et al., 2009). In the cat, we took samples from different regions of V1, to examine whether Ube3A might play a role in the loss of peripheral vision that occurs in normal aging (Sekuler et al., 2000). In the monkey and human, we took additional samples from dorsal (V3/V3a) and ventral (V4) visual processing streams. In the cat, we took additional samples from auditory and frontal cortex to examine Ube3A expression in other sensory and non-sensory cortical areas. Finally, to understand the expression of Ube3A in the context of other synaptic proteins over the lifespan, we quantified the expression of synapsin, synaptophysin, gephyrin, and PSD-95.

The human tissue samples were collected over a range of postmortem intervals (4-23 hours), but we found no significant correlation between postmortem interval and expression of any of the proteins examined (all ps > 0.1): Ube3A (R= 0.23), GAPDH (R= 0.02), Synapsin (R= 0.22), Synaptophysin (R=0.04), Gephyrin (R= 0.29), and PSD-95 (R= 0.08).

Changes in Ube3A Expression in Primary Visual Cortex (V1)

In human primary visual cortex (V1), we measured expression of Ube3A across the lifespan using Western blot analysis. No previous studies have examined the normal pattern of Ube3A expression in human V1, although other studies have shown that the loss of Ube3A

expression during development leads to mental retardation (Angelman's Syndrome), and later disruptions in ubiquitin-mediated proteasomal degradation are linked with neurodegenerative disorders. We plotted Ube3A expression as a function of age (range from 20 days to 80 years), normalized relative to the expression level of young adults (19-32 years old) and fit with a locally weighted average curve (Fig. 1). The average Ube3A expression in human V1 was relatively constant in children, teenagers, and young adults, but there was substantial inter-individual variability in Ube3A expression at these younger ages. After the young adult years (> mid 30s) there was a clear loss of Ube3A expression with aging; Ube3A expression in older adults (>50 years) fell to about 50% of the younger adult levels ($p < 0.01$). For the oldest case (79 years), the loss of Ube3A expression was substantial, with about 85% less expression relative to young adult levels. In addition, there was much less inter-individual variability in older adults.

In macaque monkey V1, we quantified changes in Ube3A, and compared expression levels in young and older adults. The Ube3A expression levels were plotted relative to young adult expression, and a locally weighted average curve was fit to the data (Fig. 2a). There was a significant loss (approximately 25% ± 5%) of Ube3A expression ($p<0.05$) in old monkeys (> 20 years), though, this loss was less than that found for human V1. The loss of Ube3A in aging monkey V1 occurred between 13 and 20 years of age when expression dropped by 25% and then remained at that level.

In cat V1, we quantified changes in Ube3A expression using multiple samples from different regions of V1, anticipating the possibility of regional differences related to the loss of peripheral vision in aging. Despite this expectation, we found no significant differences in

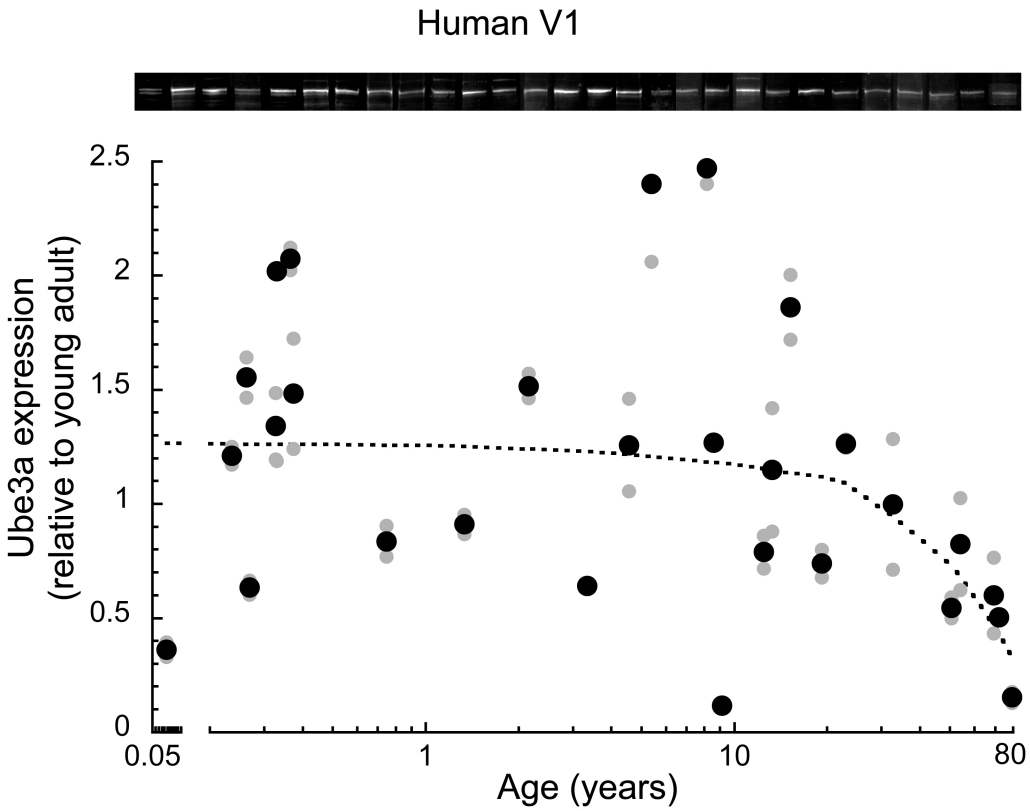

Figure 1. Ube3A expression level in human primary visual cortex across the lifespan. Expression levels are relative to young adults, and a weighted average was fit. Grey symbols indicate values from individual runs, black symbols represent the average between runs. Age is plotted on a logarithmic scale. Representative western blots are pictured above. Expression was relatively constant through childhood to teenage to young adult levels. There was a clear loss of Ube3A in aging when expression fell nearly 50% from young adult levels ($p<0.01$). In the oldest case (79 years) expression fell approximately 85%.

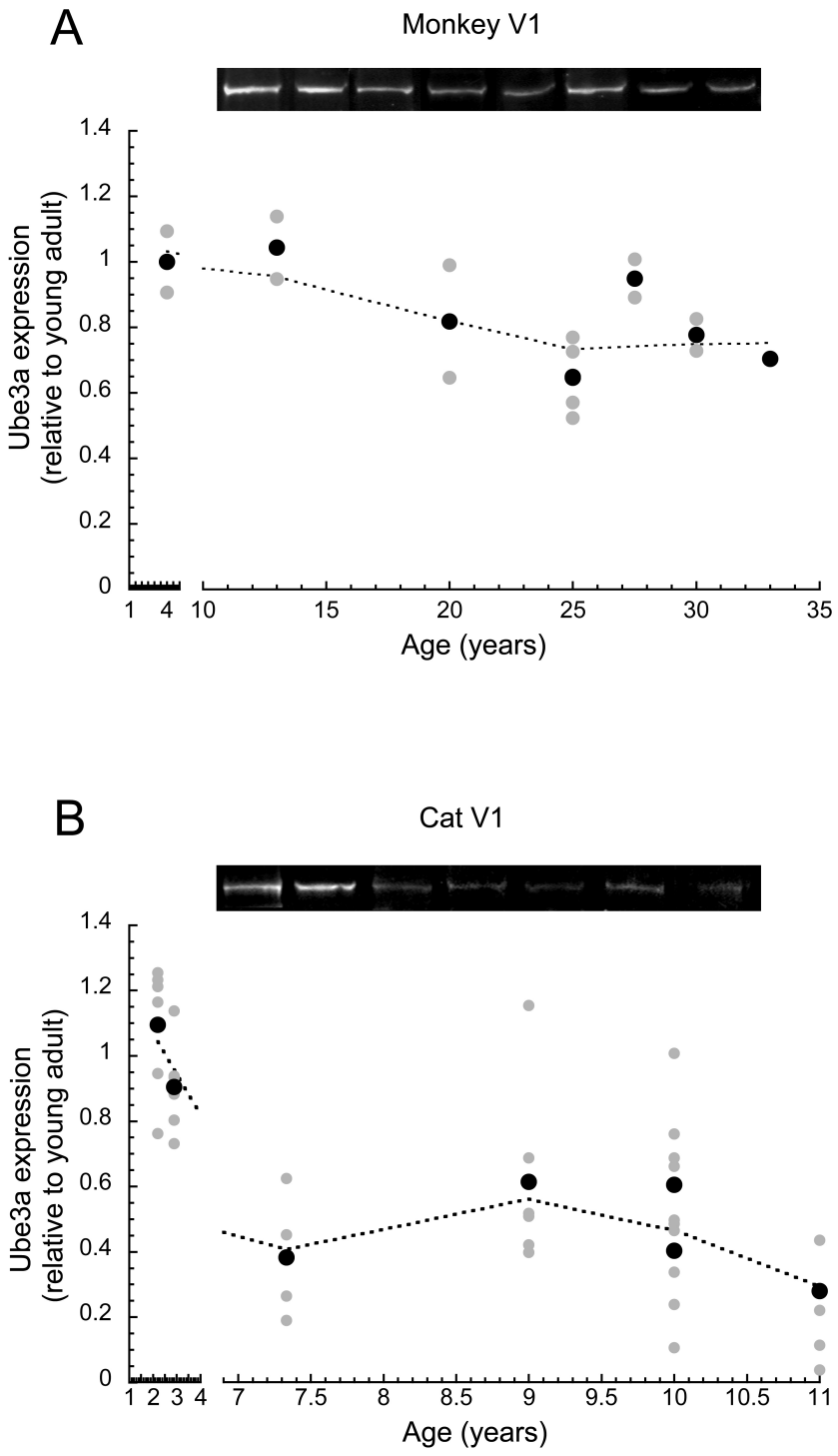

Figure 2. Ube3A expression level in primary visual cortex across the lifespan of cat and macaque monkey. Expression levels are relative to young adults, and a weighted average was fit. Grey symbols indicate values from individual runs, black symbols represent the average between runs. Representative western blots are pictured above. In monkey, expression fell approximately 25% ($p<0.05$) in old monkeys (>20 yrs). This occurred between 13 and 20 years of age. (A). In cat, expression fell approximately 45% during aging ($p<0.001$). This occurred between 2.5 and 7 years of age (B).

Ube3A expression at any age between samples from parts of V1 that represent the central, peripheral, or monocular visual field ($p>0.1$). Therefore, Ube3A expression from all regions of cat V1 were combined, normalized relative to the young adult cat level (2.5 years), and fit with a locally weighted average curve (Fig. 2b). We found a substantial loss of Ube3A expression after 2.5 years of age ($p<0.001$). In cat V1, Ube3A expression levels fell an average of 45% (±7%) during aging. The magnitude of the age-related loss of Ube3A expression in cat V1 was greater than monkeys and comparable to the loss found in older human V1.

Changes in Ube3A Expression in Extrastriate areas V3 and V4

To examine whether there were age-related losses of Ube3A expression in extrastriate visual areas we quantified expression in areas V3 and V4v of macaque monkey and human cortex. These areas represent early stages in the dorsal (V3) and ventral visual streams (V4v). The Ube3A expression levels were plotted relative to young adult expression, and a weighted average was fit to the data (Fig. 3).

In monkey V3, there was no significant difference in Ube3A expression between the young (4 year old) and older monkeys (>20 years). Closer inspection of the results (Fig. 3a) showed that one older monkey (30 years) had no loss, but the expression levels for the other 5 older monkeys had declined by about 50% ($p<0.06$). In monkey V4, Ube3A expression declined steadily during aging (Fig 3a) falling an average of 55% ($p<0.05$). The loss of Ube3A in monkey V4 was about twice as large as the loss in V1, and it occurred by 13 years of age, which was earlier than the onset of the loss in monkey V1.

The analysis of Ube3A expression in human V3 and V4 included samples covering ages from 8 to 80 years of age. The Ube3A expression levels in both V3 and V4 were relatively

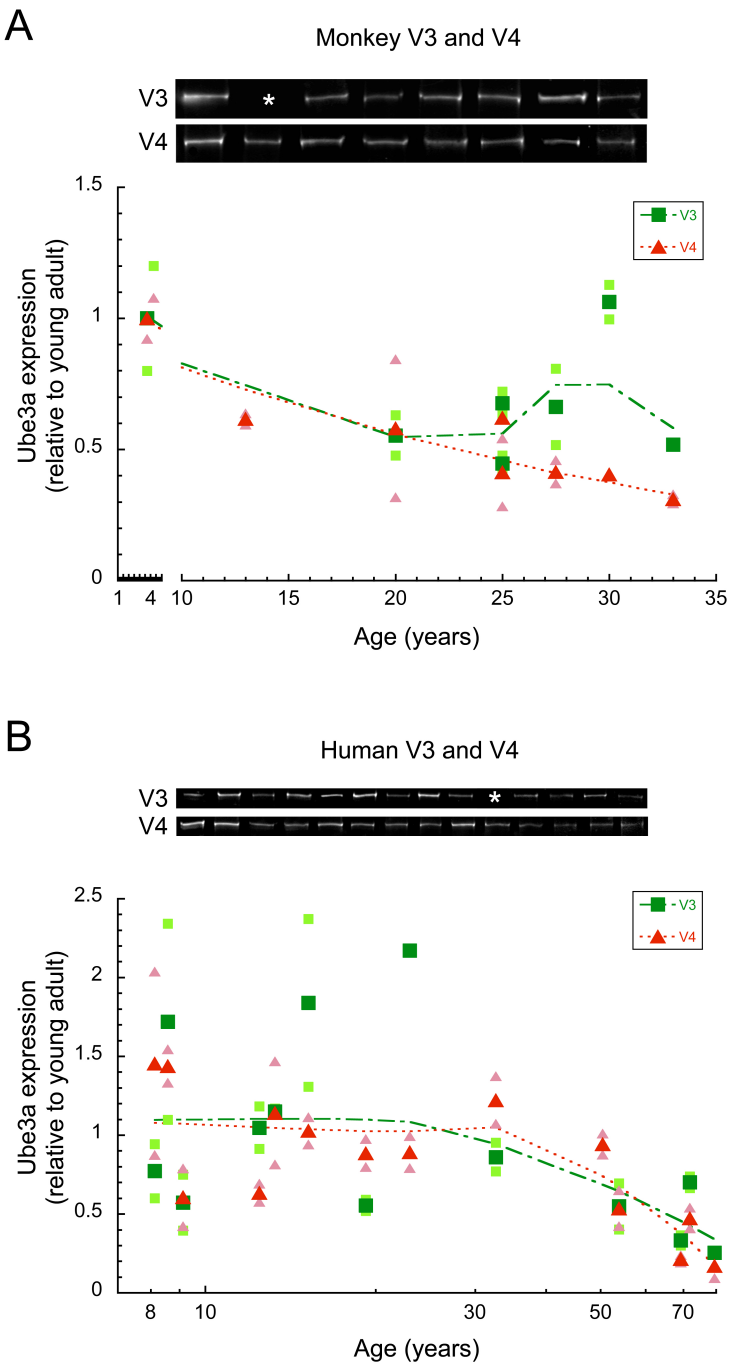

Figure 3. Ube3A expression in V3 and V4 of human and macaque monkey. Expression levels are relative to young adults, and a weighted average was fit. The larger green squares represent V3 and the larger red triangles represent V4. Individual runs are represented in the corresponding lighter shades. Age is plotted on a logarithmic scale for human V3,V4. Representative western blots are pictured above. White asterisks indicate that no sample was acquired for that age. In monkey V3, there was no significant difference between young (4 yr old) and older monkeys (<20 yrs). In monkey V4, Ube3A expression steadily declined during aging falling on average by 55% in older monkeys ($p<0.05$) (A). In human, Ube3A expression in both V3 and V4 remained relatively constant through childhood to teenage to young adult. There was a steady loss into aging ($p<0.05$). (B).

constant through childhood, teenage, young adults years (Fig. 3b). Similar to the expression pattern in V1, there was substantial inter-individual variability in Ube3A expression in areas V3 and V4 for these younger cases. After the young adult years there was a steady loss of Ube3A expression in both V3 ($p<0.05$) and V4 ($p<0.01$) of human cortex (Fig. 3b) and less inter-individual variability. When compared to the young adults, the Ube3A expression in V3 and V4 of the oldest case (79 years) had fallen by 75-80%.

Ube3A Expression in Auditory and Frontal cortex

In cat auditory and frontal cortex, we quantified the expression of Ube3A in order to determine whether other sensory and non-sensory cortical areas had age-related losses similar to V1. Ube3A expression was plotted relative to young adult (2.5 years) expression, and a locally weighted average curve was fit to the data (Fig. 4). In auditory cortex, the loss of Ube3A began between 7.5 and 9 years of age, when it dropped about 60% (±8%) relative to young adult cat levels ($p<0.01$). In frontal cortex Ube3A expression had already declined by 7.5 years of age and continued to decline in the older cats. Expression of Ube3A in older cat frontal cortex was about 60% (± 10%) less than levels in young adults ($p<0.05$). In both frontal and auditory cortex, the magnitude of the loss was comparable to the loss in cat V1.

Ube3A Expression Relative to Other Synaptic Proteins

To address whether the age-related losses we observed were specific to Ube3A expression, or reflected a more general loss of synaptic proteins, we calculated an index of Ube3A expression relative to the expression of 4 other synaptic proteins (Synapsin, Synaptophysin, Gephyrin, PSD-95). For this index, positive values indicate relatively more Ube3A and negative values indicate less Ube3A relative to the other synaptic proteins.

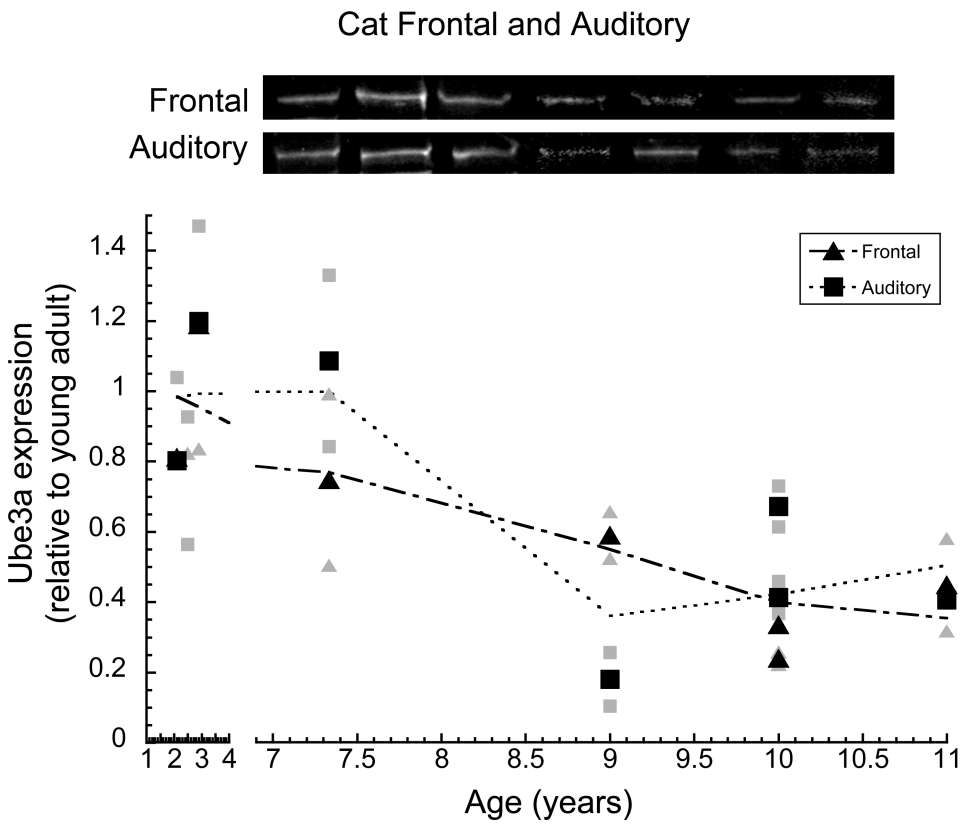

Figure 4. Ube3A expression in frontal and auditory cortex of cat. Expression levels are relative to young adults, and a weighted average was fit. The larger black triangles represent frontal cortex and the larger black squares represent auditory cortex. Individual runs are represented in the corresponding grey symbols. Representative western blots are pictured above. Expression in frontal cortex began to decline after 2.5 yrs of age and continued to decline in aging where it fell by approximately 60% ($p<0.05$). Expression in auditory cortex began to decline after 7.5 years where it fell by approximately 60% ($p<0.05$). Expression at age 2.5 for both auditory and frontal cortex was so similar the symbols completely overlap.

In monkey V1, there was no selective loss of Ube3A relative to the other synaptic proteins (Fig. 5a). The index remained close to 0, indicating that Ube3A expression and the other synaptic proteins were roughly balanced across younger and older monkeys. A similar pattern was observed for cat auditory and frontal cortex (Fig. 5b) with a slight trend towards less relative Ube3A expression in aging. A different pattern was found for cat V1, where at the oldest age (11 years) there was a significant loss of Ube3A relative to the other synaptic proteins ($p<0.05$), dropping by an average of 40% (Fig. 5c). This indicates a selective loss of Ube3A during aging of cat V1.

In human V1, there was a clear developmental change in the expression of Ube3A relative to other synaptic proteins (Fig. 6a). At the youngest ages (<1 year), there was more Ube3A; in children through to pre-teen years, Ube3A expression was roughly in balance with other synaptic proteins; in teens and young adults, relative Ube3A expressions dropped slightly, and then fell off substantially in aging ($p<0.0001$). A very similar pattern was found for human V3 and V4

(Fig. 6b). In teens and young adults, the expression of Ube3A was roughly balanced with expression of other synaptic proteins and then there was a shift to relatively less Ube3A expression in older human V3 and V4 ($p<0.01$). These results show a selective loss of Ube3A in human visual areas during aging.

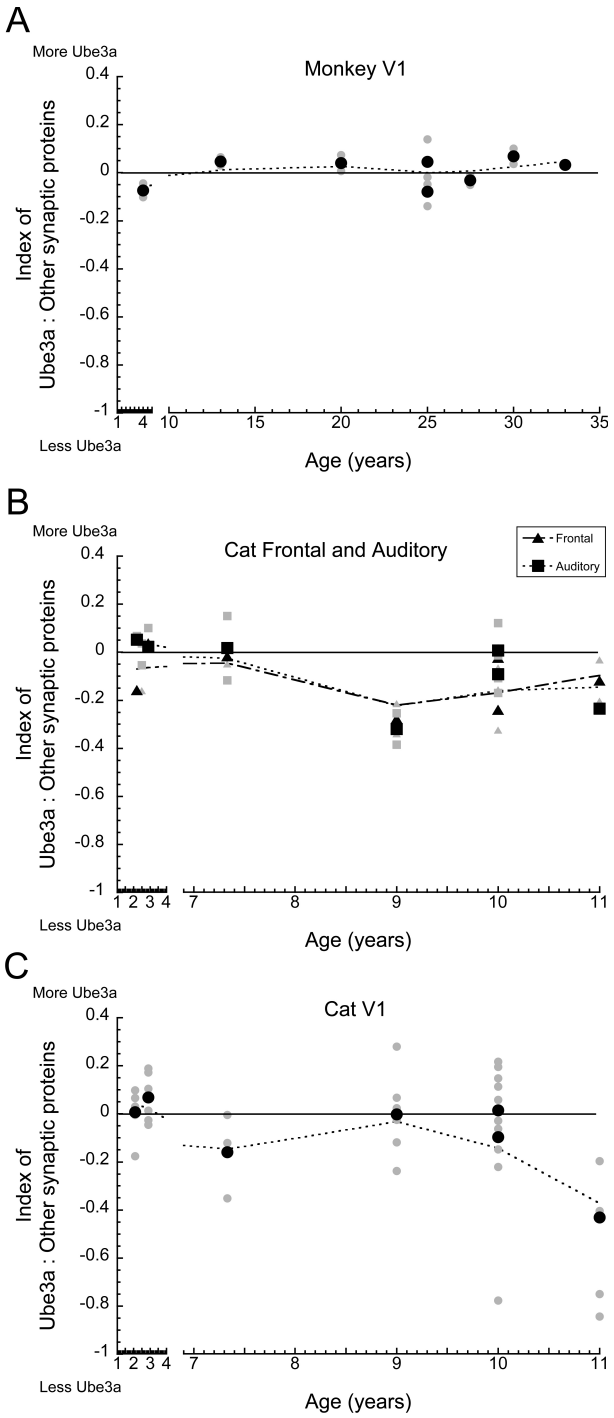

Figure 5. Index between Ube3A and other synaptic proteins (synapsin, gephyrin, PSD-95) for macaque monkey V1, cat frontal and auditory cortex, and V1. Positive values indicate relatively more Ube3A expression, negative values indicate relatively less Ube3A expression. Grey symbols indicate values from individual runs, black symbols represent the average between runs. A weighted average was fit to the data. In monkey V1, there was no selective loss in Ube3A (A). Both frontal and auditory cortex showed a slight trend towards relatively less Ube3A expression with age (B). In V1, there was a significant loss of Ube3A in the oldest cat (11 years of age) relative to other synaptic proteins, dropping on average 40% ($p<0.05$) (C).

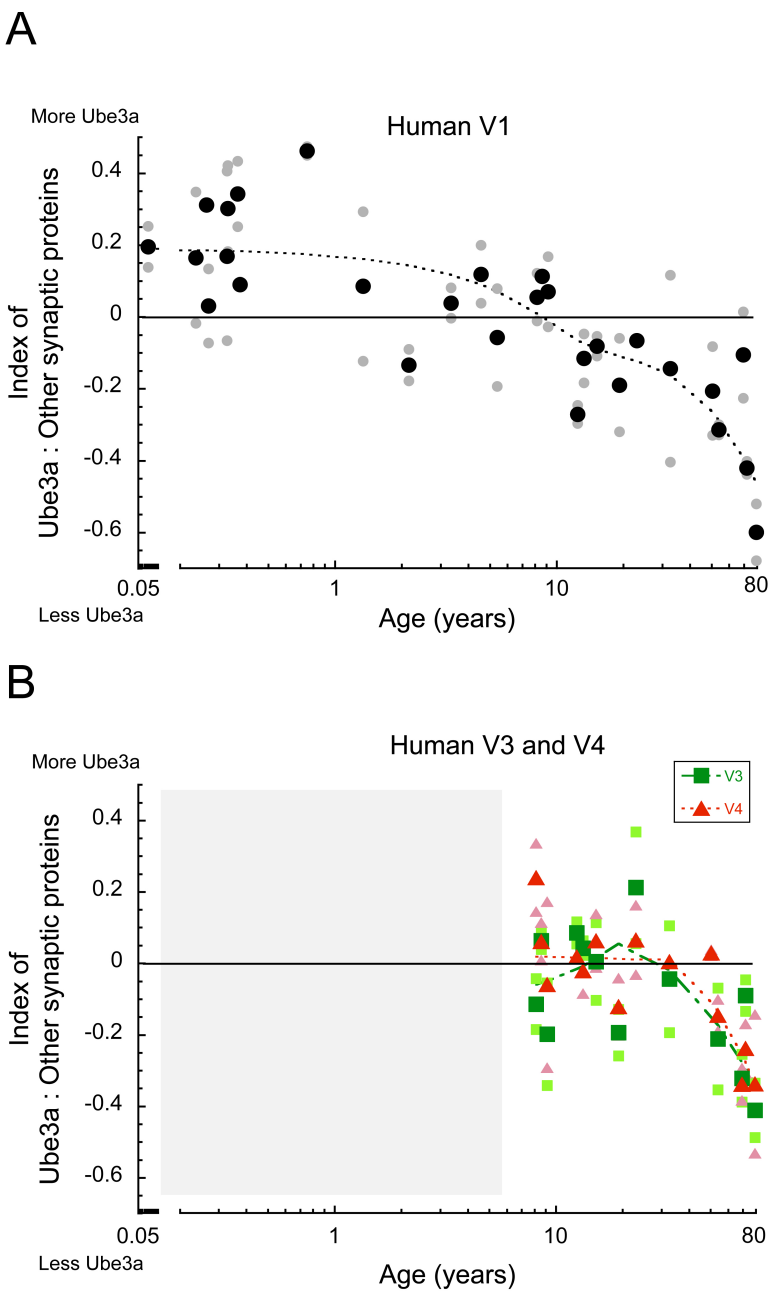

Figure 6. Index between Ube3A and other synaptic proteins (synapsin, gephyrin, PSD95, synaptophysin) in human V1,V3,V4. Positive values indicate relatively more Ube3A expression, negative values indicate relatively less Ube3A expression. The larger green squares represent V3 and the larger red triangles represent V4. Individual runs are represented in the corresponding lighter shades. A weighted average was fit to the data. Age is plotted on a logarithmic scale. The shaded area represented ages we do not have data for. In human V1, at the youngest ages there was more Ube3A, in children to pre-teens the expression was balanced, in teens to young adults it shifts in favour of less Ube3A, and falls off in aging (> 50 years old) ($p<0.0001$) (A). In V3,V4 expression was balanced in teens and young adults, and fell to less Ube3A in older humans (> 50 years old) ($p<0.01$) (B).

3.4 Discussion

Our study found that Ube3A expression declines across the lifespan in human, monkey, and cat cortex. This occurred in visual areas V1, V3, and V4, as well as frontal and auditory cortex. Moreover, in human areas V1, V3, and V4 there was a much greater loss of Ube3A relative to other synaptic proteins. This is the first study to address how the E3 ubiquitin ligase, Ube3A, changes in healthy aging. Our findings identify a fundamental change in Ube3A expression in the aging cortex and give new insights into age-related changes that lead to the loss of synaptic plasticity.

Ube3A loss: similarities and differences

We have identified many similarities, and a key difference, in age-related changes of Ube3A expression among the cortical areas, animal models, and human cortex. The large number of samples for human V1, ranging from 20 days to 80 years of age, give a comprehensive view of the trajectory of Ube3A expression in human cortex. There was a clear loss of Ube3A expression with aging in all 3 species and in all cortical areas. A common notion is that the whole synaptic proteome goes down during aging. In the animals models, much of the loss can be explained by a general loss of synaptic proteins. In human cortex, however, there was a selective loss of Ube3A. For human visual cortical areas, the loss of Ube3A was substantially greater than the loss of other synaptic proteins. These results are suggestive of a general cortical pattern of synaptic change that may help to explain age-related functional losses in learning and memory, as well as other sensory systems. A diffuse loss of Ube3A across the human cortex may underlie a common mechanism affecting synaptic plasticity and mediating

age-related functional losses. The loss of Ube3A during normal aging may lead to rigid synapses similar to Ube3A KO mice that lack synaptic plasticity (Yashiro et al., 2009).

A richer picture of the aging synapse is emerging from recent studies examining changes in synaptic proteins. The pattern of change is one of selective losses rather than a general decline in synaptic proteins. For example, there are selective pre- and post-synaptic changes in GABAergic signaling mechanisms during aging that will affect the functioning of cortical inhibition (Pinto et al., 2010, submitted). These selective changes at aging synapses have specific implications for dynamic synaptic function, as the relative balance between proteins changes and reduces experience-dependent plasticity.

Ube3A loss reduces experience-dependent plasticity

Although Ube3A is required for experience-dependent plasticity during development of the visual cortex (Yashiro et al., 2009), the precise role that Ube3A plays is unclear. Ube3A knockout mice have deficits in LTP, despite normal baseline neurotransmission, and they display deficits in context dependent learning (Jiang et al., 1998). In addition, the development of dendritic spines is abnormal with fewer spines and abnormal spine morphologies in Ube3A knockout mice (Dindot et al., 2008). Together, these results point to a role for Ube3A in local regulation of synaptic plasticity. An important insight into the function of Ube3A comes from studying ocular dominance plasticity in the Ube3A knockout mice (Yashiro et al., 2009). They lack ocular dominance plasticity when reared in the light, but when dark reared and then brought into the light, these animals show the typical shift of ocular dominance in response to closing one eye. This experience-dependent plasticity, however, is lost after just a few days in the light and once again the synapses become rigid, unable to adapt to experience (Yashiro et al., 2009).

Simply using the synapses depletes them of plasticity. This leaves neural connections in the cortex rigid, unable to be fine tuned and to develop normal function. Ube3A mediates plasticity at excitatory synapses by controlling the degradation of Arc which effects the internalization of AMPA receptors. Thus, disrupting Ube3A function causes an increase in Arc expression leading to greater internalization of AMPA receptors and loss of AMPA receptor at excitatory synapses (Greer et al., 2010). Perhaps the rigid synapses found in the Ube3A knockout mice are the key to linking the loss of Ube3A expression during normal aging of human cortex with the rigid behaviors that often accompany aging.

Visual changes in aging

The time course of loss in Ube3A expression is similar to the decline of visual abilities in aging. There are a wide range of visual deficits in aging which suggests that widespread neural changes occur across the central visual pathway. Certain visual perceptions, however, are more vulnerable to the effects of aging (Faubert, 2002; Billino et al., 2008). Specifically, the processing of complex visual stimuli declines more than the perception of simple visual stimuli, suggesting that complex neural integration may be more affected by aging (Habak & Faubert, 2000). For example, the perception of a moving stimulus that is surrounded by other moving stimuli is particularly affected in aging (Betts et al., 2005), raising the possibility that aging affects dynamic neural processing in the visual system. In this framework, the loss of Ube3A during aging may reduce the capacity for experience driven dynamic synaptic changes that are necessary to integrate and adapt physiological response in the visual cortex (Fu et al., 2002).

Links between Ube3A and GABAergic changes in aging

The most common explanation for age-related changes in visual perceptual is a loss of GABA-mediated inhibition (Betts et al., 2005). This idea comes from studies showing that the

direct application of GABA onto cortical neurons improves the neurophysiological responses of individual V1 neurons in senescent monkeys (Leventhal et al., 2003). Additionally, the loss of GABA-mediated inhibition in aging is further supported by a loss of GABAergic neurons in visual cortex of older cats (Hua et al., 2008), and a loss of specific synaptic components of GABAergic signaling during aging of human visual cortex (Pinto et al., 2010, submitted). The current findings of a loss of Ube3A, broadens our understanding of the age-related changes in visual cortex by showing synaptic compromises beyond changes in GABAergic signaling mechanisms.

It seems likely that changes at the aging synapse are as complex as those during development. The challenge for understanding aging visual perception will be to determine if particular perceptual losses are linked with specific changes in visual cortical synapses. There are interesting parallels in the development of experience-dependent plasticity between mice lacking GAD65 or Ube3A. Mice lacking GAD65, the enzyme that synthesizes the on-demand pool of GABA, have reduced inhibitory transmission and are insensitive to monocular deprivation (Hensch et al., 1998). But ocular dominance plasticity is re-established in GAD65 knockout mice, at any age, by infusion of diazepam into the visual cortex to increase GABA-mediated activity. Similarly, Ube3A knockout mice fail to express ocular dominance plasticity in development, but this plasticity can be restored by dark rearing (Yashiro et al., 2009). Perhaps future studies that compare the similarities and differences in experience-dependent plasticity in these mouse models will help to inform our understanding of the functional changes in aging synapses.

What is the nature of Ube3A loss in aging?

While the rigid synaptic plasticity found in Ube3A knockout mice provides a promising model for understanding experience-dependent changes in aging synapses there is an important difference to consider. Plasticity is preserved in Ube3A mice by *restricting* experience. At first glance, this seems counter to our current thinking that the best way to preserve brain function during aging is to engage in activities to keep the mind active (Fratiglioni et al., 2004). Clearly, the age-related loss in Ube3A must be more complex than simply losing Ube3A at all synapses. An intriguing possibility is that age-related Ube3A loss is synapse-specific, and it is lost at some synapses and retained at others. This would lead to a situation where some synapses have reduced capacity for experience-dependent plasticity and become rigid, while others maintain a normal level of plasticity. The changes could be widespread and random, or particular circuits may be more vulnerable to the loss of Ube3A. If the latter is true, then insights into what makes a particular circuit or synapse more likely to lose Ube3A becomes a new and important question for understanding healthy aging. This is particularly interesting in light of a recent study in *C. elegans* showing that an E3 ligase homologous to Ube3A is necessary for caloric restriction to extend longevity (Carrano et al., 2009).

The current results raise questions about what drives the loss of Ube3A in the cortex during aging. It will be important to address this while considering strategies to prevent the loss or increase expression of Ube3A in the aging cortex. The loss may reflect a generalized down-regulation of cellular function and the ubiquitination machinery during aging. It seems likely that this will account for some of the loss but probably not all of it since we found a much greater loss of Ube3A relative to other synaptic proteins in human cortex. Another possibility is that

age-related degradation of sensory and/or cognitive functions drives an experience-dependent loss of Ube3A. Two recent studies provide some insights about the impact of experience on Ube3A expression. One study found that enriched experience in adult mice increases Ube3A expression in the hippocampus (Greer et al., 2010) while the other study showed that early monocular deprivation does not lead to a loss of Ube3A (Sato & Stryker, 2010). These contrasting results indicate that more work is needed to determine the full scope of experience-dependent changes in Ube3A expression in the brain. One possibility is that the role of experience in regulating Ube3A expression changes across the lifespan so that it has greater impact in the adult brain.

The progressive loss of Ube3A expression during cortical aging is an important new finding for understanding age-related losses in experience-dependent synaptic plasticity. Furthermore, the selective loss of Ube3A in human cortex highlights a particular vulnerability in human brain aging that may signify a shift in cortical function and selective loss of synaptic plasticity.

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Chapter 4. Loss of Glutamatergic and GABAergic Synaptic Proteins in Visual Cortex During Aging

Abstract

Visual perception declines across the lifespan. Age-related deficits include poor acuity, contrast sensitivity, and motion perception. Neurobiological studies are beginning to link these functional changes in vision with changes in the physiological properties of neurons in primary visual cortex. The prevailing view is that reduced GABAergic inhibition underlies these changes. Glutamatergic activity, however, plays a key role shaping receptive field properties of neurons in visual cortex. Furthermore, activation of the AMPA and NMDA glutamate receptors mediates much of the feedforward and feedback visual processing mechanisms in visual cortex that underlie visual perception. In a previous study we showed an age-related decline in the ubiquitin ligase, Ube3A, in aging cat visual cortex. Ube3A regulates expression of AMPARs at synapses, where reduced Ube3A leads to reduced AMPAR expression. Taken together, this led us to ask whether normal aging is characterized by a loss of glutamate receptors. We used Western blot analysis to quantify the expression of a group of pre-and post-synaptic proteins at glutamatergic (GluA2, GluN1, PSD95, VGLUT1) and GABAergic ($GABA_A$ α1, $GABA_A$ α3, Gephyrin, VGAT, GAD65, GAD67) synapses across the lifespan of cat visual cortex. Using samples taken from the region of V1 representing the central, peripheral, and monocular visual field, we found substantial losses in almost all glutamatergic and GABAergic synaptic proteins. Furthermore, the losses were widespread across all regions of visual cortex. These findings provide new insight into the neural basis of vision loss during aging.

4.1 Introduction

Vision declines during normal, healthy aging (Faubert, 2002; Owsley, 2011; Andersen, 2012). The wide range of age-related visual deficits includes those limited by processing in the central visual field such as reduced visual acuity (Wood & Bullimore, 1995; Li et al., 2000) and contrast sensitivity (Owsley et al., 1983; Wood & Bullimore, 1995; Del Viva & Agostini, 2007), but also include deficits in motion perception (Bennett et al., 2007), peripheral acuity (Collins et al., 1989) and complex visual tasks such as feature tracking (Kennedy et al., 2009; Allard et al., 2013). Most of these deficits occur despite normal retinal and subcortical processing, indicative that changes in visual cortex (V1) underlie age-related decline in vision. Furthermore, the range of visual deficits suggests there are widespread changes in aging V1.

Animal models have provided insight into the neurobiological changes in V1 that occur during normal, healthy aging. Neurons in V1 of old cats and macaques show degradation of neuronal properties such as reduced contrast sensitivity (Hua et al., 2006; Zhou et al., 2011), spatial frequency tuning (Zhang et al., 2008; Hua et al., 2011), and orientation and direction selectivity (Schmolesky et al., 2000; Fu et al., 2013). These physiological changes have primarily been linked with reduced GABAergic inhibition (Leventhal et al., 2003), which is further supported by a loss of GABAergic neurons in aging cat V1 (Hua et al., 2008) and reduced expression of GABAergic synaptic proteins in aging human V1 (Pinto et al., 2010).

Age-related changes in V1, however, are not restricted to the GABAergic system. We previously showed an age-related loss of the ubiquitin ligase, Ube3A, in V1 of cats, macaques, and humans (Williams et al., 2010). Ube3A regulates the insertion of the AMPA glutamate receptor, such that when Ube3A is reduced, AMPARs become over-internalized, resulting in loss

of synaptic AMPARs, and reduced excitatory transmission (Greer et al., 2010). Taken together, the loss of Ube3A we previously observed in aging may drive a loss of AMPARs. Importantly, recent studies have shown that AMPA and NMDA glutamate receptors mediate much of the visual processing mechanisms that underlie visual perception (Self et al., 2012). AMPARs convey the majority of feedforward activity, which underlies the main stimulus driven responses in V1, while NMDARs mediate feedback or recurrent activity, which modulates activity in V1 necessary for complex visual processing. Thus, glutamate receptors play a key role mediating visual perception.

To better understand the full range of age-related changes in components of the glutamatergic and GABAergic system, we quantified the expression of a group of pre- and post-synaptic proteins found at glutamatergic (GluA2, GluN1, PSD-95, VGLUT) and GABAergic synapses ($GABA_A$ α1, $GABA_A$ α3, Gephyrin, VGAT, GAD65, GAD67) in V1 of young and old cats. We used cats as an animal model because of their well developed central visual field, which allowed us to quantify changes in the regions of V1 representing the central, peripheral, and monocular visual field to determine if there is regional vulnerability in aging. Overall, we found a significant age-related loss of all glutamatergic, and most GABAergic proteins. These losses were widespread, as they were present across most regions of V1. Our study supports the current view that a loss of GABAergic inhibition occurs in aging, but also extends it by showing losses in the glutamatergic system. These findings provide new insight into the neural basis of vision loss during aging.

4.2 Methods

Tissue Collection

Tissue samples were collected from primary visual cortex (V1) of 7 cats (age range: 2.5-11 years old). Cats were euthanized with Euthanol (165mg/kg), and transcardially perfused with cold 0.1M phosphate buffered saline (PBS) (4˚C; 80-100 ml/min) until circulating fluid was cleared. The brain was quickly removed from the skull and immersed in cold PBS. To facilitate quantification of regional differences within V1, the area was divided into a series of small tissue samples (approx. 2 X 2mm) representing the center of vision (<5˚, n=2)), the visual periphery (~10˚-~50˚, n=2-3), and the monocular field of vision (>60˚, n=1) (Tusa et al., 1978). For each cat, 2 samples of both central and peripheral, and 1 sample from the regions were used. Each cortical tissue sample was rapidly frozen on dry ice and stored at -80˚C. All experimental procedures were approved by the McMaster University Animal Research Ethics Board.

Tissue Sample Preparation

Frozen tissue samples were suspended in cold homogenization buffer (1 ml buffer:50 mg tissue, 0.5m*M* DTT, 1m*M* EDTA, 2 m*M* EGTA, 10 m*M* HEPES, 10mg/L leupeptin, 100n*M* microcystin, 0.1 m*M* PMSF, 50 mg/L soybean trypsin inhibitor) and homogenized using a glass-glass Dounce tissue homogenizer. A subcellular fractionation procedure (synaptoneurosomes) (Hollingsworth et al., 1985; Quinlan et al., 1999) was used to obtain protein samples that were enriched for synaptic proteins. The homogenate was passed through a 5um pore hydrophillic filter (Millipore, Billerica, MA) and then centrifuged at 4°C for 10 minutes at x 1000g to obtain the synaptic fraction of the membrane. The supernatant was removed, leaving a pellet enriched for synaptic proteins, the synaptoneurosome. The synaptic pellet was re-suspended in boiling

1% sodium-dodecyl-sulfate (SDS) and stored at -80°C. Protein concentrations were determined using bicinchoninic acid (BCA) assay guidelines (Pierce, Rockford, IL.)

Immunoblotting

Samples (25 μg) were separated on polyacrylamide (SDS-PAGE) mini-gels (Precise Protein Gels) (Pierce Biotechnology Inc, Rockford, IL) and transferred to polyvinylidene difluoride (PVDF-FL) (Millipore, Billerica, MA) membranes. Each sample was run twice. Blots were pre-incubated in blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) for 1 hour (Li-cor Biosciences; Lincoln, NE), after which blots were incubated in primary antibody overnight at 4°C using the following concentrations: PSD-95; 1:32000 (Chemicon International, Temecula, CA), GluN1; 1:8000 (BD Pharmingen; Mississauga, ON), GluA2; 1:2000 (Invitrogen, Carlsbad, CA), VGLUT1; 1:1000 (Synaptic Systems, Goettingen, Germany), Gephyrin; 1:2000 (Imgenex, San Diego, CA), GAD65; 1:1000 (Chemicon International, Temecula, CA), GAD67; 1:1000 (Imgenex, San Diego, CA), $GABA_A$ α1; 1:1000 (Imgenex, San Diego, CA), $GABA_A$ α3; 1:1000 (Imgenex, San Diego, CA), VGAT; 1:1000 (Synaptic Systems, Goettingen, Germany), GAPDH; 1:4000 (Imgenex, San Diego, CA). The blots were washed with PBS containing 0.05% Tween (Sigma, St. Louis, MO) (PBS-T) (3 x 10 min), incubated (1 hour, room temperature) with the appropriate IRDye labeled secondary antibody, (Anti-Mouse, 1:8000, Anti-Rabbit, 1:10,000) (Li-cor Biosciences; Lincoln, NE), and washed in PBS-T (3 x 10 min). The blots were visualized using the Odyssey scanner (Li-cor Biosciences; Lincoln, NE). The IRDye secondary antibodies and Odyssey scanner system have a wide linear dynamic range so that strong and weak bands could be accurately quantified on the same blot. The blots were stripped

and prepared to be reprobed with additional antibodies (Blot Restore Membrane Rejuvenation kit, Chemicon International, Temecula, CA).

Analysis

To analyze the expression levels we scanned the blots (Odyssey Infrared Scanner) and quantified the bands using densitometry (Licor Odyssey Software version 3.0; Li-cor Biosciences; Lincoln, NE). Density profiles were determined by performing a subtraction of the background, integrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in band size. GAPDH normalization was used as the loading control and for each sample expression of the synaptic proteins was divided by GAPDH expression. We verified that GAPDH expression was not correlated with age for each region (Fig 1, all p values n.s) and thus was an appropriate loading control for this study. A control sample (a mixture of all the samples) was run on all of the gels and the density of each sample was measured relative to that control (sample density/control density). We made scatterplots showing all the results (grey symbols) and the average expression level for each cat (black symbols). To facilitate comparisons the results were normalized to the average expression of young cats (2.5 years old) for each region of V1. The changes in protein expression as function of age were best fit by linear functions using least squares analysis, and the goodness of fit (R), and the statistical significance of the fit (p value) were reported. We used ANCOVAs to determine if the slopes were significantly different between regions of V1. Finally, statistical comparisons in protein expression levels were made between the young (n=2, ages: both 2.5 years old) and the older group of cats (n=5, ages: 7-11 years old), and were calculated using Wilcoxon-Mann Whitney tests.

Principal Component Analysis

To assess what factors effect the pattern of changes in synaptic proteins we ran a multivariate analysis using principal component analysis (PCA). Protein expression was compiled into an mxn matrix, where the rows (m=10) represent proteins (GluA2, GluN1, PSD-95, VGLUT1, $GABA_A$ α1, $GABA_A$ α3, Gephyrin, VGAT, GAD65, GAD67), and the columns (n=68) represent all of the samples (central=28, peripheral=28, monocular=12). The data were centered by subtracting the mean column vector, and then singular value decomposition (SVD) was applied to calculate the principal components in Matlab (The MathWorks, Inc, Natick, MA). The SVD represents the expression of all proteins for each sample as a vector in high dimensional space. The PCA identifies the directions in "protein expression space" that represent the variance in the data.

4.3 Results

GAPDH expression is not correlated with age

As a loading control, we first verified that expression of GAPDH was not correlated with age in any region of V1 (Fig 1A, R=0.0067, p=0.97; Fig 1B, R=0.34, P=0.08; Fig1C, R=0.35, P=0.3).

Age-related loss of glutamatergic proteins is gradual and widespread

To examine age-related changes in the glutamatergic system, we quantified the expression of a group of proteins (GluA2, GluN1, PSD-95, VGLUT1) in samples taken from the central, peripheral, and monocular regions of V1. GluA2 and GluN1 are subunits of AMPA and NMDA glutamate receptors, respectively (Cull-Candy et al., 2006). PSD-95 anchors and stabilizes glutamate receptors (El-Husseini et al., 2000; Beique et al., 2006) and its expression is a good marker for the number of excitatory synapses (El-Husseini et al., 2000; Cohen-Cory, 2002; Keith & El-Husseini, 2008). Finally, VGLUT1 is located presynaptically, where it loads glutamate into synaptic vesicles (Fremeau et al., 2004; Wilson et al., 2005). Together, the expression of these proteins gives insight into the functional state of glutamatergic synapses. We found a steady loss in the expression of all proteins as a function of age, a loss that was well fit by linear functions (Fig 2). Furthermore, the losses were similar across all regions of V1, which was statistically determined by ANCOVAs for linear fits across the regions for each protein. We compared the average expression in the young (n=2, 2.5 years old) and old cats (n=5, 7-11 years old) to calculate the average magnitude of the loss in aging and to make statistical comparisons (Wilcoxon-Mann Whitney) (Fig 3).

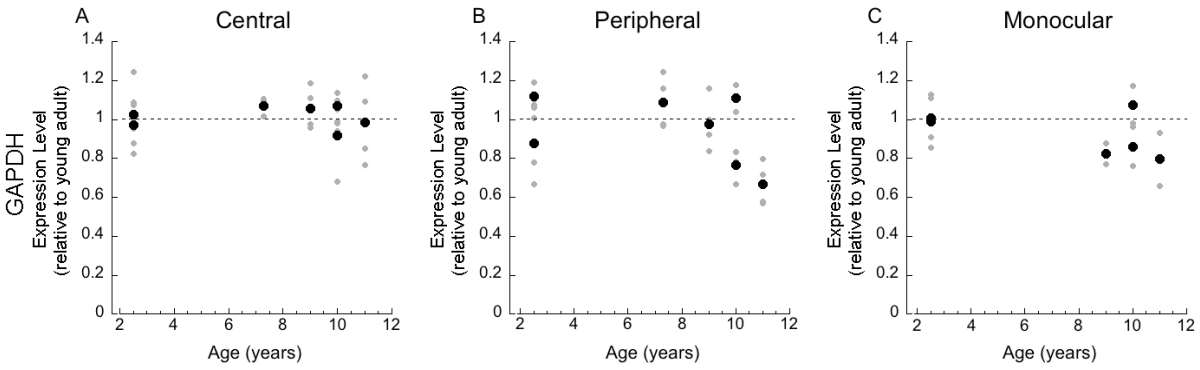

Figure 1. GAPDH expression. Expression levels are relative to young adults (dotted line). Grey symbols are results from all runs, and black symbols represent the average for each animal. GAPDH expression is not correlated with age in the central (p=0.97, A), peripheral (p=0.08, B), or monocular (p=0.3, C) regions of V1.

There was a steady loss of GluA2 expression in the central (Fig 2Ai, $R=0.76$, $p<0.001$), peripheral (Fig 2Aii, $R=0.61$, $p<0.001$), and monocular (Fig 2Aiii, $R=0.85$, $p<0.001$) regions of V1. Overall, the average expression of the older cats (7-11 years old) was reduced by ~ 50% in the central (SEM ± 5%, $p<0.001$), peripheral (SEM ± 9%, $p<0.01$), and monocular regions (SEM ± 8%, $p<0.01$) (Fig 3A). GluN1 expression followed a similar pattern of loss in aging (Fig 2B). Expression declined in the central (Fig 2Bi, $R=0.77$, $p<0.001$), peripheral (Fig 2Bii, $R=0.58$, $p<0.01$), and monocular (Fig 2Biii, $R=0.75$, $p<0.05$) regions, where the average expression in the older cats (7-11 years old) was reduced by ~60% in the central (SEM ± 3%, $p<0.001$), and 50% in the peripheral (SEM ± 9%, $p<0.01$), and monocular regions (SEM ± 9%, $p<0.01$) (Fig 3B). PSD-95 expression followed a similar pattern of loss in aging (Fig 2C). There was a steady loss in expression in the central (Fig 2Ci, $R=0.64$, $p<0.001$), peripheral (Fig 2Cii, $R=0.67$, $p<0.001$), and monocular regions (Fig 2Ciii, $R=0.86$, $p<0.001$), where the average expression in the older cats (7-11 years old) was reduced by ~50% in the central (SEM ±3%, $p<0.01$), peripheral (SEM ± 6%, $p<0.001$), and monocular (SEM ± 7%, $p<0.01$) regions (Fig 3C). Finally, VGLUT1 expression also followed a similar pattern of loss in aging (Fig 2D). There was a gradual loss in the central (Fig 2Di, $R=0.74$, $p<0.001$), peripheral (Fig 2Dii, $R=0.76$, $p<0.001$), and monocular (Fig 2Ciii, $R=0.74$, $p<0.01$) regions. Compared to the other proteins, however, the amplitude of the loss was greater as the average expression of the older cats (7-11 years old) was reduced by ~80% in the central (SEM ± 4%, $p<0.001$), 60% in the peripheral (SEM ± 6%, $p<0.001$), and 70% in the monocular region (SEM ± 7%, $p<0.01$) (Fig 3D). Overall, the effect of aging on the expression of each protein was very similar across regions, and the slopes of the linear functions

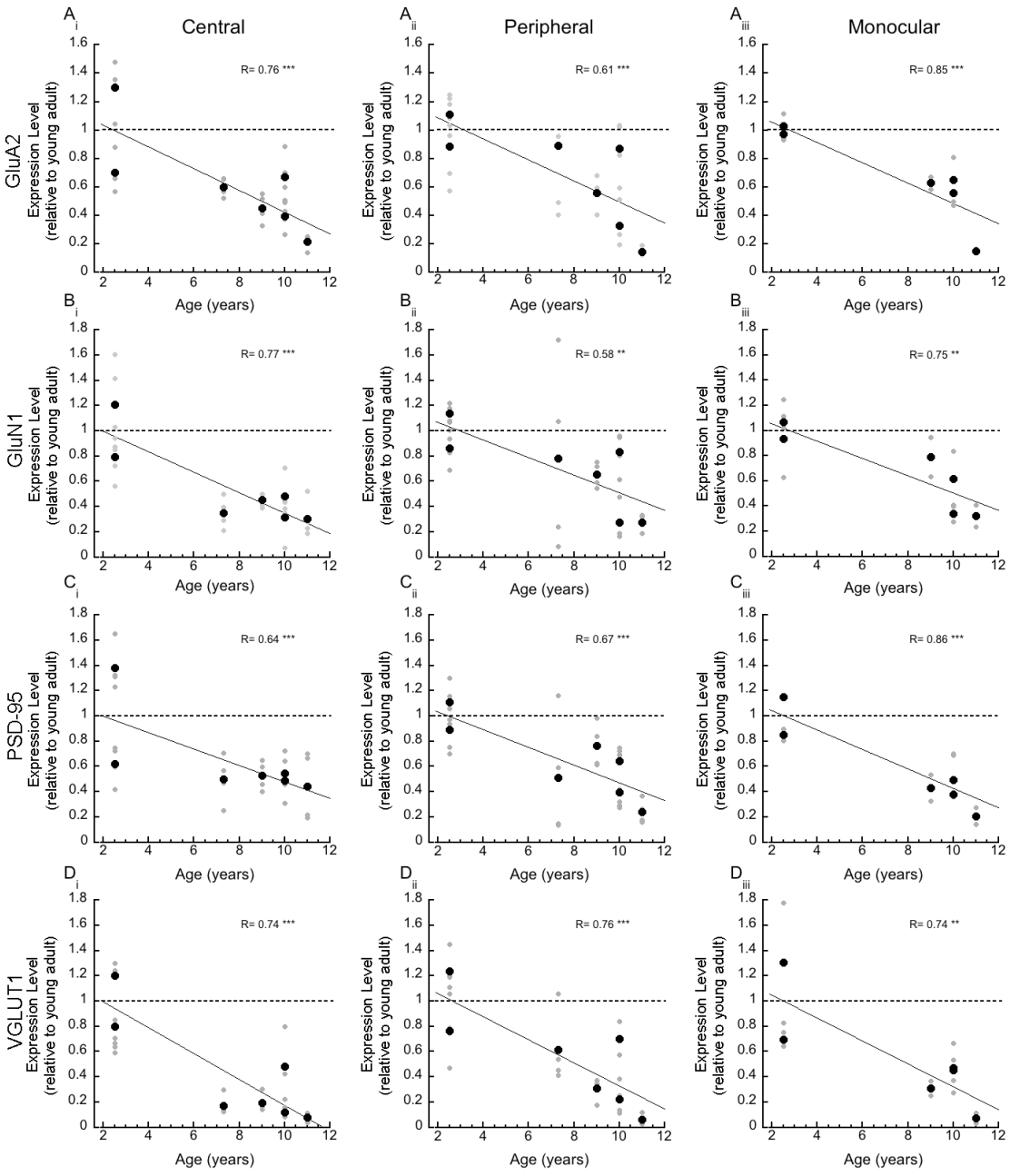

Figure 2. Age-related changes in the expression of glutamatergic synaptic proteins. Expression levels are relative to young adults (dotted line). Grey symbols are results from all runs, and black symbols represent the average for each animal. GluA2 expression decreased linearly in aging in the central ($R=0.76$, $p<0.001$, Ai), peripheral ($R=0.61$, $p<0.001$, Aii), and monocular ($R=0.85$, $p<0.001$, Aiii) regions of V1. GluN1 expression decreased linearly in aging in the central ($R=0.77$, $p<0.001$, Bi), peripheral ($R=0.58$, $p<0.01$, Bii), and monocular ($R=0.75$, $p<0.01$, Biii) regions of V1. PSD95 expression decreased linearly in aging in the central ($R=0.64$, $p<0.001$, Ci), peripheral ($R=0.67$, $p<0.001$, Cii), and monocular ($R=0.86$, $p<0.001$, Ciii) regions of V1. VGLUT1 expression decreased linearly in aging in the central ($R=0.74$, $p<0.001$, Di), peripheral ($R=0.76$, $p<0.001$, Dii), and monocular ($R=0.74$, $p<0.01$, Diii) regions of V1.

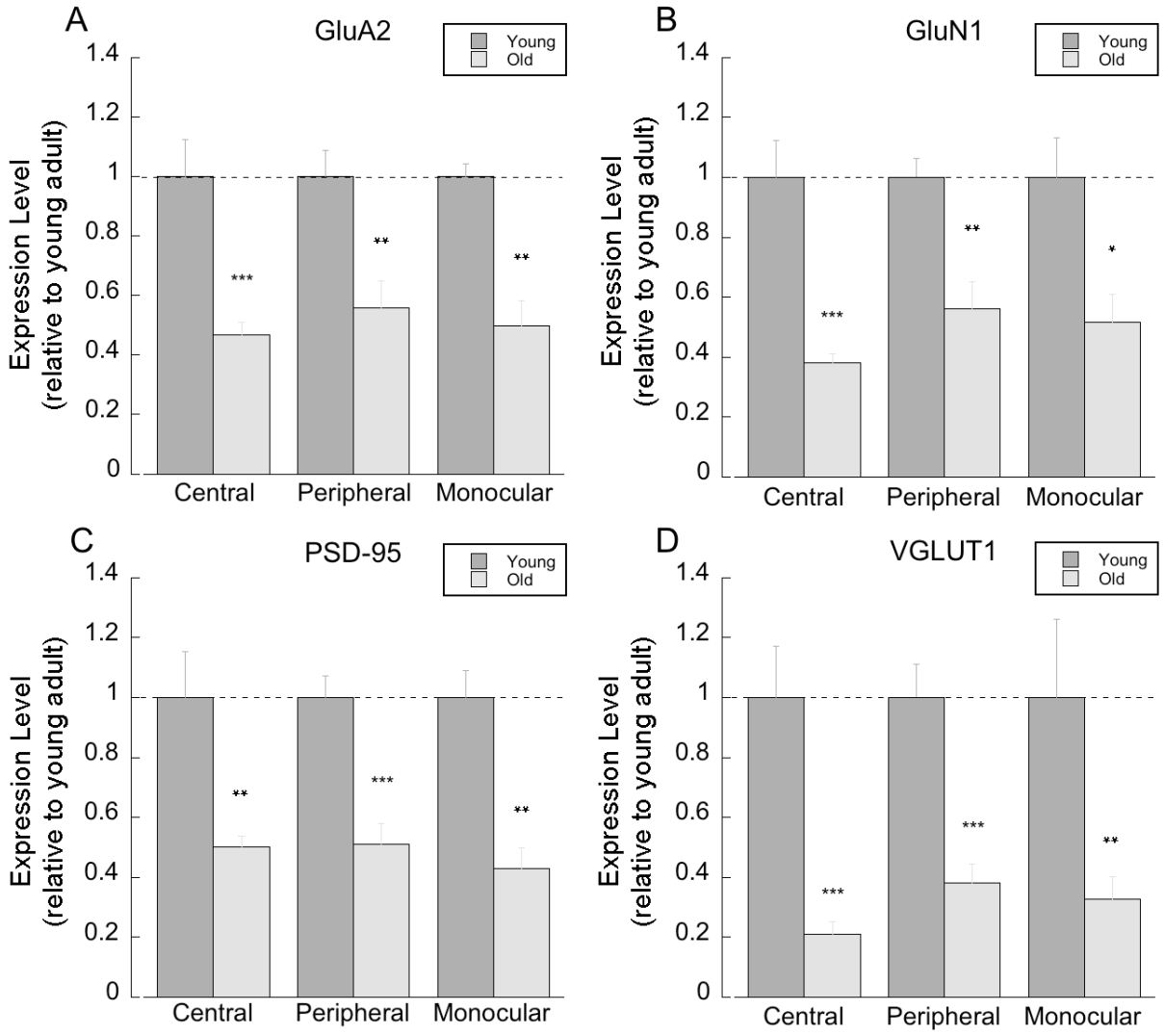

Figure 3. Average expression in young adult (n=2, 2.5 years old) and old animals (n=5, 7-11 years old). Expression levels are relative to young adults (dotted line). GluA2 expression was ~50-60% less in the central ($p<0.001$) peripheral ($p<0.01$) and monocular ($p<0.01$) regions of V1 (A). GluN1 expression was 40-60% less in the central ($p<0.001$) peripheral ($p<0.01$) and monocular ($p<0.05$) regions of V1. PSD95 expression was ~50% less in central ($p<0.01$) peripheral ($p<0.001$) and monocular ($p<0.01$) regions of V1. VGLUT1 expression was 60-80% less in the central ($p<0.001$) peripheral ($p<0.001$) and monocular ($p<0.01$) regions of V1.

were not different across regions of V1 (ANCOVA: GluA2 p=0.6; GluN1 p=0.2; PSD95 p=0.9; VGLUT1 p=0.3).

Age-related loss of GABAergic proteins is gradual and selective

A loss of GABAergic inhibition has been shown in aging V1, but the nature of the loss is unclear (Leventhal et al., 2003). A decrease in inhibition could be due to fewer $GABA_A$ receptors, reduced GABA release, and/or the production of GABA. To investigate these possibilities, we quantified the expression of a group of inhibitory synaptic proteins ($GABA_A$ α1, $GABA_A$ α3, Gephyrin, VGAT, GAD 65, GAD67). $GABA_A$ α1 and $GABA_A$ α3 are subunits of $GABA_A$ receptors: $GABA_A$ α1 is primarily found in mature receptors that typically have faster kinetics (faster current decay), whereas α3-containing $GABA_A$Rs are primarily found early in development, where they confer slower kinetics (slower current decay) to the receptor (Fritschy et al., 1994; Bosman et al., 2002). Gephyrin anchors $GABA_A$ Rs, and its expression is a good marker of inhibitory synapses (Keith & El-Husseini, 2008). VGAT is a pre-synaptic protein that loads GABA into synaptic vesicles (McIntire et al., 1997; Chaudhry et al., 1998). Finally, we quantified expression of the GABA synthesizing enzymes, GAD65 and GAD67. GAD65 is located at the synapse and supplies the on-demand pool of GABA, while GAD67 is primarily located in the cell body, providing the basal pool of GABA (Feldblum et al., 1993; 1995). We saw a similar pattern of loss as we did with the glutamatergic proteins, however, often the amplitude of losses was not as large, and for some proteins there was no clear pattern of loss in the monocular region of V1.

There was a steady loss of $GABA_A$α1 expression in the central (Fig 4Ai, $R=0.65$, $p<0.001$) and peripheral (Fig 4Aii, $R=0.57$, $p<0.01$) regions of V1, where the average expression in the

older cats (7-11 years old) was reduced by ~50% in the central (SEM ± 5%, $p<0.001$) and 40% in the peripheral (SEM ± 7%, $p<0.01$) regions of V1 (Fig 5A). In contrast, there was only a trend towards a loss in the monocular region of V1 (Fig. 4Aiii, $R=0.49$, $p=0.09$), and overall no significant loss of expression in the older cats ($p=0.15$) (Fig 5A). $GABA_A$ α3 expression gradually decreased in aging in the central (Fig 4Bi, $R=0.63$, $p<0.001$), peripheral (Fig 4Bii, $R=0.57$, $p<0.01$), and monocular (Fig 4Biii, $R=0.73$, $p<0.01$) regions, where the average expression level in the older cats (7-11 years old) was reduced by ~50% in the central (SEM ± 5%,$p<0.001$), 40% in the peripheral (SEM ± 6%, $p<0.001$), and 50% in the monocular region (SEM ± 6%, $p<0.05$) (Fig 5B). Gephyrin expression decreased gradually in the central (Fig 4Ci, $R=0.57$, $p<0.01$) and peripheral (Fig 4Cii, $R=0.44$, $p<0.05$) regions, where the average expression of the older cats (7-11 years old) was reduced by ~50% in the central (SEM ± 4%, $p<0.001$) and 40% in the peripheral regions (SEM ± 10%, $p<0.05$) (Fig 5C). In contrast, there was no clear trend in gephyrin expression in the monocular region (Fig 4Ciii, $R=0.36$, $p=0.23$), and overall no significant loss of expression in the older cats ($p=0.4$). This is likely due to the substantial variability within the older group of cats, as some samples had expression levels above normal, and some below normal. Finally, VGAT expression gradually decreased in the central (Fig 4Di, $R=0.74$, $p<0.001$), peripheral (Fig 4Dii, $R=0.67$, $p<0.001$), and monocular regions (Fig 4Ciii, $R=0.68$, $p<0.05$), with the average expression of older cats (7-11 years old) reduced by ~80% in the central (SEM ± 6%, $p<0.001$) and 60% in the peripheral (SEM ± 6%, $p<0.001$) (Fig 5D). In contrast, there was only a trend towards a loss of expression in older cats (7-11 years old) in the monocular region ($p=0.07$), which is likely due to the substantial inter-individual variability in the older group of animals. Again, the slopes of the linear functions were

not different across regions of V1 (ANCOVA: $GABA_A$ α1 p=0.1 $GABA_A$ α3 p=0.2; Gephyrin p=0.5; VGAT p=0.08).

Finally, we quantified the expression of GAD65 and GAD67. There were distinct differences in the expression pattern of each protein with aging. GAD65 expression decreased gradually in the central (Fig 6Ai, R=0.44, p<0.05) and peripheral regions of V1 (Fig 6Aii, R=0.60, p<0.001), where the average expression in the older cats was reduced by ~40% in both the central (SEM ± 9%, p<0.05) and peripheral regions (SEM ± 8%, p<0.01) (Fig 7A). In contrast, there was no clear trend in the monocular region (Fig 6Aiii, R=0.29, p=0.4), and no significant change in the average expression of older cats (p=0.5). Notably, GAD65 expression in the oldest cat (11 years old) was extremely low in the monocular region (~90% less than young adults), which was also observed in the central and peripheral regions. Unlike GAD65, the expression of GAD67 was relatively constant in aging (Fig 6B; central: p=0.10; peripheral: p=0.62; monocular: p=0.95), where the average expression was reduced by ~20% in the central region (SEM ± 5%, p<0.05) but there was no loss in the peripheral (p=0.5) or monocular (p=0.9) regions (Fig 7B). Overall, the widespread loss of both pre- and post-synaptic GABAergic proteins fits well with a loss of GABAergic inhibition in aging V1 (Leventhal et al., 2003; Hua et al., 2008; Pinto et al., 2010).

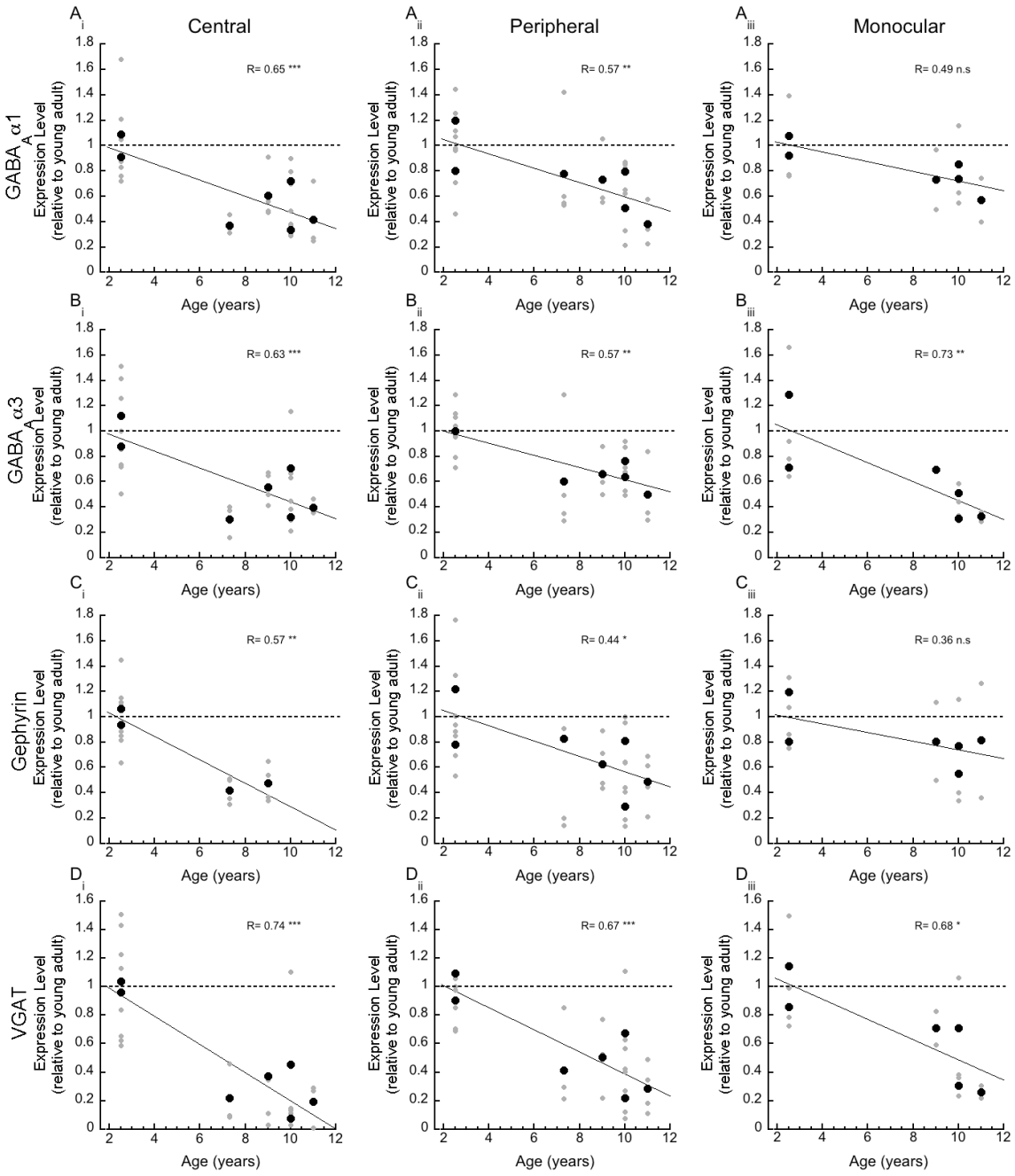

Figure 4. Age-related changes in the expression of GABAergic synaptic proteins. Expression levels are relative to young adults (dotted line). Grey symbols are results from all runs, and black symbols are the average for each animal. $GABA_A$ α1 expression decreased linearly in aging in the central ($R=0.65$, $p<0.001$, Ai) and peripheral ($R=0.57$, $p<0.01$, Aii) regions of V1, but not monocular ($R=0.49$, $p=0.09$, Aiii) regions of V1. $GABA_A$ α3 expression decreased linearly in aging in the central ($R=0.63$, $p<0.001$, Bi), peripheral ($R=0.57$, $p<0.01$, Bii), and monocular ($R=0.73$, $p<0.01$, Biii) regions of V1. Gephyrin expression decreased linearly in aging in the central ($R=0.57$, $p<0.01$, Ci) and peripheral regions of V1 ($R=0.44$, $p<0.05$, Cii), but not monocular ($R=0.36$, $p=0.2$, Ciii) regions of V1. VGAT expression decreased linearly in aging in the central ($R=0.74$, $p<0.001$, Di), peripheral ($R=0.67$, $p<0.001$, Dii), and monocular ($R=0.68$, $p<0.05$, Diii) regions of V1.

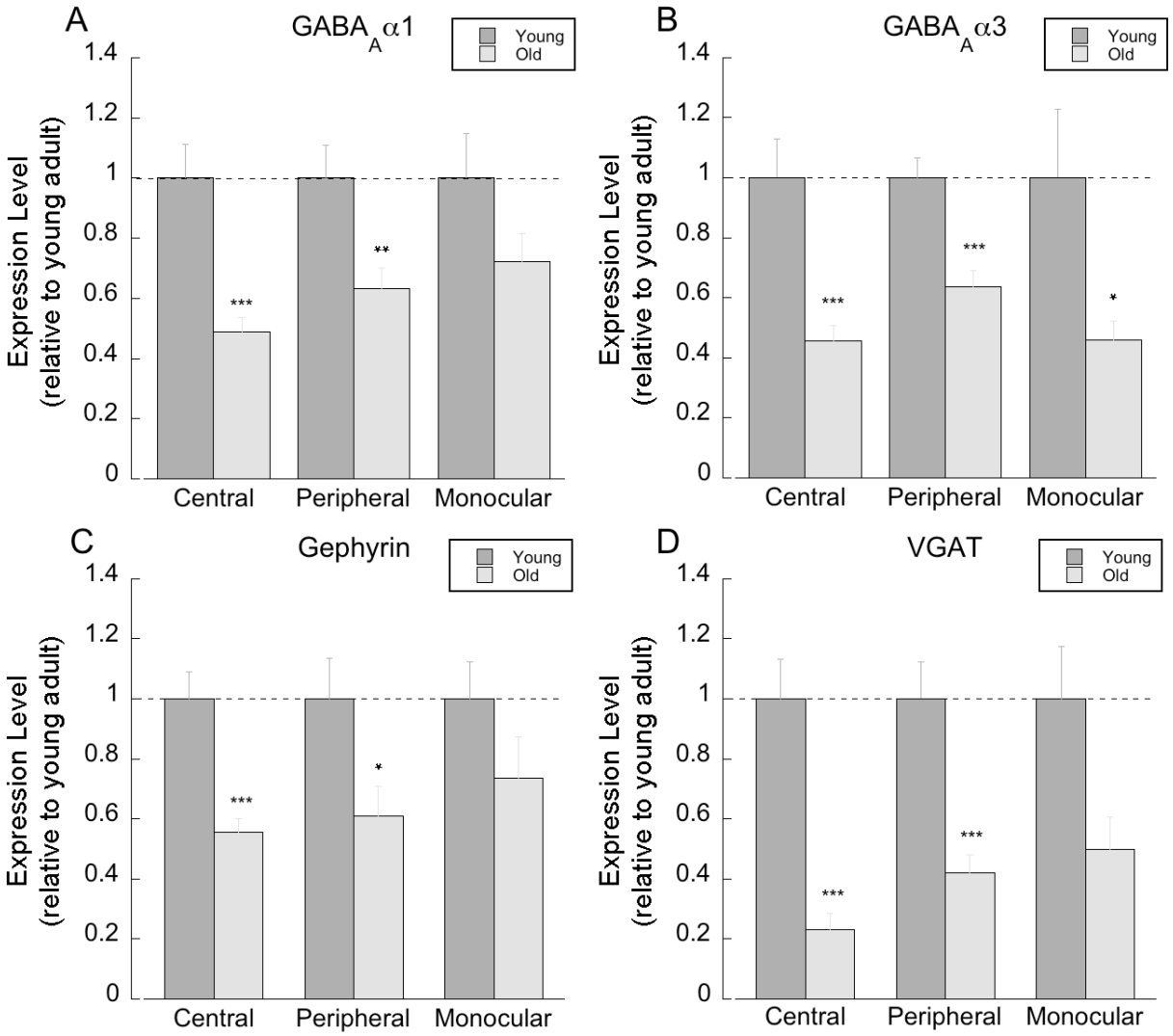

Figure 5. Average expression in young adult (n=2, 2.5 years old) and old animals (n=5, 7-11 years old). Expression levels are relative to young adults (dotted line). $GABA_A$ α1 expression was ~40-50% less in the central ($p<0.001$) and peripheral ($p<0.01$) regions of V1 (A). $GABA_A$ α3 expression was 40-50% less in the central ($p<0.001$) peripheral ($p<0.01$) and monocular regions of V1 ($p<0.01$). Gephyrin expression was ~40-50% less in central ($p<0.001$) and peripheral ($p<0.05$) regions of V1. VGAT expression was 60-80% less in the central ($p<0.001$) and peripheral regions of V1 ($p<0.001$).

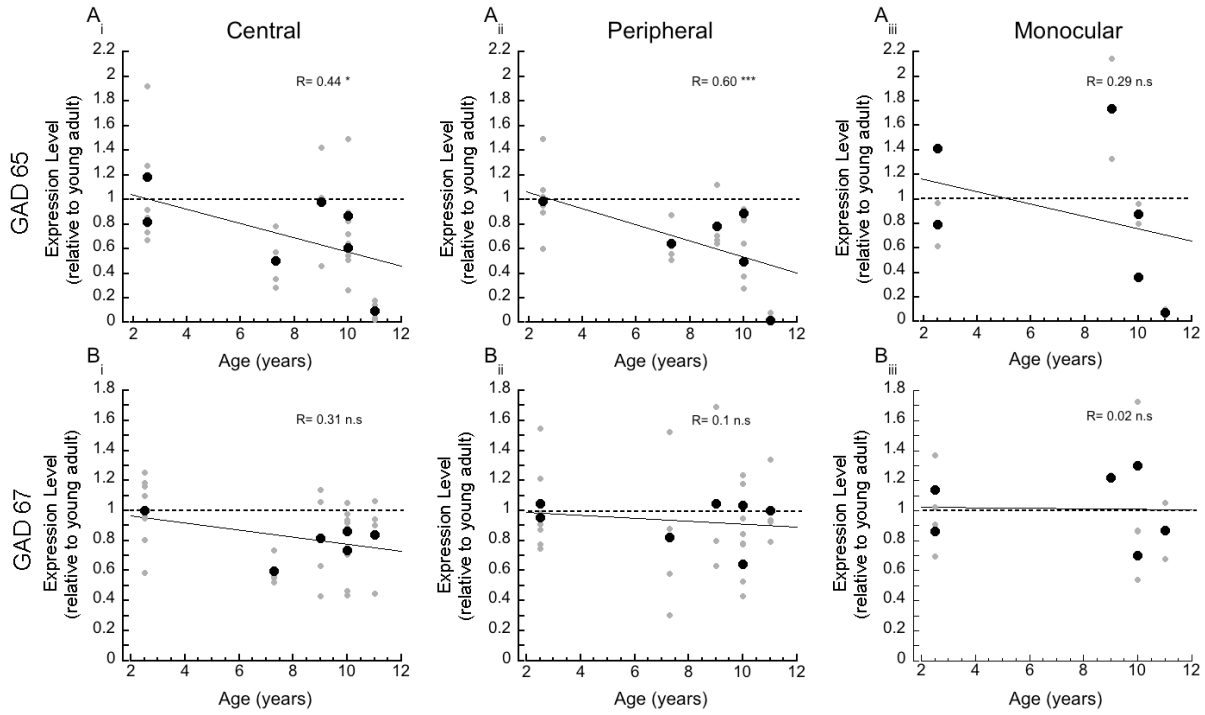

Figure 6. Age-related changes in the expression of GABA synthesizing enzymes, GAD65 and GAD67. Expression levels are relative to young adults. Grey symbols are results from all runs, and black symbols are the average for each animal. GAD65 expression decreased linearly in aging in the central ($R=0.44$, $p<0.05$, Ai) and peripheral regions of V1 ($R=0.6$, $p<0.001$, Aii), but not in monocular ($R=0.3$, $p=0.4$, Aiii). In contrast, GAD67 expression remained fairly constant in aging in the central ($R=0.31$, $p=0.1$, Bi), peripheral ($R=0.1$, $p=0.62$, Bii), and monocular ($R=0.02$, $p=0.95$, Biii) regions of V1.

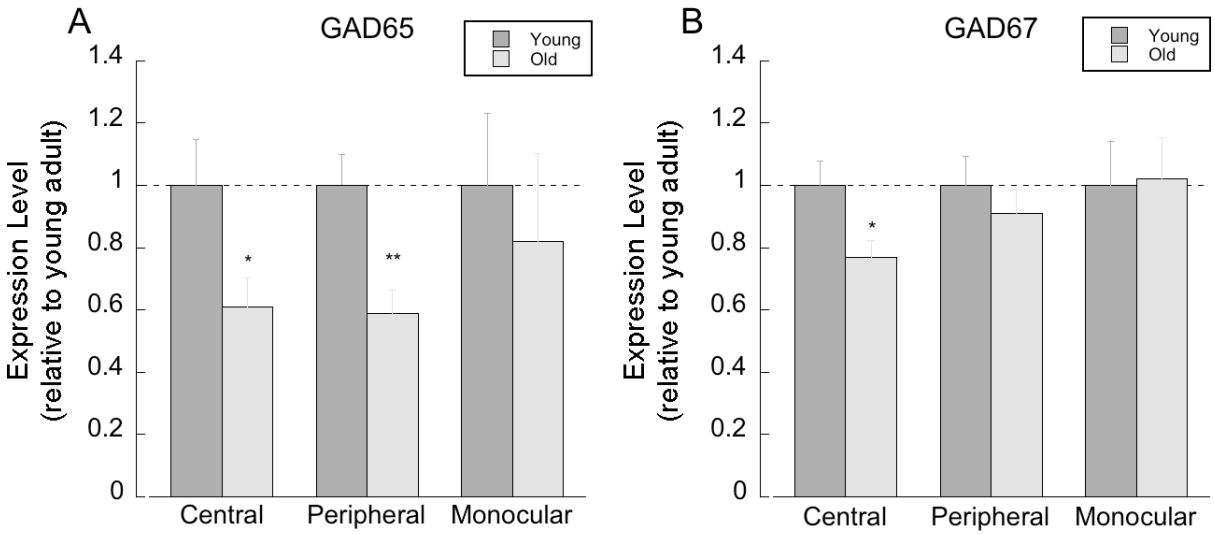

Figure 7. Average expression of GAD65 and GAD67 in young and old cats. Expression levels are relative to the average expression of young cats (dotted line). GAD65 expression was ~40% less in the central ($p<0.05$) and peripheral ($p<0.01$) regions of V1, but no change in the monocular ($p=0.5$) (A). GAD67 expression was ~20% less in the central region of V1 ($p<0.05$), but did not change in the peripheral ($p=0.5$) and monocular regions ($p=0.9$).

Total protein expression declines in aging

The expression pattern for almost all proteins was strikingly similar, suggesting that aging has a similar impact on synaptic proteins across all regions of V1. To determine what factors are influencing the data, we took a data driven approach, and ran a multivariate analysis using principal components analysis (PCA). The PCA revealed that >70% of the variance in the data was accounted for by a single component (PCA 1) (Fig 8A). Only PCA1 met the Guttman-Kaiser criterion (an eigenvalue >1) and was the only component used for further analysis. PCA 1 represents a linear combination of the expression of all the proteins, and the influence of each protein on PCA 1 was reflected by the relative amplitude of their basis vectors. To determine biological factors for PCA 1, we plotted the basis vectors for PCA1 and found that the weights for all proteins were positive and had similar amplitudes. Notably, there were greater contributions from the vesicular transporter proteins, VGLUT1 and VGAT, and much less by GAD67. The weights of the basis vectors suggested that PCA 1 is being driven by total protein expression, which was supported by a very strong correlation between PCA 1 and total protein expression ($R=0.99$, $p<0.001$). We summed the total protein expression for each sample, taking into account the weighted differences for each protein, and plotted total protein expression as a function of age for each region of V1 (Fig 8C). Total protein expression decreased linearly with age (central, $R=0.77$, $p<0.001$; peripheral, $R=0.68$, $p<0.001$; monocular, $R=0.79$, $p<0.001$), indicating that aging has a similar, non-selective effect on the expression of this group of synaptic proteins across all regions of V1.

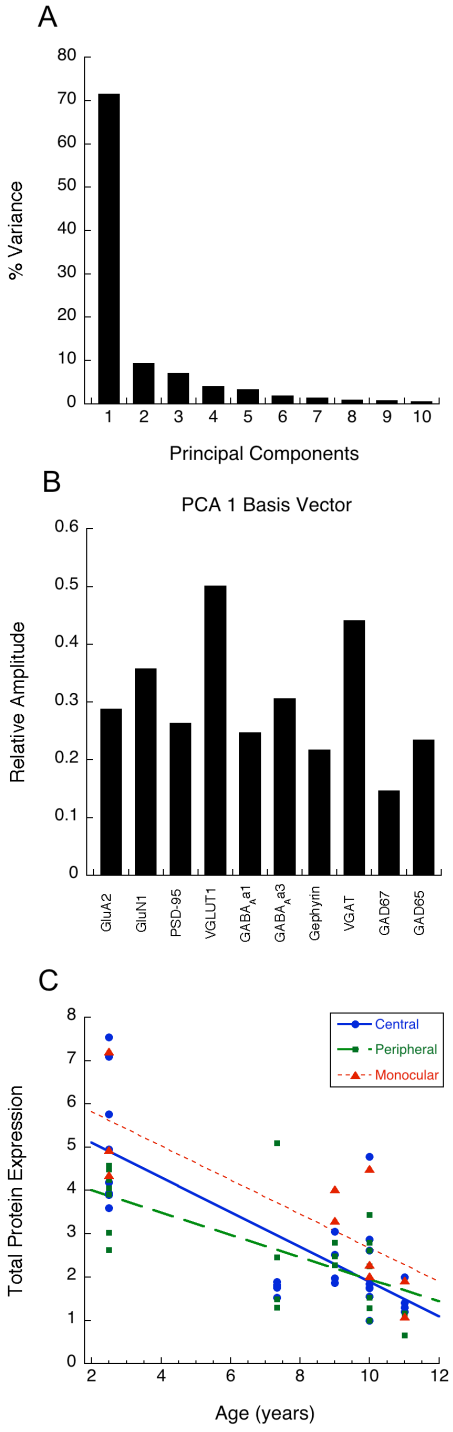

Figure 8. Principal component analysis. The percent variance captured by each component of the SVD analysis of all protein expression across all regions of V1 (A). The first principal component (PCA 1) accounted for 71.5% of the variance in the data. The influence of each protein on PCA 1 was reflected by the relative amplitude in the basis vectors (B). Weighted total protein expression decreased linearly as a function of age (C).

4.4 Discussion

This study characterized the impact of aging on a group of proteins associated with glutamatergic and GABAergic synapses in cat V1, and found a gradual age-related decline in the expression of both glutamatergic and GABAergic proteins. The losses were widespread, and generally occurred across all regions of V1. This is the first study to characterize changes in both glutamatergic and GABAergic synaptic proteins in an animal model of aging, and these findings provide new insights into the changes in V1 that may contribute to age-related vision loss.

Functional impact of losses on vision

We observed a widespread loss in expression of each of the glutamatergic proteins we quantified. The loss of the post-synaptic proteins, PSD95, GluA2, and GluN1 likely reduces the strength of excitatory inputs, and the loss of the pre-synaptic vesicular glutamate transporter protein, VGLUT1, likely reduces the amount of glutamate loaded into vesicles, thus affecting the amount of glutamate released, and further reducing excitatory transmission. Reduced excitatory activity in V1 will likely impact vision because glutamate receptors have been linked with mediating specific visual processing mechanisms (Self et al., 2012). For example, AMPARs mediate the main feedforward, stimulus-driven response of neurons in V1, while NMDARs mediate modulatory, feedback activity onto neurons in V1. Blocking these receptors in macaques reduces the neuronal responses for each type of visual processing (Self et al., 2012). Although the impact of acute receptor blockade versus chronic receptor loss (during aging) on visual processing may be different, it seems likely that reduced receptors during aging will impact visual functions dependent on feedforward and feedback visual processing (Lamme &

Roelfsema, 2000). Modulatory feedback onto V1 neurons typically only occurs if the neurons are well-driven by feedforward activity (Ekstrom et al., 2008) and although we measured a similar loss of both the AMPAR subunit, GluA2, and the NMDAR subunit, GluN1, the impact of a loss of AMPARs on feedforward activity may be subtle compared to the impact on downstream NMDAR-dependent modulatory functions. Because NMDAR-dependent modulatory activity typically underlies more complex visual functions this fits with the view that more complex visual functions are vulnerable in aging (Habak & Faubert, 2000).

Next we characterized the impact of aging on a group of GABAergic synaptic proteins. Though a reduction in GABAergic inhibition has been measured in aging V1, it remains unclear what drives the loss (Leventhal et al., 2003). We found a significant loss of both pre-and post-synaptic inhibitory proteins that may underlie a reduction in GABAergic inhibition. Specifically, reduced expression of the post-synaptic proteins, gephyrin, $GABA_A$ α1, and $GABA_A$ α3, may reduce the strength of inhibitory inputs, whereas reduced expression of the pre-synaptic proteins GAD65 and VGAT may reduce the capacity for GABAergic neurons to synthesize and release GABA. Different from the group of glutamatergic proteins, there were some selective losses in GABAergic synaptic proteins during aging. For example, although GAD65 expression declined in aging, for the most part, GAD67 expression was unchanged. Interestingly, this same pattern has been shown in aging human V1, demonstrating that there are common age-related changes between species (Pinto et al., 2010). Furthermore, because GAD67 is located in the cell body, the selective loss of GAD65 may reflect a *synaptic* vulnerability in aging. GABAergic inhibition plays a key role in shaping receptive field properties in V1 (Alitto & Dan, 2010; Lee et al., 2012) and reduced GABAergic inhibition has been proposed to account for degradation of receptive

field properties of neurons in V1 of old cats and macaques (Leventhal et al., 2003). This includes reduced signal-in-noise, and selectivity for orientation, direction (Hua et al., 2006; Schmolesky et al., 2008), and spatial frequency (Hua et al., 2011). Interestingly, application of a GABA agonist can only *partially* restore selectivity of neurons, indicating that more than just inhibitory mechanisms maintain these physiological properties (Leventhal et al., 2003). Some properties like orientation selectivity, can be generated completely by excitatory inputs (Nelson et al., 1994), raising the possibility that the degradation of neuronal properties in aging is likely due to a combination of the loss of both excitatory and inhibitory mechanisms.

What is the nature of the loss?

In order to link the losses with specific visual functions, it is crucial to know what types of neurons are vulnerable to age-related loss of synaptic proteins. For example, the impact of a reduction in glutamate receptor expression will be quite different if the reduction occurs mainly on glutamatergic neurons or GABAergic neurons. A recent study has shown that activation of parvalbumin-positive (PV+) inhibitory interneurons improves orientation tuning of V1 neurons, leading to improved visual perception (Lee et al., 2012). A reduction in the activity of PV+ interneurons is a likely candidate for the age-related loss of inhibition underlying vision loss, and our results support this in several ways. First, reduced activity of PV+ interneurons could be due to reduced ability of the neurons to synthesize and release GABA, as reflected by reduced GAD and VGAT expression. Second, reduced activity of PV+ interneurons could be due to reduced excitatory input *onto* PV+ interneurons. Because the main glutamate receptors on PV+ interneurons are GluA2- containing AMPARs (Kooijmans et al., 2014), our results support this possibility by showing a loss of GluA2 expression. These possibilities highlight the importance

of knowing *where* the losses occur. There is accumulating evidence that changes in aging V1 are not random, but rather reflect cell- and layer-specific vulnerability. For example, complex cells in V1 of old macaques have reduced orientation and direction selectivity, whereas simple cells are spared (Liang et al., 2012). Furthermore, physiological properties such as signal timing are similar for neurons in layer IV in both young and old monkeys, but outside layer IV, they are worse in old monkeys (Wang et al., 2005). Finally, studies in human V1 have revealed a selective loss of dendritic spines on spiny stellate neurons restricted to layer IVC (Mavroudis et al., 2012). Further anatomical studies will be needed to determine which neurons are vulnerable to a loss of synaptic proteins in aging. This information will be crucial for predicting the impact on specific circuits that underlie visual functions, and will provide great insight into why some visual functions are impaired, preserved, or even *improved* with aging (Betts et al., 2005).

Losses are gradual and widespread across visual cortex

Much of the age-related visual deficits reported in humans include reduced acuity and contrast sensitivity: both visual functions that are limited by the central visual field (Owsley et al., 1983; Wood & Bullimore, 1995; Li et al., 2000; Del Viva & Agostini, 2007). Anticipating the possibility of regional differences, we measured protein expression in regions of V1 representing the central, peripheral, and monocular visual field representations. Surprisingly, the losses of protein were relatively similar across all areas. The widespread losses across the peripheral and monocular regions of V1 may contribute to age-related loss of visual functions that rely on peripheral vision such as motion perception (Trick & Silverman, 1991; Bennett et al., 2007) and peripheral acuity (Collins et al., 1989). Furthermore, complex visual tasks dependent on visual integration across the visual field have also been shown to decline linearly in aging

(Del Viva & Agostini, 2007; Kennedy et al., 2009). Interestingly, the lack of regional differences contrasts with the regional vulnerability of V1 during development. We have previously shown that abnormal visual experience during development causes a regional loss of glutamatergic and GABAergic proteins in the central region of cat V1 (Murphy et al., 2004; Beston et al., 2010). The changes that occur during aging are likely just as complex as those during development, but perhaps the regional vulnerability during development reflects region-specific plasticity that isn't available in aging V1.

Interestingly, the losses in aging were linear, which matches well with reports of gradual, steady declines in visual acuity and contrast sensitivity that begin early in adulthood (Sekuler et al., 1982; Wood & Bullimore, 1995). However, because of our small sample size, and few animals representing "middle age" it is difficult to draw a firm conclusion that protein loss is linear. Certainly future experiments that quantify the trajectory across the lifespan will gain more insight into the pattern of the loss.

The widespread losses suggest that aging causes a non-specific, down-regulation of proteins. This seems unlikely, however, because not all visual functions are affected by age. Rather, the selective loss of visual functions during aging likely reflects more subtle and selective changes in V1 (Billino et al., 2008). The proteins we studied are a small subset of the large and complex synaptic proteome, and it is possible that quantifying different synaptic proteins will yield selective losses in aging. For example, large-scale proteomic studies in aging rodents (Yang et al., 2008) and humans (Chen et al., 2003) reveal selective losses in the aging cortex. Another possibility is that the amount of inter-individual variability in aging may occlude regional vulnerability. Recent physiological studies have shown that degradation of neuronal

properties is even more variable during early senescence for macaques (Fu et al., 2013). The oldest cats in this study ranged between 7-11 years old, whereas the oldest cats in physiological studies have typically been between 12-14 years of age (Hua et al., 2006; 2008; 2011; Zhou et al., 2011), raising the possibility that an older group of animals might yield more selective changes. Taken together, the widespread and steady loss of both glutamatergic and GABAergic synaptic proteins is an important new finding for understanding age-related changes in V1. These findings provide new insight into the neural basis of vision loss during aging, and provide a strong foundation for further anatomical studies to determine the nature of the losses in aging.

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Chapter 5. General Discussion

5.1 Summary of Main Findings

In this thesis, I have made significant advances toward our understanding of the neural basis of vision loss during amblyopia and aging. The first part of this thesis examined the impact of monocular deprivation (MD) on the expression of AMPAR and AMPAR-trafficking proteins in the regions of V1 representing the central, peripheral, and monocular visual fields. Brief MD caused a rapid loss of all AMPAR-associated proteins in all regions of V1, but longer deprivation revealed a selective recovery in the peripheral and monocular regions. To correlate the regional changes with vision, I used behavioural experiments and showed that both short and long term MD caused long lasting binocular acuity deficits that were most severe in the center of vision. Furthermore, MD has an impact on vision of *both* eyes. Taken together, these findings (chapter 2) showed that disrupting binocular vision early in development leads to synaptic and behavioural changes that are greatest in the center of vision. The persistent loss of AMPAR protein expression in the central region of V1 may provide a neural basis for binocular vision loss in the center of vision.

The second part of this thesis examined the impact of aging on synaptic protein expression in different cortical areas, across a range of species. During aging there was a steady loss in the expression of Ube3A, an E3 ligase necessary for cortical plasticity, in V1, but also in other sensory (V3,V4, A1) and non-sensory cortical areas (frontal cortex) (chapter 3). Importantly, by comparing the pattern of loss across a range of species, I revealed a selective loss of Ube3A relative to other synaptic proteins that occurred only in aging human cortex. Because Ube3A is tightly linked with AMPARs, the findings from chapter 3 provided the foundation for chapter 4, where I examined the impact of aging on the expression of glutamatergic synaptic proteins in cat

V1. Examination of aged V1 revealed a large loss in expression of both glutamatergic and GABAergic synaptic proteins, which supports and extends the current view that aging V1 is characterized by reduced GABAergic inhibition. Furthermore, in contrast to the regional vulnerability of V1 during development, aging had a similar impact on synaptic protein expression across all regions of V1 (central, peripheral, and monocular). Taken together, the findings from chapters 3 and 4 provide new insight into age-related changes in V1 that may contribute to age-related changes in visual function and plasticity.

5.2 Methodological Approach

Since the pioneering studies of Hubel & Wiesel, cats have been used to study the role of visual experience on the functional development of V1. More recently, cats are being used to study the impact of aging on physiological properties of neurons in V1. Cats have been selected as a model for studying the visual system because their visual system is similar to that of humans in many ways. First, like humans cats have forward facing eyes, which means a large area of their visual field is binocular. Second, cats have an area of specialization within the retina, called the area centralis, where specific neuronal properties permit high spatial acuity. This area of specialization is comparable to the fovea in humans and monkeys. Third, cats have a well-mapped representation of their visual field across V1. Fourth, cats can easily be trained to perform visual detection tasks, allowing for a behavioural measurement of their acuity. Finally, the high spatial acuity of cats means a range of amblyopic conditions can be created using varying lengths of monocular deprivation, creating mild, moderate, or severe acuity deficits.

Although the central and peripheral visual field representation in cat V1 both contain binocular neurons, the areas are functionally distinct, and this warranted especially important consideration in the studies that make up this thesis. For example, the center of vision can support binocular fusion, but the peripheral cannot. Furthermore, neurons in the central region of V1 are characterized by smaller receptive fields that largely overlap, and account for high spatial resolution in center of vision. In the peripheral region, receptive fields of neurons are large, and acuity is much worse. In many ways the peripheral vision of cats is similar to that of rodents, whereas the central vision of cats is closer to that of monkeys and humans. These

distinctions are especially important for bridging findings between rodents, cats, macaques, and humans.

By taking advantage of the well mapped visual field in cat V1, I was able to use Western blot, a robust technique that allowed quantification of a number of proteins over a large number of samples, to quantify changes in protein expression across the regions of V1 representing the central, peripheral, and monocular visual fields. By quantifying expression of proteins that are linked with both synaptic plasticity and visual functioning, this approach aimed to bridge the gap between synaptic changes and functional changes in vision. Importantly, this technique allowed a comparison of findings across cats, macaques, and humans. Western blot analysis, however, cannot capture cell-type or layer-specific changes. The findings from this thesis, however, provide a strong foundation for subsequent anatomical and physiological studies that will be needed to gain a complete picture of the cortical changes that emerge following monocular deprivation, and during aging.

5.3 Significance

Taking a second look at amblyopia

Despite early treatment up to 50% of children with amblyopia have recurrent or residual vision loss (Birch, 2013). This strikingly high statistic highlights a need for better treatment, but new treatments rely on a better understanding of amblyopia. The findings from chapter 2 challenge the long-standing view that following MD, the non-deprived eye is normal (Dews & Wiesel, 1970; Giffin & Mitchell, 1978). Binocular acuity is limited by acuity in the better eye, or in the case of MD, the non-deprived eye. We found that all the previously deprived animals had persistent binocular acuity deficits despite re-opening their deprived eye during the critical period, and receiving months of binocular vision with daily vision testing. Although poor vision in the fellow eye of human amblyopes has been reported, it has generally been thought to be caused by aggressive patching therapy. Several studies, however, have found vision deficits in the fellow eye of *untreated* human amblyopes, including reduced contrast sensitivity (Chatzistefanou et al., 2005) and abnormal eye movements (Kelly & Buckingham, 1998). The findings from chapter 2 show that MD can impact the vision of *both* eyes, even without patching. Furthermore, the binocular deficits were greatest in the center of vision, which fits well with reports of severe vision loss in the fovea of human amblyopes (Johnson et al., 1979; Hess & Pointer, 1985; Bowering et al., 1993; Agervi et al., 2010). The findings from chapter 2 may have great impact on the current treatment paradigms for amblyopia which still rely on patching the "good" eye to make the amblyopic eye work harder. Rather, the findings from chapter 2 fit well with the current view that binocular visual experience is an essential ingredient for achieving good, permanent vision recovery (Kind et al., 2002; for review see Mitchell & Duffy, 2014).

Perceptual learning paradigms that use binocular approaches to balance activity in both eyes elicit good recovery (Hess et al., 2010; Knox et al., 2012; Ooi et al., 2013) and perhaps a large part of their success is that binocular paradigms facilitate recovery of vision in *both* eyes. Incredibly, despite our advances in amblyopia research, the same patching methods initially described in the 1700s continue to be the main course of treatment for children today (Barrett et al., 2004). It is clear that more insight into the neural basis of binocular vision loss will be crucial for moving binocular-based treatments from research studies into clinical practice.

Exciting new findings in aging

Seniors make up the fastest-growing age group in Canada: over the next 25 years the Canadian population aged 65 and older is expected to double. With a rapidly aging population there is a great priority to understand the impact of aging on the cortex. The findings from chapters 3 and 4 add to a growing body of research that characterizes age-related changes in V1 that may explain functional changes in vision and plasticity. The studies in chapters 3 and 4 are the first to provide a comprehensive characterization of age-related changes in the expression of glutamatergic and GABAergic synaptic proteins in the cortex of a range of species. Importantly, chapter 3 revealed a selective loss of Ube3A in human V1 that wasn't found in the animal models, highlighting a vulnerability in the human aging process. This finding has important implications for translating findings from animal models, and emphasizes the value in running parallel studies between animals models and humans.

A common observation across all species was that the loss of synaptic protein expression was progressive over the lifespan, as opposed to a sudden loss in aging. This suggests the functional impact of cortical changes on vision may begin even earlier than the typical age range

that most perceptual studies use to study vision loss in aging (60+ years). Rather the findings suggest that vision loss may begin earlier in life, when most humans are still working, driving, and when the impact of reduced vision on quality of life is great. Therefore, understanding the mechanisms underlying age-related vision loss will be critical for developing interventions that can *slow* or reverse age-related loss of vision. Additionally, a full understanding of the capacity for plasticity in the adult cortex will be important for designing perceptual learning paradigms to improve age-related vision loss. For example, recent studies have used perceptual learning in adults over the ages of 50 (Polat et al., 2013) and 65 years old (Andersen et al., 2010) to improve age-related vision loss. Understanding the age-related changes in the cortical mechanisms that support plasticity and visual perception in V1 will be crucial for optimizing perceptual learning paradigms for older individuals (Deloss et al., 2013), as it is likely that a perceptual learning paradigm that works well in young adults may not for an older individual. Finally, characterizing age-related changes in V1 that occur during normal, healthy aging is the first step for understanding the impact of *abnormal* aging on vision and V1. For example, neurodegenerative disorders such as Alzheimer's and Parkinson's Disease are characterized by changes in vision (for review see Jackson & Owsley, 2003). Recent studies are beginning to tease apart the cortical differences in V1 of humans with and without neurodegenerative diseases (Mavroudis et al., 2011; Brewer & Barton, 2014), but more studies will be needed to fully understand the impact of normal and abnormal aging on vision and V1.

5.4 Future Directions

How does monocular deprivation impact the circuitry that supports vision?

The studies described in this thesis lay the foundation for future studies to advance our understanding of the neural basis of vision loss. The findings from chapter 2 showed that MD caused a sustained loss of GluA2 in the central region of V1. However, in order to understand the functional consequence of a sustained loss of GluA2, a crucial next study will be determining which neurons are losing GluA2. This will give insight into the impact of monocular deprivation on the circuitry that supports vision. For example, activation of PV+ interneurons sharpens orientation selectivity, which translates to improved orientation perception. If the loss of GluA2 primarily occurs on PV+ interneurons, persistent inactivity may lead to degradation of receptive field properties, thus driving changes in visual perception (Lee et al., 2012). Alternatively, if the loss of GluA2 is largely restricted to pyramidal neurons in layer IV, which receive the majority of thalamocortical input, a reduction in GluA2 may reduce the amount of feedforward activity. Because feedforward activity sets up much of the activity necessary to shape receptive field properties, this may also drive vision loss. It will be crucial to perform anatomical and physiological studies within the central region of V1 to distinguish between these possibilities. The impact of MD on the cortical circuitry supporting vision likely differs across the regions of V1, and performing these studies will give insight into the neural basis of vision loss across the visual field. Furthermore, by comparing the nature of the loss across regions, this will give insight into experience-dependent plasticity mechanisms that drive regional changes in V1.

What limits recovery of vision following monocular deprivation?

Despite re-opening the deprived eye during the critical period and receiving months of binocular vision, all of the deprived animals had persistent vision deficits. Furthermore, for most of the deprived animals, acuity in the deprived eye was similar to binocular acuity, suggesting recovery of vision is limited by binocular acuity. This raises an important question: what cortical mechanism *limits* recovery of vision? Because the deficits were most severe in the center of vision, it raises the possibility that the sustained loss of AMPARs has an impact on experience-dependent plasticity mechanisms necessary for recovery. Interestingly, rodent studies have suggested that recovery of vision associated with perceptual learning is tied with LTP-like plasticity mechanisms (Frenkel et al., 2006; Cooke & Bear, 2010; Sale et al., 2011) that depend on NMDAR activation and AMPAR trafficking (Frenkel et al., 2006). The loss of AMPARs in the central region of V1 suggests the capacity for plasticity (and therefore recovery) is reduced. Although there is evidence for regional plasticity in the binocular and monocular cortex of rodents (Nataraj & Turrigiano, 2011; Kuo & Dringenberg, 2012) it is not known whether the *capacity* for plasticity differs across regions of V1 following MD. A crucial next study will be to determine the potential for plasticity across the central, peripheral, and monocular regions of V1 following MD. This will yield important information for designing optimal paradigms to recover vision. For example, if the capacity for LTP is reduced in the central region of V1, but preserved in the peripheral region, then perceptual learning paradigms that take advantage of plasticity in the peripheral region may be a useful strategy to train the periphery to compensate for poor central vision. This raises an interesting possibility for the well documented phenomenon of eccentric fixation of the amblyopic eye in humans (Malik et al., 1969). If the peripheral region

of V1 retains AMPAR-dependent plasticity mechanisms, then eccentric fixation may manifest as an adaptive strategy to compensate for poor central vision. Interestingly, some amblyopes not only have *higher* acuity at their eccentric fixation point (compared to their amblyopic fovea), but also have higher *peripheral acuity* in their amblyopic eye than in their normal eye (Avetisov, 1979).

Interestingly, perceptual learning paradigms that target the visual periphery are being used for those with central vision loss due to age-related macular degeneration (AMD) (Yu et al., 2010). Patients with AMD can be trained to develop a peripheral fixation point in order to optimize their residual vision in the periphery. Using the new peripheral fixation point, perceptual learning leads to better visual performance on tasks that typically rely on central vision, such as reading (Nilsson et al., 2003; Tarita-Nistor et al., 2009). Interestingly, cat models of AMD have proposed the ability of the peripheral visual field to compensate for central vision is rooted in increased GluA2 expression in the peripheral region of V1, an adaptive response to reduced activity in the central region of V1 (Hu et al., 2011). In some ways the daily testing of grating acuity is similar in both the length and intensity to perceptual learning paradigms, however it is clear that the grating detection paradigm used in chapter 2 is *not* an ideal training paradigm to improve binocular acuity, as the animals with longer MD (5 and 6 weeks) had severe acuity deficits even after months of training. This, however, opens up the possibility that a different training paradigm (one that actively engages the periphery) can yield better recovery of acuity.

What mechanism underlies reduced inhibition in aging?

We are still far from a complete understanding of how V1 changes with age. Compared to what we know on the development of V1, our knowledge of how V1 changes during aging lags. The studies in this thesis lay the foundation for future studies which can advance our understanding of the neural basis of vision loss in aging. The dominant view is that aging V1 is characterized by reduced GABAergic inhibition (Leventhal et al., 2003), but it is still unclear what mechanisms drive a loss of inhibition. A specific class of inhibitory neurons, PV+ interneurons, are a critical component of the neural circuitry that supports vision (Atallah et al., 2012; Lee et al., 2012). Activation of PV+ interneurons sharpens receptive field properties, such as orientation and direction selectivity, which translates to improvement on orientation discrimination tasks. Thus, activity of PV+ interneurons plays a key role in visual perception (Lee et al., 2012). This raises the possibility that reduced activity of PV+ interneurons is one mechanism that may drive an age-related reduction in inhibition, leading to poor vision. Future studies are needed to determine whether PV+ interneuron activity is altered during aging, and if so, what mechanism underlies reduced activity. The findings from chapter 4 support several possibilities: First, an age-related reduction in GAD65 and VGAT expression may reduce the capacity for PV+ neurons to produce and release GABA, thus reducing their inhibitory activity. Second, because the majority of AMPARs on PV +interneurons contain GluA2 subunits (Kooijmans et al., 2014), the loss of GluA2 expression in aging may reduce the excitatory drive onto PV+ neurons, thus reducing their activity. It seems likely that a combination of a loss of both components will impact the circuitry necessary to support vision, but it remains unknown which components of the neural circuitry underlying vision are vulnerable in aging.

How do age-related cortical changes underlie vision loss?

There are many changes that occur at the aging synapse, but how, or which of these changes gives rise to the array of age-related visual deficits is unclear. A great challenge for aging research is that unlike developmental milestones that are fairly conserved (for example the development of acuity), humans and animals simply don't age the same. It is not uncommon for the visual perception of an elderly person to be similar or identical to that of a young adult, creating large inter-individual variability in aging (Johnson & Choy, 1987). Similarly, physiological studies in macaques have shown a large amount of inter-individual variability in the degradation of neuronal properties, especially in early senescence (Fu et al., 2013). This variability among individuals can make it challenging to link the physiological and anatomical changes in aging animal models with age-related visual deficits in humans. For example, physiological studies in old macaques and cats have shown neurons with reduced orientation selectivity (Schmoleskey et al., 2000; Hua et al., 2006), but orientation selectivity in the visual system of older humans appears unaffected (Delahunt et al., 2008; Govenlock et al., 2009). The large amount of inter-individual variability, however, can be used as an advantage. If the selective loss of a visual function in aging can be linked with a specific cortical change within the same animal, it will be a powerful tool to determine the neural basis of the age-related visual deficit. Future studies where physiological, anatomical, and behavioural measures are performed within the same animal will be critical for linking age-related visual deficits with age-related cortical changes. Ideally, observed changes in visual function should guide neurobiological studies by studying changes in specific circuits known to underlie the affected visual function. Finally, the range of inter-individual variability in aging raises an important question: what

makes one individual age "well" (normal vision) while another one doesn't? Understanding the environmental factors that contribute to the long term outcome of aging will be critical for 'optimizing' the aging process. For example, something as simple as diet may have a great impact on the synaptic mechanisms that mediate plasticity and vision (Spolidoro et al., 2011).

Summary

The findings in this thesis provide new insight into the cortical changes that underlie vision loss. Using an animal model of amblyopia and aging, the studies in this thesis revealed many similarities and differences between two different conditions that share similar features of vision loss. The loss of proteins associated with glutamatergic and GABAergic synapses may underlie a common mechanism that affects the synaptic machinery necessary to support synaptic plasticity and the physiology underlying vision. To gain a full understanding of the neural basis of vision loss, more experiments that use physiological, anatomical, and behavioural work will be needed to fully understand the impact on the cortical circuitry in V1 that underlies experience-dependent plasticity and visual perception. Together, this will not only yield important information for developing new treatments and preventative measures for vision loss, but also important information regarding our basic understanding of the development of the visual system, and the neuronal mechanisms that support vision.

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