Functional connectivity favors aberrant visual network c-Fos expression accompanied by cortical synapse loss in amyloidosis.

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Abstract

While Alzheimer's disease (AD) has been extensively studied with a focus on cognitive networks, sensory network dysfunction has received comparatively less attention despite compelling evidence of its significance in both Alzheimer's disease patients and mouse models. We recently found that neurons in the primary visual cortex of an amyloid mouse model exhibit an imbalance of postsynaptic structures favoring neuronal hyperactivity alongside increased c-Fos expression, which regulates plasticity and memory. Here, we investigate aberrant visual network and brain-wide c-Fos expression and functional connectivity patterns, network responses to light deprivation, and visual system presynaptic deficits of a mouse model of Alzheimer's disease. We found that the mouse model of AD exhibits aberrant c-Fos expression and functional connectivity patterns across multiple brain regions, and functional connectivity between brain regions is a significant predictor for aberrant c-Fos expression. We also show that one week of light deprivation increases c-Fos expression across the brain in nonpathological controls but not the AD model, indicating experience-dependent plasticity deficits in multiple brain regions. Using *in vivo* and *ex vivo* imaging of presynaptic termini, we found that aberrant visual cortical c-Fos expression is associated with selective loss of excitatory cortical but not inhibitory or subcortical synapses. Our findings reveal novel structural and functional connectivity deficits in the visual network pre-plaque amyloidosis.

Introduction

Accumulating evidence underscores the coincidence of amyloid accumulation with cellular and synaptic loss within the visual cortex in individuals affected by Alzheimer's disease (AD) (Armstrong, 2009; Beach, Walker and McGeer, 1989; Cui et al., 2007; Frisoni et al., 2009; Hill et al., 2014; Hof and Morrison, 1990; Hwang et al., 2021; Ikonomovic et al., 2005; Jorge et al., 2020; Kurucu et al., 2022; Leuba and Kraftsik, 1994; Lewis et al., 1987; Mavroudis et al., 2011; Metsaars et al., 2003; Morrison, Hof and Bouras, 1991). AD patients exhibit visual impairments in contrast sensitivity, depth perception, and facial recognition (Cormack, Tovee and Ballard, 2000; Kirby, Bandelow and Hogervorst, 2010; Kusne et al., 2017; Mandal, Joshi and Saharan, 2012; Murphy, 2019; Zhang et al., 2023). In some patients, visual system disruption and visuospatial deficits emerge early in the disease progression, particularly in those developing the posterior cortical atrophy variant of AD (Best, Chapleau and Rabinovici, 2023; Chen et al., 2019; Crutch et al., 2012; Holden, Bettcher and Pelak, 2020; Maia da Silva et al., 2017; North et al., 2021; Yerstein et al., 2021). In these patients, cortical areas of the visual system frequently display irregular patterns of activation and functional connectivity (Hao et al., 2005). Mouse models of AD have also shown structural and functional visual system disruption (Chen et al., 2022; Chiquita et al., 2019; Criscuolo et al., 2018; Grienberger et al., 2012; Liebscher et al., 2016; Maya-Vetencourt et al., 2014; Niraula et al., 2023; Niraula, Yan and Subramanian, 2023; Papanikolaou et al., 2022; Rosales Jubal et al., 2021; Rudinskiy et al., 2012; van Veluw et al., 2020; William et al., 2012; William et al., 2021). Consistently, we also observed an increase in the expression of the immediate early gene c-Fos in the primary visual cortex of an amyloid mouse model (Niraula et al., 2023).

c-Fos is rapidly and transiently expressed following membrane depolarization and calcium influx in some neurons (Barth, Gerkin and Dean, 2004; Kawashima, Okuno and Bito,

2014; Mahringer et al., 2022; Morgan et al., 1987; Zangenehpour and Chaudhuri, 2002). Its low baseline expression, widespread expression profile, and close associations with learning and memory recall have made c-Fos^{+} cell labeling an attractive tool for mapping and manipulating experience and memory associated neuronal ensembles (Barth, Gerkin and Dean, 2004; Guenthner et al., 2013; Liu et al., 2012; Reijmers et al., 2007). c-Fos^{+} neurons fire more frequently and are more likely to share direct and indirect connections than c-Fos^{-} neurons (Yassin et al., 2010). Due to these properties, correlated c-Fos expression between brain regions has frequently been used to represent functionally connected networks in a variety of contexts (Borcuk et al., 2022; Silva, Burns and Graff, 2019; Tanimizu et al., 2017; Teles et al., 2015; Vetere et al., 2017; Wheeler et al., 2013). Our recent observations of elevated visual cortical c-Fos expression, pro-excitatory shifts in postsynaptic structures, and impaired visual memory in an amyloid mouse model are suggestive of a structurally and functionally impaired visual network (Niraula et al., 2023).

Here, we used a pre-plaque (5-6-months-old) mouse model of AD (J20 line (hAPP mice)) (Mucke et al., 2000) with human-like cortical amyloid deposition (Whitesell et al., 2019) to test how amyloid pathology disrupts visual network connectivity and whether functional connectivity contributes to aberrant c-Fos expression patterns. We also investigated the structural changes to excitatory and inhibitory presynaptic boutons in the visual network (primary visual cortex and dorsal lateral geniculate nucleus). We found that c-Fos^{+} cell density is selectively increased in cortical regions of the visual network of hAPP mice, which showed strengthened functional interconnectivity. Furthermore, a region's functional connectivity strength under nonpathological conditions predicts its shifts in c-Fos^{+} cell density in hAPP mice. Intriguingly, one week of light deprivation increased c-Fos^{+} cell density in the visual network of nonpathological (wild type

(WT)) mice but not hAPP mice. In addition to functional disruptions of the visual network, we found structural evidence of subtype-specific loss of excitatory presynaptic termini in the visual cortex but not in the subcortical lateral geniculate nucleus. However, short-term bouton dynamics were unchanged in hAPP mice.

Results

Aberrant c-Fos$^+$ cell distribution emerges in cortical regions of the visual network in hAPP mice.

Using *in vivo* calcium transient imaging in awake mice, we recently found that neurons in the visual cortex of ~5-month-old hAPP-overexpressing mice exhibit aberrant activity (Niraula et al., 2023). Consistently, we also found increased c-Fos$^+$ cell density in the primary visual cortex compared to WT littermates. To test whether increased c-Fos$^+$ cell density is a general feature of the visual network and brain at large, we performed c-Fos immunohistochemistry on 24 evenly spaced coronal slices spanning the brain of hAPP mice and WT littermate controls and mapped them to the Allen Mouse Brain CCFv3 for regional analysis (Figure 1A-C, see Supplementary Table 1 for region abbreviations). Cells were automatically counted with consistent parameters for size range, circular shape, and a local brightness threshold, which was set such that dim cells with near baseline c-Fos expression were excluded (Supplementary Figure 1A). Increases in c-Fos$^+$ cell density were nonuniform in hAPP mice (brain region × genotype interaction $P < 0.0001$, Supplementary Table 2), with posterior dorsal cortical regions showing sharper increases in c-Fos$^+$ cell density (~2-3-fold increase) than thalamic and midbrain regions (~1.2-2-fold increase). Increased c-Fos$^+$ cell density was also observed in olfactory and hippocampal areas, which commonly exhibit amyloidosis and functional deficits in mouse models of AD (Reale et al., 2018; Wesson et al., 2010; Wu et al., 2013). Notably, the visual cortex displayed, on average,

the densest c-Fos$^+$ cells of any region in WT mice (260.7 cells/mm^2) and the largest average magnitude of increased c-Fos$^+$ cell density in hAPP mice (+442.7 cells/mm^2).

We next asked whether aberrant c-Fos$^+$ density is localized to specific layers or subregions of cortical visual areas. Within the primary visual cortex, we found that all layers containing neuronal cell bodies, including layer 4, which receives dense subcortical inputs from the dorsal part of the lateral geniculate nucleus (Ji et al., 2016; Seabrook et al., 2017), show elevated c-Fos$^+$ cell density in hAPP mice (Supplementary Figure 2). We additionally found a statistically significant brain region × genotype interaction across visual network regions ($P < 0.0001$, Supplementary Table 3) and that c-Fos$^+$ cell density was significantly higher in most higher-order visual cortical areas in hAPP mice (Figure 1D, Table 2). To test whether subcortical visual network regions upstream of the visual cortex display dense neuronal activation patterns, we next quantified c-Fos$^+$ cell density in the lateral geniculate nucleus and superior colliculus. However, we found no significant difference between hAPP and WT mice in these regions (Figure 1D, Table 2). These results indicate that aberrant patterns of neuronal activation are widespread across cortical but not upstream subcortical areas of the visual system.

To investigate the contribution of light experience to aberrant c-Fos expression in the hAPP visual network, we housed WT and hAPP mice in complete darkness for one week and quantified c-Fos$^+$ cell density. Interestingly, c-Fos$^+$ cell density was significantly increased in WT mice across many areas of the brain (Figure 1C, Table 1), including cortical areas of the visual system (Figure 1D, Table 2). In contrast, c-Fos$^+$ density in hAPP mice was almost entirely unchanged after one week of light deprivation. Notably, the superior colliculus, which receives direct retinal projections (Ito and Feldheim, 2018; Seabrook et al., 2017), uniquely showed reduced c-Fos$^+$ cell density following light deprivation in hAPP mice (Figure 1D, Table 2).

These results suggest that aberrant c-Fos$^+$ cell density in the visual network is localized to the cortex in early-stage amyloidosis and that c-Fos expression is sensitive to light deprivation.

We tested if the c-Fos$^+$ cell density increase in the primary visual cortex during long-term light deprivation persists when we reduce the deprivation duration to minimize circadian rhythm impact (Kress et al., 2018). This involved a shift from a 12-hour light/dark cycle to a 24-hour dark cycle, resulting in ~40 hours of continuous darkness, including the 12-hour dark cycle from the previous day and 4 hours of darkness on the following day. WT mice (318.50 ± 86.71 c-Fos+ cells/mm^2) showed nearly a 2-fold increase over density observed in the hAPP mice (115.60 ± 22.46 c-Fos+ cells/mm^2). Though this trend did not reach statistical significance (P = 0.059, student's *t* test with Welch's correction; n = 7 WT and 5 hAPP mice), the variance was nearly four times as high in the WT group (P = 0.011, F test to compare variances), indicating that shorter duration light deprivation increases c-Fos in some but not all WT mice.

Functional connectivity favors aberrant c-Fos$^+$ cell density across the brain of hAPP mice

Correlated c-Fos$^+$ cell density in a pair of regions indicates functional connectivity between those regions (Silva, Burns and Graff, 2019; Tanimizu et al., 2017; Teles et al., 2015; Vetere et al., 2017; Wheeler et al., 2013). Previous studies have shown that hyperconnectivity is associated with excessive neuronal activation in early AD networks (Corriveau-Lecavalier et al., 2021; Koelewijn et al., 2019). To investigate whether hAPP mice display hyperconnectivity, we calculated functional connectivity strength for every pair of imaged regions (Pearson's r of c-Fos$^+$ density) both within the visual network and across the brain. Visual network (Figure 2A, left) functional connectivity matrices revealed an increase in pairwise correlation strength between brain regions in hAPP (bottom) relative to WT (top) mice, represented by warmer colors across the hAPP matrix. One week of light deprivation, which increases c-Fos$^+$ density, also increased functional connectivity across the WT visual network, including subcortical

regions (Figure 2B, top left). In contrast, hAPP visual network functional connectivity was largely unaltered by light deprivation (Figure 2B, bottom left). Overall, the average functional connectivity of the visual network, measured as the mean of z values (Fisher's r-to-z transformation) of each brain region in the network, showed a significant effect of genotype, light, and their interaction (Figure 2C, left).

We extended this analysis across the entire brain (Figure 2A, B, right) and observed a significant effect of genotype and light, but not their interaction, indicating that dark adaptation does not increase average functional connectivity across the entire brain in hAPP mice (Figure2C, right). Intriguingly, the increase in dark-associated connectivity in hAPP whole brain network was qualitatively different from that of the WT network. In the WT network, visual deprivation increased functional connectivity between some lateral cortical and subcortical regions, whereas in hAPP mice, it was distributed across nearly all cortical and subcortical regions (Figure 2B, bottom right). Interestingly, the dentate gyrus, which displayed the lowest average functional connection strength in WT mice, was the only region to yield a negative average z across the hAPP brain. Progressive depletion of calcium-dependent proteins and reductions in c-Fos^{+} cells have been observed in the dentate gyrus J20 mice of this age (Palop et al., 2003), likely contributing to decoupled functional connectivity with other regions. These findings indicate that both amyloid pathology and light deprivation increase visual network functional connectivity in mice.

Abnormal c-Fos^{+} cell density, observed in multiple brain regions of hAPP mice (Figure 1C-D), may emerge independently or may arise in part due to the propagation of abnormal activity in other regions via functional connectivity. Thus, we next investigated whether a given region's average network connection strength under nonpathological conditions is predictive of

the increase in its c-Fos$^+$ cell density in amyloidosis. We correlated each region's average connection strength across the WT brain-wide or visual network (average z) with the effect size of hAPP overexpression on c-Fos$^+$ density (Cohen's *d*) for that region. A significant positive correlation emerged between these factors, both within the visual (Figure 2D, left) and the whole brain (Figure 2D, right) networks. Furthermore, nonpathological functional connection strength explained ~38% and ~28% of the variance in the effect size of c-Fos$^+$ density in the hAPP visual network and the whole brain, respectively. These results indicate that regions with stronger functional connections in nonpathological conditions are predisposed to display aberrantly dense c-Fos$^+$ cells in amyloid pathology.

Amyloidosis selectively weakens an excitatory presynaptic terminal subtype in the visual cortex.

We previously observed functional connectivity alterations that coincide with pro-excitatory structural postsynaptic alterations *in vivo* in layer 2/3 visual cortical neurons of pre-plaque J20-hAPP mice (Niraula et al., 2023). To test whether pro-excitatory presynaptic structural changes also occur in the visual cortex in this model, we immunolabeled excitatory presynaptic vesicular transporters (VGluT1 and VGluT2) and inhibitory presynaptic vesicular transporters (VGAT) in two visual network regions of standard-housed (12h light/dark cycle) mice: the primary visual cortex and the dorsal part of the lateral geniculate nucleus (see Supplemental Figure 2B for low magnification expression patterns). Automated puncta identification (Figure 3A, Supplementary Figure 2C) revealed a significant decrease in VGluT1$^+$ but not VGluT2$^+$ or VGAT$^+$ puncta densities in the primary visual cortex (with elevated c-Fos expression Figure 1D, Table 2) of hAPP mice, while the dorsal part of the lateral geniculate nucleus (without significant c-Fos elevation, Figure 1C) showed no significant changes in puncta densities (Figure 3B-D). Note that

the puncta densities do not reflect the absolute number of synapses but rather indicate relative differences between genotypes (See Supplementary Figure 2C-D for synapse detection details).

Amyloidosis induces morphology-specific weakening and loss of excitatory presynaptic boutons *in vivo* but does not influence short-term dynamics.

Though the decrease in above threshold immunolabeled VGluT1^{+} puncta is indicative of excitatory synapse loss in the visual cortex, the high density of cortical excitatory terminals necessitates alternate validation. To further confirm that early amyloidosis induces excitatory presynaptic loss in the visual cortex, we analyzed the density and dynamics of presynaptic boutons from datasets of excitatory layer 2/3 visual cortical neurons imaged *in vivo* using two-photon microscopy. Neurons of anesthetized head-fixed mice were imaged in two sessions separated by one week of normal 12h light/dark cycle, and axons were manually identified by shaft morphology and absence of labeled postsynaptic structures (Figure 4A). We counted presynaptic boutons >3 standard deviations brighter than the axonal shaft and manually classified them as en passant or terminaux (Figure 4B). We found that en passant bouton density in hAPP mice was not different from that in WT mice (Figure 4C, left), but terminaux bouton density was significantly reduced (Figure 4C, right). However, en passant boutons were dimmer (Figure 4D, left), while terminaux bouton fluorescence did not differ (Figure 4D, right). These results are consistent with VGluT1 immunostaining and indicate that excitatory presynaptic terminals are weakened or lost in amyloidosis.

To test whether the structural dynamics of presynaptic boutons are altered in amyloidosis, we calculated the gain and loss of layer 2/3 visual cortical boutons in mice between two imaging sessions separated by one week of standard 12h light/dark housing (Figure 4E). The percentage of stable, gained, and lost boutons were similar between the genotypes for both en passant and terminaux boutons (Figure 4F-H). Furthermore, en passant and terminaux boutons present in

both imaging sessions showed no alterations in their brightness between the sessions (Figure 4I). These results suggest that the loss of boutons of layer 2/3 visual cortical neurons occurs at an earlier age or that bouton loss occurs gradually enough to be undetectable over one week. Alternatively, low bouton density may not represent bouton loss per se but rather developmental deficit due to early expression of the hAPP transgene.

Discussion

AD is described as a synaptic and network disease due to extensive synapse loss and network abnormalities. The main goal of this study is to map network reorganization and synaptic vulnerability in the visual system in a mouse model of AD. The study is motivated by a relatively limited understanding of visual system dysfunction in AD-linked pathology despite multiple visual abnormalities observed in AD patients, particularly those with posterior cortical atrophy (Crutch et al., 2012). Similarly, AD mouse models have repeatedly shown that increased amyloid is associated with aberrant neural activity and calcium levels, including in the visual cortex (Arbel-Ornath et al., 2017; Asavapanumas et al., 2019; Bai et al., 2017; Brawek and Garaschuk, 2014; Busche et al., 2012; Calvo-Rodriguez and Bacskai, 2021; Chakroborty et al., 2012; Lerdkrai et al., 2018; Zhang et al., 2015). Since neural activity elicits c-Fos expression (Morgan et al., 1987) and has recently been exploited to map brain-wide functional connectivity, we quantified c-Fos expression across multiple brain regions. We found that cortical but not subcortical areas of the visual system exhibit increased c-Fos$^+$ cell density and reduced excitatory synapses and that nonpathological network functional connectivity patterns are predictive of pathological c-Fos expression in the pre-plaque stage of amyloid pathology.

Aberrant c-Fos expression in the visual system.

We found that the highest increases in c-Fos$^+$ cell density occur in posterior cortical brain regions involved in the processing of visual information that develops high amyloid plaque

density at an older age in this mouse model (Whitesell et al., 2019). If visual experience evoked activity contributed to c-Fos$^+$ cell density, we reasoned that manipulating visual experience would alter this density. Prolonged visual deprivation (≥5 days) triggers the reorganization of visual circuits in adult rodents, including increased NR2b/NR2a ratio, reduced GluR2/$GABA_A$ ratio (He, Hodos and Quinlan, 2006), and retraction of inhibitory dendrites or synapses (Chen et al., 2011; Keck et al., 2011; van Versendaal et al., 2012), which together may lower the threshold for neuronal activation (Bridi et al., 2018). Additionally, we recently found that one week of visual deprivation increased inhibitory synapse loss in WT but not hAPP mice (Niraula, Yan and Subramanian, 2023). We now show that c-Fos$^+$ cell density increased in WT but not hAPP mice following one week of visual deprivation. We speculate that inhibitory synapse loss elicited by visual deprivation in WT mice creates a permissive environment for the plasticity of non-visual inputs, including cross-modal inputs, to the visual cortex. Consistently, we found that one week of visual deprivation broadened spontaneous but not visually evoked calcium transients in WT mice (Niraula, Yan and Subramanian, 2023). Such cross-modal plasticity may serve as a novelty signal in the visual system and beyond, leading to higher c-Fos expression. In contrast, the lack of inhibitory synapse loss in the visual cortex of hAPP mice following light deprivation would restrict the plasticity of non-visual inputs, as evidenced by the lack of broadening of spontaneous activity (Niraula, Yan and Subramanian, 2023). Therefore, hAPP mice with higher c-Fos$^+$ cells in normal light housing conditions would not show further increases in c-Fos levels during light deprivation. Higher c-Fos in hAPP mice during normal light housing could be due to a defect in habituation to repeated everyday visual experiences. Consistent with this notion, we previously found that visual cortical neurons do not adapt to repeated experiences of the same stimulus (Niraula et al., 2023). Together, we propose that the visual system of hAPP mice senses novelty

regardless of light experience, whereas that of WT mice habituate to home cage visual experiences under light housing and sense novelty in extended periods of darkness, presumably due to cross-modal plasticity.

Functional connectivity and aberrant c-Fos expression.

Under normal visual experience, c-Fos$^+$ cell density is increased in multiple brain regions in hAPP mice compared to WT mice. Neuronal hyperactivity may emerge independently in different brain regions due to localized instances of imbalanced synaptic excitation and inhibition (Bi et al., 2020; Maestu et al., 2021; Petrache et al., 2019; Ruiter, Herstel and Wierenga, 2020; Vico Varela, Etter and Williams, 2019), potentially leading to higher c-Fos$^+$ cell density. However, in both AD patients and mouse models of amyloidosis, there is considerable heterogeneity in the extent and type of synaptic disruption across brain regions (Berchtold et al., 2013; Kirvell, Esiri and Francis, 2006; Kurucu et al., 2022). The interconnected nature of brain networks allows for hyperactivity to spread to multiple brain regions without requiring local synaptic disruption. Therefore, variations in the extent of increased c-Fos$^+$ cell density may partly be attributable to variations in functional connectivity of brain regions. A hub region with stronger functional connectivity may display a larger increase in c-Fos$^+$ cell density compared to a brain region with weaker functional connectivity. Consistently, we found that functional connectivity in WT mice can predict ~38% of the variance in effect sizes of hAPP overexpression on c-Fos$^+$ cell density increases, indicating that at least a part of the increase in c-Fos$^+$ cells in these regions could stem from the spread of aberrant activity through functional connections.

Differential sensitivity of presynaptic termini subtypes

Using *in vivo* multiphoton structural imaging of postsynaptic structures in the visual cortex of hAPP mice, we recently found a pro-excitatory shift in the ratio of excitatory to inhibitory postsynaptic structures (Niraula et al., 2023). Therefore, we expected pro-excitatory shifts in presynaptic structures in the form of increased VGluT1/2$^+$ puncta and/or decreased VGAT$^+$ puncta density. However, we found that VGluT1$^+$ puncta, representing excitatory synapses of cortical origin, are reduced in the visual cortex of hAPP mice.

Presynaptic termini are highly vulnerable at the initial stages of amyloid accumulation (Barthet and Mulle, 2020; de Wilde et al., 2016; Hark et al., 2021; He et al., 2019; Russell et al., 2012; Sze et al., 2000; Trujillo-Estrada et al., 2014). Reduced excitatory presynaptic density has repeatedly been shown to accompany amyloid pathology (Bell, Bennett and Cuello, 2007; Canas et al., 2014; Kashani et al., 2008; Kirvell, Esiri and Francis, 2006; Mitew et al., 2013; Mucke et al., 2000; Naito et al., 2017). Inhibitory synapses have also been shown to be vulnerable to amyloid toxicity, but the results are inconsistent (Agarwal, Tannenberg and Dodd, 2008; Canas et al., 2014; Hollnagel et al., 2019; Kurucu et al., 2022; Mitew et al., 2013; Naito et al., 2017; Niraula et al., 2023; Palop et al., 2007; Ruiter, Herstel and Wierenga, 2020; Verret et al., 2012). Across excitatory synapses, we found VGluT1$^+$ structures to be more vulnerable than VGluT2$^+$ structures in amyloid pathology. One possible cause could be higher amyloid accumulation in VGluT1$^+$ boutons than in VGluT2$^+$ boutons (Sokolow et al., 2012). An alternative possibility is that VGluT1$^+$ puncta predominantly reflect presynaptic termini from cortical neurons, which display elevated c-Fos$^+$ cell density, whereas VGluT2$^+$ structures in the mammalian visual cortex represent projections of the dorsal lateral geniculate nucleus (Balaram, Hackett and Kaas, 2013; Balaram, Takahata and Kaas, 2011; Fremeau et al., 2001; Garcia-Marin et al., 2013; Nahmani

and Erisir, 2005), which in contrast does not show significant changes to c-Fos^{+} cell density. Further experiments are needed to test whether aberrant activity is associated with presynaptic weakening and whether subcortical inputs carrying VGluT2 display less aberrant activity than VGluT1 expressing cortical inputs.

Interestingly, within excitatory synapses of cortical origin, we observed morphology-specific alterations in hAPP mice; en passant boutons appear dimmer in hAPP mice but are equally dense as WT mice, whereas terminaux boutons are sparser without appearing dimmer in hAPP mice. Though functional differences between en passant and terminaux boutons are not well understood, morphology-based modeling suggests that boutons on extremely small terminal branches are especially sensitive to changes in membrane potential (Luscher and Shiner, 1990). Additionally, terminaux boutons are less likely than en passant boutons to harbor mitochondria, which are more commonly found in stable boutons than unstable boutons (Lees, Johnson and Ashby, 2019). These findings are also consistent with a recent study showing reduced terminaux bouton density but unaltered postsynaptic spine density in the barrel cortex of the same mouse model (Stephen et al., 2019). A reduction in presynaptic structure density but normal or elevated postsynaptic structure density indicates that each presynaptic structure may be associated with multiple postsynaptic structures. Thus, early stages of amyloidosis could be associated with increased multi-synaptic boutons (Martinez-Serra et al., 2022).

Hyperconnectivity and synapse loss in the visual system in amyloidosis

How can excitatory cortical synapse loss be reconciled with hyper-functional connectivity based on the correlated increase in c-Fos expressing neurons in the visual network? We recently found

that an increased fraction of neurons become non-responsive to visual stimuli in hAPP mice. Simultaneously, the remaining responsive neurons became hyperactive in these mice (Niraula et al., 2023). One possibility is that excitatory synapse loss is an adaptation to neuronal hyperactivity, reducing the propensity for neurons to activate their neighbors, in turn producing non-responsive neurons. Neurons that remain hyperactive could strongly express c-Fos and become hyperconnected. Alternatively, excitatory synapse loss could also emerge due to chronic hyperconnectivity. Functional connectivity positively correlates with amyloid levels across the brain in humans (Sepulcre et al., 2017). Neuronal hyperactivity also promotes local amyloid accumulation (Cirrito et al., 2005; Jones et al., 2016; Leal et al., 2017; Stargardt, Swaab and Bossers, 2015; Yamamoto et al., 2015), which in turn promotes hyperactivity (Bero et al., 2011; Fogel et al., 2014; Harris et al., 2020; Kazim et al., 2017; Kazim et al., 2021; Mattson et al., 1992; Targa Dias Anastacio, Matosin and Ooi, 2022) in a vicious cycle (Zott et al., 2019). Synaptic failure, which is ameliorated by reducing neuronal hyperactivity (Haberman, Branch and Gallagher, 2017; Sanchez et al., 2012), could be an eventual outcome of this vicious cycle enabled by the spread of hyperactivity through functional connectivity.

Materials and Methods

Mice

All animal procedures are approved by the University of Kansas Institute of Animal Use and Care Committee and meet the NIH guidelines for the use and care of vertebrate animals. J20 mice (B6.Cg-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799Vas/Mmjax, RRID: MMRRC_034848-JAX) was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at The Jackson Laboratory, an NIH-funded strain repository, and was donated to the MMRRC by Robert Vassar, Ph.D., Northwestern University. They were maintained as heterozygotes for the hAPP transgene by breeding heterozygous J20-hAPP male mice with WT

female mice. A maximum of five mice were housed in a standard cage but individually housed after the cranial window surgery. Mice were housed on a 12h-light/12h-dark cycle except for the group that went through a period of light deprivation (24h-dark). For light deprivation, mice were placed in a ventilated, lightproof cabinet inside of a dark room for seven days. Infrared goggles with a low-intensity 850nm wavelength emission source were used at all times while maintaining mice in dark housing to prevent light exposure. All mice used for c-Fos immunohistochemistry were housed with 2-3 littermates.

Tissue Preparation and Immunohistochemistry

Mouse cages were brought to the surgical suite and remained undisturbed for at least five hours before brain extractions to avoid capturing c-Fos expression elicited by cage movement or contextual novelty. Dark-housed mice were brought to the surgical suite in a light-blocking container and anesthetized in a dark room using infrared goggles to prevent light-evoked c-Fos expression. 3.5-6-month-old (presynaptic terminal immunohistochemistry) or 5-6-month-old (c-Fos immunohistochemistry) J20-hAPP and WT littermate mice were deeply anesthetized by intraperitoneal injection of 2% avertin in phosphate-buffered saline (PBS), pH 7.4 and transcardially perfused with cold PBS followed by 4% paraformaldehyde. The brains were extracted and post-fixed in 4% PFA overnight at 4°C, followed by storage in PBS. For 40μm slicing, the brains were embedded in 4% oxidized agarose as previously described (Ragan et al., 2012) to limit artifacts during sectioning and sliced on a vibratome (Leica VT1000 S). For 20μm slicing, brains were cryoprotected overnight at 4°C in 15% (w/v) and then in 30% (w/v) sucrose in phosphate buffer (PB). The brains were sectioned coronally on a microtome (Leica SM 2010R) and collected in PBS with sodium azide (0.02%).

24 evenly spaced 40μm slices from each brain spanning the posterior midbrain to the anterior olfactory bulb were fluorescently immunolabeled for c-Fos, and 3 evenly spaced 20-

40μm slices spanning the visual cortex and dorsal part of the lateral geniculate nucleus (1-2 slices for each region per mouse) were fluorescently immunolabeled for $VGAT^+$, $VGluT1^+$, and $VGluT2^+$ puncta. Sections were permeabilized for 2h at room temperature in a 1% TritonX-100 and 10% normal goat serum (NGS) solution in PBS followed by incubation with rabbit anti-c-Fos (1:1000, CST cat. #2250S) or rabbit anti-VGAT (1:1000, Synaptic Systems cat. #131002), mouse anti-VGluT1 (1:2000, Sigma cat. #MAB5502), and guinea pig anti-VGluT2 (1:1000, Sigma cat. #AB2251-I) in a PBS solution containing 0.1% TritonX-100 and 5% NGS overnight at 4°C. Sections were then washed 3× with PBS and incubated with Alexa 555-conjugated goat anti-rabbit antibody (1:2000; Fisher cat. #PIA32732) for c-Fos immunohistochemistry or Alexa 488 conjugated goat anti-rabbit antibody (1:2000, Fisher cat. #A11008), Alexa 555-conjugated goat anti-mouse antibody (1:2000, Fisher cat. #A32727), and Alexa 647-conjugated goat anti-guinea pig antibody (1:2000, Fisher cat. #A21450) for presynaptic terminal immunohistochemistry for two hours in a PBS solution containing 0.1% TritonX-100 and 5% NGS at room temperature, followed by three washes with PBS before mounting on glass slides. Slices were imaged using an ImageXpress Pico automated imaging system (Molecular Devices, San Jose, CA) with a 10× objective for c-Fos acquisition (Leica HC PL FLUOTAR 10x/0.32) or a 63× objective for presynaptic termini acquisition (Leica HC PL FLUOTAR 63x/0.70 CORR).

Immunofluorescence Quantification and Statistical Analysis

Slice registration, cell/synapse detection, puncta fluorescence collection, and brain region area measurements were performed using NeuroInfo software (MBF Bioscience, Williston, VT). 12-bit slice images were first mapped in 3D to the Allen CCF v3 to allow automated cell/synapse detection and area measurement by region. A total of 31 regions covering the entire cerebral cortex, cerebral nuclei, interbrain, and midbrain, and 17 subregions of the visual network were mapped for analysis. Bright circular objects against a darker background were automatically

detected using a scale-space analysis of the response to Laplacian of Gaussian (LoG) within the expected size range and shape of labeled cell body or presynaptic puncta diameters as described (Kong, Akakin and Sarma, 2013). Briefly, cells/synapses were filtered out from all identified objects with a user-defined threshold based on the strength of the LoG response within an expected range of cell body or presynaptic terminal diameters. The LoG threshold value was set at LoG threshold = 55 for cell bodies and 101 for all presynaptic puncta (range 0-255). Only objects above the respective LoG strength threshold were included in the analysis, and identified objects were manually proofread to eliminate false positives. The c-Fos cell detection threshold was set such that dimly labeled cells that represent near baseline c-Fos expression were not counted as "c-Fos$^+$". All regions in both hemispheres of the 24 brain-wide slices were analyzed for c-Fos$^+$ cell detection. Cell/synaptic puncta density for each mouse was calculated by dividing the total number of cells or synapses per region by the area per region across all slices for each brain (24 slices for c-Fos, 2-3 slices for synapses). Pearson's r was calculated between each pairwise combination of brain regions either within the whole brain or visual network for light and dark conditions, and average z values were calculated following Fisher's r-to-z transformation for each region within its respective network. 8 WT light (5 males, 3 females), 9 WT dark (4 males, 5 females), 10 hAPP light (4 males, 6 females), and 8 hAPP dark housed mice (3 male, 5 females) were used for c-Fos$^+$ cell density analysis.

For presynaptic terminal quantification, 1-2 ~230 × 900μm columns per mouse spanning all layers of one hemisphere of the visual cortex was analyzed, and 1-2 ~230 × 375μm columns per mouse vertically spanning one hemisphere of the dorsal lateral geniculate nucleus were analyzed. Synaptic puncta fluorescence was determined by collecting the fluorescence from a 0.2μm (1.9 pixel) radius around the center of each identified puncta. Localized areas containing

artifacts were excluded from the analysis. 14 WT (9 males, 5 females) and 12 hAPP mice (6 males, 6 females) housed under the standard 12h light/dark cycle were used for presynaptic density and fluorescence analysis. Synaptic puncta fluorescence analysis in the visual cortex included a total of 759,230 WT & 550,683 hAPP $VGAT^+$ puncta, 386,322 WT & 223,032 hAPP $VGluT1^+$ puncta, and 330,103 WT & 239,168 hAPP $VGluT2^+$ puncta. For presynaptic terminal clustering percentage evaluation, a 10µm × 900µm column for each mouse spanning all layers of one hemisphere of the visual cortex was analyzed. All detected puncta in the columns were evaluated for incorrect clustering, and initially uncounted puncta were added. The percentage (%) of undercounted puncta was calculated by dividing the number of uncounted puncta by the number of automatically detected puncta. Representative images were generated in FIJI (NIH, Bethesda, MD), and all figures and statistical tests were performed in GraphPad Prism (GraphPad Software, San Diego, CA).

In Vivo Presynaptic Structural Analysis

The presented in vivo presynaptic structural data is an independent analysis performed from the same raw dataset acquired as previously described (Niraula et al., 2023; Niraula, Yan and Subramanian, 2023). Briefly, Cre-dependent TdTomato cell fill (pFudioTdTomatoW), Teal-gephyrin (pFudioTealgephyrinW), and PSD95-venus (pFudioPSD95venusW) were injected into the right lateral ventricle of E15.5-16.5 embryos to fluorescently label excitatory L2/3 neurons as previously described (Tabata and Nakajima, 2001). A pair of platinum electrodes (Protech International) and a square wave electroporator (ECM830, Harvard Apparatus) were used to target these plasmids toward the visual cortex. 4-6-month-old J20 and WT mice received a cranial window over the visual cortex of the right hemisphere, and optical intrinsic signal imaging was performed 14 days later to map the location of the visual cortex (Kalatsky and Stryker, 2003; Niraula et al., 2023). L2/3 excitatory visual cortical neurons were imaged in

anesthetized head-fixed mice in two sessions separated by one week of normal 12h light/dark cycle. TdTomato-filled axons were first manually identified based on branch morphology and absence of postsynaptic markers, then en passant and terminaux boutons were manually labeled based on morphology using a modified version of the ObjectJ plugin (Villa et al., 2016) for FIJI (Schindelin et al., 2012). Puncta on or protruding from axonal shafts were scored as boutons only if they were present in two consecutive z-frames. After proofreading of the bouton identification and classification in both sessions by another investigator, the marked boutons were subjected to an unbiased intensity-based threshold relative to the nearby axonal shaft. 5-pixel × 5-pixel measurement ROIs were placed over the identified boutons and along their respective axonal shafts, and the fluorescent intensity of each bouton and its nearest 10-20 neighboring axonal shaft ROIs were collected using the modified ObjectJ plugin. Using the modified ObjectJ plugin, bouton IDs and locations were transferred from S1 to S2 image stacks, and newly formed and lost boutons were manually identified. Identified objects were excluded if their fluorescence did not exceed three times the standard deviation of fluorescence of the nearest neighboring axonal shaft ROIs. Bouton gain and loss were calculated as the number of gained or lost boutons between S1 and S2 divided by the number of boutons present in S1. The percentage of stable boutons was calculated as the number of boutons that persisted into S2 divided by the number of boutons in S1. The percentage of fluorescence change in stable boutons between S1 and S2 was calculated as S2 fluorescence divided by S1 fluorescence for each stable bouton where a bouton's fluorescence is represented by the number of standard deviations above the average fluorescence of the nearest 10-20 neighboring shaft ROIs in its respective session. Nearest neighbor and threshold calculations were performed using custom MATLAB scripts. All bouton metrics were calculated separately for en passant and terminaux boutons. All in vivo bouton data was obtained

from 7 WT cells (4 mice, 2 males and 2 females) and 8 hAPP cells (7 mice, 5 males and 2 females). For S1 bouton fluorescence distribution, a total of 347 en passant and 62 terminaux boutons were identified from WT cells, and 287 en passant & 19 terminaux boutons were identified from hAPP cells.

Figure Legends

Figure 1: **Amyloid or light deprivation increases c-Fos$^+$ cell density. A)** Representative images of 24 evenly spaced landmark slices used for brain-wide c-Fos$^+$ cell density quantification. **B)** Representative images of c-Fos fluorescence in the primary visual cortex of hAPP mice (right) and wildtype (WT) littermate controls (left). The blue box is zoomed in on each right panel. Blue circles indicate c-Fos$^+$ cells identified by automatic cell detection. The identification threshold was set such that dim cells representing close to baseline c-Fos expression were excluded. Scale bar: 1mm (left half of each panel), 100µm (right half). **C)** c-Fos$^+$ cell density across the brain for hAPP and WT mice housed under a standard 12h light/dark cycle (Light) and one week of light deprivation (Dark). See Table 1 for mixed-effects ANOVA results. **D)** c-Fos$^+$ cell density across regions of the visual network for hAPP and WT mice under a standard 12h light/dark cycle (Light) and one week of light deprivation (Dark). See Table 2 for mixed-effects ANOVA results. Data are presented as mean ± SEM. n = 8 WT light, 9 WT dark, 10 hAPP light, and 8 hAPP dark housed mice.

Figure 2: **Functional connectivity in nonpathological conditions correlates with amyloid or light deprivation associated with aberrant c-Fos expression. A)** Functional connectivity matrices showing pairwise correlation (Pearson's *r*) of c-Fos$^+$ density between regions in the visual (left) and the brain-wide (right) networks for WT (top) and hAPP (bottom) mice reared under 12h light/12 dark conditions (light). Region label color indicates anatomical brain area. Warmer colors indicate stronger pairwise functional connections between regions. **B)** Visual

network (left) and brain-wide (right) functional connectivity matrices for WT (top) and hAPP (bottom) mice following one week of light deprivation (dark). C**)** Each region's average correlation strength (average z following r-to-z transformation) across the visual (left) and whole brain (right) networks in WT and hAPP mice for light and dark conditions. Each data point represents the average functional connectivity strength (z) of one brain region across its respective network. Mixed-effects ANOVA with genotype (G), light exposure (L) and interaction (G×L) P-values shown. Data are presented as mean ± SEM. n = 8 WT light, 9 WT dark, 10 hAPP light, and 8 hAPP dark housed mice. D) Correlation of each region's average z across WT networks (visual, left; whole brain, right) with the effect size (Cohen's *d*) of hAPP on that region's c-Fos$^+$ cell density. n = 8 WT light and 10 hAPP light mice. Linear regression with 95% confidence intervals, correlation P-values, and R^2 are shown.

Figure 3: **Amyloid-associated synapse loss in the visual system is selective for VGluT1$^+$ presynaptic terminals. A)** Representative images of synaptic immunohistochemistry labeling VGluT1$^+$, VGluT2$^+$, VGAT$^+$, and merged puncta channels in layer 2/3 of the primary visual cortex in WT (top) and hAPP (bottom) mice housed under 12h light/dark cycle. Circles indicate puncta identified by automated synapse detection. Some circles contain dim puncta that have sufficient fluorescence above local background to meet the detection threshold. Scale bar: 10µm (merge), 5µm (individual channels). **B-D)** Density of VGluT1$^+$ (B), VGluT2$^+$ (C), and VGAT$^+$ (D) puncta in the primary visual cortex (VISp) and dorsal part of the lateral geniculate nucleus (dLGN), *P < 0.05, unpaired Student *t* tests.

Figure 4: **Amyloidosis induces excitatory terminaux bouton loss in L2/3 of the primary visual cortex *in vivo*. A)** Experimental timeline for *in vivo* presynaptic structural imaging. Imaging sessions (S1 and S2) were separated by one week. IUE – in utero electroporation, OIS –

optical intrinsic signal imaging to identify the visual cortex. **B)** Representative *in vivo* image of presynaptic boutons of excitatory layer 2/3 primary visual cortical neurons in WT (top) and hAPP mice (bottom). Example terminaux (filled arrowhead) and en passant (open arrowhead) boutons. Scale bar: 5µm. **C)** En passant (left) and terminaux (right) bouton density of excitatory layer 2/3 primary visual cortical neurons *P<0.05 Student's *t*-tests. **D)** Cumulative frequency distributions of S1 en passant (left) and terminaux (right) bouton brightness, represented as the number of standard deviations above nearby axonal shaft fluorescence. Kolmogorov-Smirnov tests, P-values displayed. **E)** Representative images of boutons that are gained (left, filled arrowhead) and lost (right, open arrowhead) between imaging sessions. Scale bar: 2µm. **F-I)** En passant (left) and terminaux (right) bouton dynamics, including percent stable (F), gained (G), lost (H), and fluorescence change (I) of boutons in S2 relative to S1. Data are presented as mean ± SEM, and individual data points represent cell averages. n = 7 cells from 4 WT mice, 8 cells from 7 hAPP mice for all.

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Author contributions: OJL, ASS, and JS designed the experiments and analysis. OJL, JM, SN, and GD performed the experiments and analyzed the data. OJL and JS wrote the manuscript. JS supervised the research.

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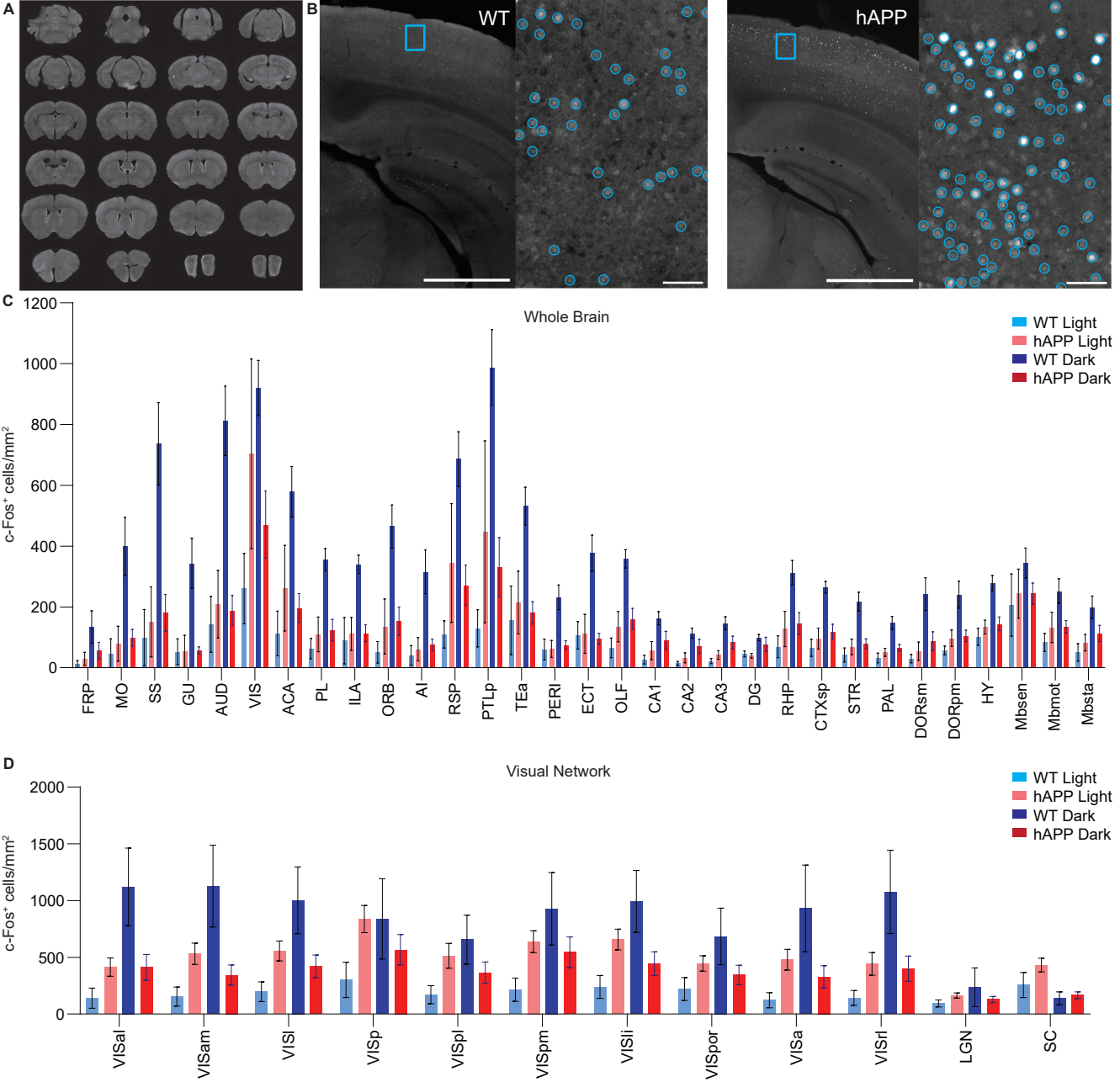
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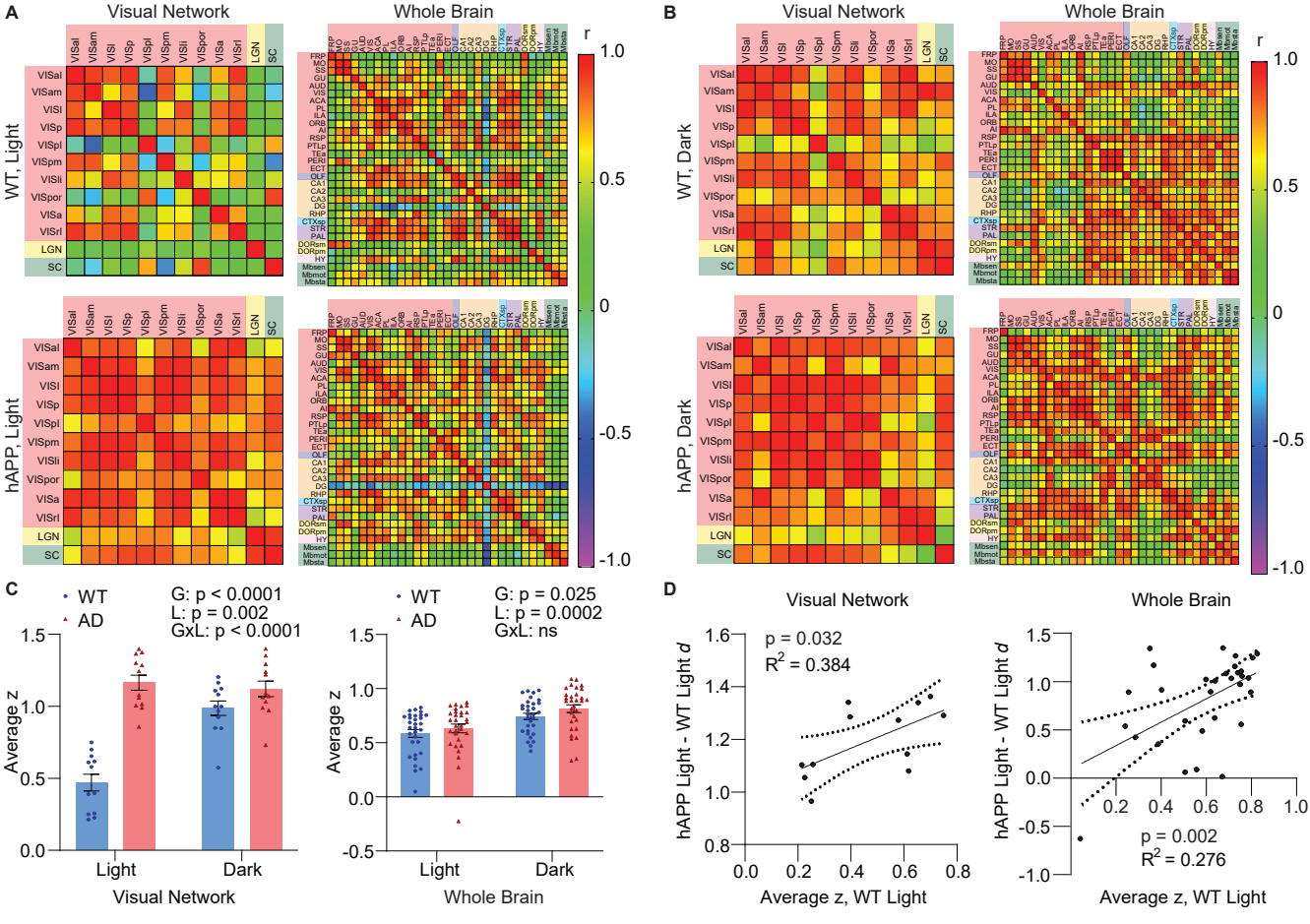
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C
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AD
G: p < 0.0001
L: p = 0.002
GxL: p < 0.0001
Average z
Light
Dark
Visual Network
G: p = 0.025
L: p = 0.0002
GxL: ns
Whole Brain
D
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p = 0.032
R2 = 0.384
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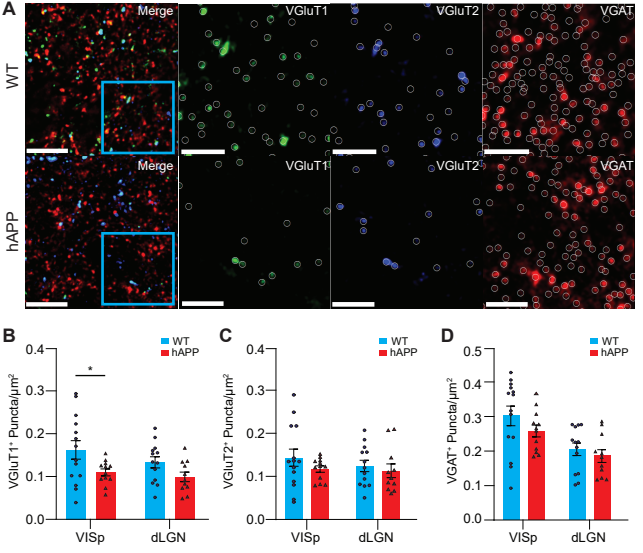
Average z, WT Light
Whole Brain
p = 0.002
R2 = 0.276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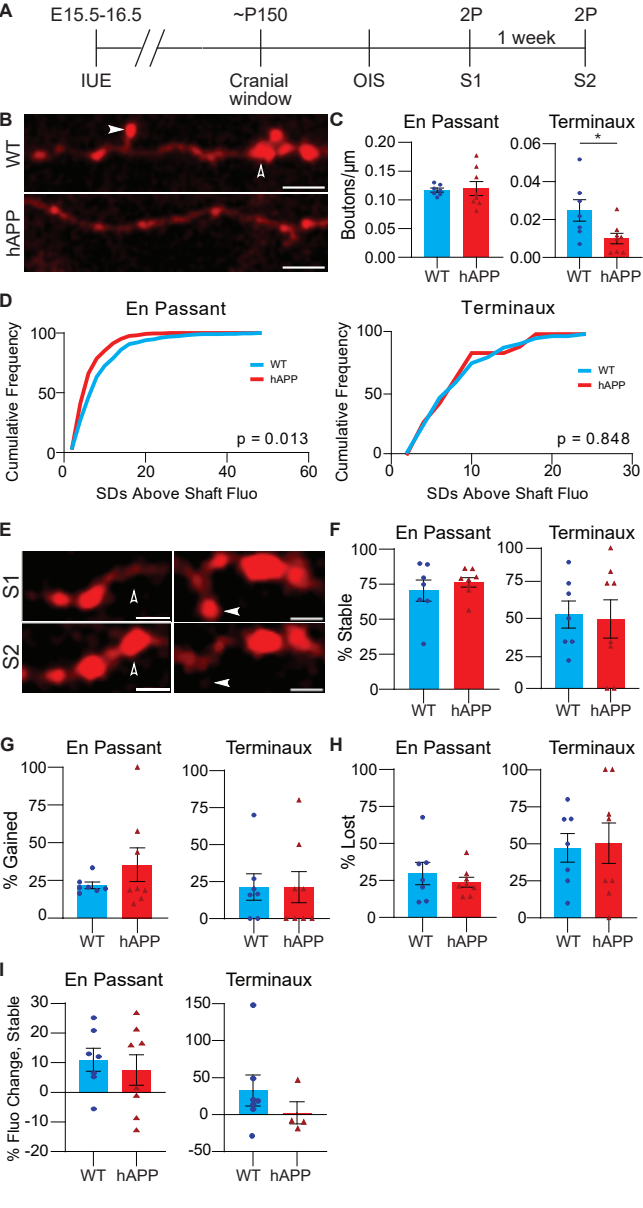
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C
En Passant
Terminaux
Boutons/µm
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0.05
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0.02
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Terminaux
Cumulative Frequency
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WT
hAPP
p = 0.013
p = 0.848
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20
40
60
10
20
30
SDs Above Shaft Fluo
E
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F
En Passant
Terminaux
% Stable
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75
50
25
0
WT
hAPP
G
En Passant
Terminaux
% Gained
H
En Passant
Terminaux
% Lost
I
En Passant
Terminaux
% Fluo Change, Stable
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20
10
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-10
-20
150
100
50
0
-50