

Characterizing microglial interactions with cortical neurons during development of the healthy brain



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Abstract

Recently, microglia were found to have roles in synaptic pruning, synaptic stripping, and neuroprotection. Through these studies an interesting phenomenon of microglia making soma-to-soma contact, “hugging”, with cortical neurons was observed. The goal of this study was to use transgenic mice to define hugging behavior throughout post-natal neuronal development. Specifically, how many soma-to-soma contacts were being made by microglia and what types of neurons were being contacted. I further investigated the role of AMIGO1, a cell adhesion molecule found in neurons and glia, in hugging behavior. Understanding normal microglial-neuronal interactions in the healthy brain is relevant to many neurodevelopmental diseases.

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Introduction

Microglia are the resident immune cells of the CNS. Having qualities similar to macrophages, microglia perform phagocytic actions on damaged nervous tissue and cell debris and are found activated in the brain under most pathological conditions (Davalos et al. 2005). Until recently, not much was known about microglial function within the healthy brain. It was believed that microglia existed in a dormant state until activation. However, it was shown that microglia within a healthy brain are highly motile with processes that are constantly extending and retracting, serving as a surveillance system for the rest of the brain parenchyma (Nimmerjahn et al. 2005). This continual monitoring of the brain allows the microglia to rapidly respond to any brain injury that may arise by rapid engulfment of cellular debris, and also contributes to the plasticity of the brain as a whole (Nimmerjahn et al. 2005, Davalos et al. 2005).

During the continual surveying of the brain by microglia, contacts are made with astrocytes, neuronal perikarya, axon terminals, and dendritic spines (Nimmerjahn et al. 2005, Davalos et al. 2005, Wake et al. 2009). Among these interactions it was discovered that microglia make direct contacts with neuronal synapses in an activity-dependent manner; the microglia contact active synapses more often (Wake et al. 2009). From these findings it was proposed that resting microglia monitor the functional state of these synapses. With this in mind as well as the microglia's phagocytic properties and rapid engulfment, Tremblay et al. examined the nature of these microglia processes and their relationship with synapses to find if the microglia cause any structural changes at synapses. They found that under normal visual conditions, microglia located to multiple synaptic elements, specifically small dendritic spines (Tremblay et al. 2010). In addition, many of these spines were lost after a few days and much of the microglia processes were surrounded by extracellular growth. Under light deprivation conditions, however, microglia formed phagocytic structures and apposed engulfed synaptic clefts and synapse-associated elements more frequently (Tremblay et al. 2010). These findings gave evidence that microglia actively modify or eliminate synapses in a healthy brain in an experience dependent manner.

During early development, the brain contains numerous synapses, a great number more than in a normal adult brain. Many of these synapses are eliminated while the remaining synapses are retained and strengthened in an activity-dependent manner in a process called synaptic pruning. Most of the excess synapses removed during this process are excitatory (Hua and Smith 2004). The maturation of inhibitory synapses follows the elimination and reinforcement of the excitatory synapses (Grantyn et al. 1995). Recent studies have found microglia to be a vital component in this important process of synaptic pruning (Paolicelli et al. 2011, Schafer et al. 2012). First, it was shown that microglia, during postnatal development, actively engulf synaptic material and eliminate synapses. Furthermore, upon knockout of an

important microglia specific receptor, CX3CR1, microglia numbers were reduced during development of the hippocampus causing synaptic pruning to be delayed and an increase in dendritic spines and immature synapses to remain in the brain (Paolicelli et al. 2011). Improper synapse number and altered dendritic spine morphology are hallmarks of several neurological disorders such as autism, Fragile X syndrome, and Alzheimer's disease (Spronsen and Hoogenraad 2010), demonstrating the importance of microglia in brain development.

With this latest discovery of microglia functionality, it became important to find the exact function of microglia at synapses as well as the underlying mechanisms that cause microglia and synapse interaction. Schafer et al. showed, using the retinogeniculate (RGC) system, that microglia actively engulf healthy transient synapses in the RGC and prune them with a phagocytic mechanism. This synaptic pruning was also found to be performed in an activity-dependent manner. Certain synapses in the RGC were enhanced by pharmacological manipulation and the microglia preferentially engulfed and pruned the "weaker" synapses as a result (Schafer et al. 2012). The classical complement cascade is a potential mechanism that drives the interaction of microglia and developing synapses. The C1q and C3 components of this classical cascade have been shown to localize to immature synapses and perform a necessary function in the pruning of synapses during development (Stevens et al. 2007). Defects in developmental synaptic pruning were observed upon knockout of C1q, C3, and CR3 within mice. The defects in synaptic pruning were due to flaws in the engulfment process of the microglia (Schafer et al. 2012). This provides evidence that the classical complement cascade, specifically the CR3/C3 signaling in microglia, is an underlying molecular mechanism within the engulfment of synapses by microglia during synaptic pruning. However, engulfment was only reduced by approximately 50% in CR3 and C3 knockout mice (Schafer et al. 2012) and the CR3/CR pathway may be independent from other pathways underlying the phagocytic mechanisms in microglia, proving there is still much left to be learned of synaptic pruning by microglia.

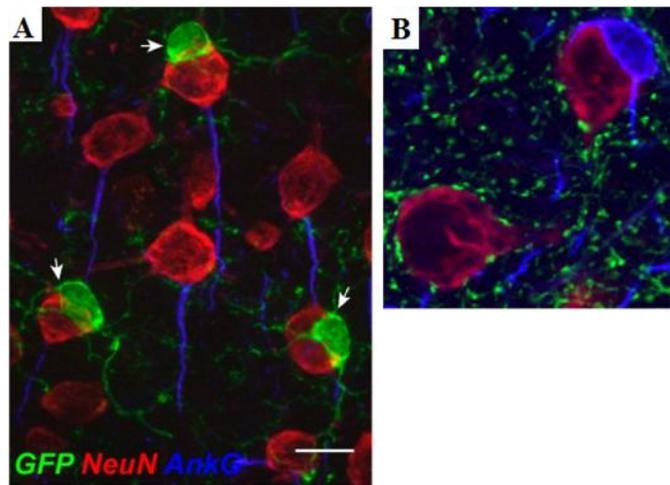


Figure 1 - Soma to soma contact of microglia and neurons. (A) Figure 1a from Baalman et al., showing cortical neurons (red) contacted by microglia (green). (B) Figure 1e from Chen et al., showing microglia (blue) enwrapping an inhibitory neuron (red)

Recently a new phenomenon of microglia making soma to soma contact with neurons has been found (Figure 1). This was first observed by Chen et al. (Figure 1b) when studying the mechanisms of microglia-mediated synaptic stripping, the displacement of pre or post synaptic components from a synapse (Blinzinger and Kreutzberg 1968). They discovered that activated microglia transiently displace inhibitory synapses from cortical neurons in adult mice (8-12 weeks old), reducing inhibitory input, through this soma to soma contact. This mechanism of microglia was described as a neuroprotective role (Chen et al. 2014). Soma-to-soma contact by microglia was also identified in a separate study by Baalman et al (Figure 1a). They found that in the cortex a specific population of microglia, termed axon initial segment- associated (AXIS) microglia, specifically associate with the axon initial segment, the site where action potentials are generated. In order for this association to occur microglia needed to be in contact with the neuronal cell body, in the hugging formation. This association was found to be mainly with excitatory neurons and to start early in development and continue into adulthood (Baalman et al. 2015).

With these two examples of microglia “hugging” of cortical neurons it became clear that more information was needed on the phenomenon. The following experiments were designed to identify how development in the mouse brain may affect this hugging relationship. Both number of instances and type, inhibitory or excitatory, of neurons were observed. It is my hypothesis that during the younger ages of neuronal development, the microglia will associate more with excitatory neurons since there is an excess of these neurons at that time and the microglia are necessary in synaptic pruning. As the mice get older, however, I believe that the microglia will begin to associate more with inhibitory neurons, while still maintaining contacts with excitatory neurons, to perform their neuroprotective or maintenance roles.

Amphoterin-induced gene and ORF (AMIGO) is a relatively recently discovered family of genes that codes for a cell adhesion molecule expressed in neuronal tracts (Kuja-Panula et al. 2003). These proteins have been shown to play a role in axon tract development as well as being an auxiliary subunit in the Kv2.1 potassium channel (Kuja-Panula et al. 2003, Peltola et al. 2011). Knockout of this gene has also been linked with schizophrenia related phenotypes (Peltola et al. 2016). These genes are expressed in many cell types throughout the CNS, including glia cells. The functional reason for the expression of AMIGO in glia cells is unknown at this time, however (Chen et al. 2011). With this in mind, I investigated microglia “hugging” in both AMIGO1 knockout mice and heterozygous mice to see if the gene has any effect on the mechanism. I predict that if AMIGO1 is involved in the “hugging” mechanism, then there will be a noticeable reduction in soma to soma contacts between microglia and cortical neurons.

With increasing functions and roles of microglia in the healthy brain being discovered, this relatively uncharacterized phenomenon of microglia “hugging” of cortical neurons remained an interesting point of focus. This study focused on the effects of development of the CNS and the gene AMIGO1 has on the relationship. The results show that the development appears to have some affect the hugging behavior of microglia. A clear trend was unable to be determined due to a low number of replicates and problems with staining, but with further research, characterization of this behavior throughout development should be within reach. It was also found that AMIGO1 likely has little effect on the sheer number of soma-to-some contacts made by microglia, but an unusual phenotype of “double hugging” did emerge in the AMIGO1 knockout and heterozygous mice and could prove as an interesting topic for future studies.

Materials and Methods

Brain Dissection

Each mouse is anesthetized by the inhalation of isoflurane. The mice are then decapitated and the brain is dissected. The brains are drop fixed in 4% paraformaldehyde in phosphate buffer (PB) (0.0019M sodium phosphate monobasic monohydrate, 0.081M sodium phosphate dibasic anhydrous) for four hours. After three washes in PB, the brains are moved to 30% sucrose solution and stored overnight at 4°C and until sectioning.

Coronal Sectioning

The brain is removed from 30% sucrose and the cerebellum and olfactory bulbs are removed. The brain is chilled on dry ice and cut in forty micron sections with the microtome. The brain is cut from back to front (cerebellum → olfactory bulbs). The sections are stored in PB with 0.01% sodium azide, for preservation, at kept at 4°C.

Immunohistochemical Staining

Brain slices containing the targeted cortical layers are created and selected for analysis as described (Schafer et al. 2012). Slices are blocked for an hour in PBTGS (10% goat serum, 0.3% triton X, 89.7% phosphate buffer). The PBTGS is removed and the primary antibodies (Table 1) are added and the slices are left to incubate overnight. The primary antibody is removed and the slices are washed three times with phosphate buffer. The secondary antibodies (Table 1) are added and the slices incubate for two hours. The secondary antibody is removed and the slices are washed three times again. The slices are then mounted on slides using Fluoroshield™ with DAPI (Sigma-Aldrich).

Table 1 - Antibody Information

Antibody	Type	Manufacturer	Product number
Rabbit - Parvalbumin	Primary	Swant	PV27
Ms x Neuronal Nuclei	Primary	Merck Millipore	MAB377
Alexa Fluor® 594 goat anti-rabbit IgG	Secondary	Invitrogen	A11037
Alexa Fluor® 647 goat anti-mouse IgG	Secondary	Invitrogen	A21236

Microscopy and Image Analysis

All microscopy was done with a Zeiss Cell Observer SD confocal microscope. Laser settings can be in table 2. Three images per hemisphere were taken under 40x magnification. Field of views in the cortex were preferentially chosen based on the number of PV-stained cells (~10 cells per FOV). Slides were blinded before imaging to prevent bias. Images were analyzed using ImageJ. Each image was counted, by hand, for number of microglia, number of PV cells, number of microglia-PV soma-to-soma contacts, and number of Microglia-NeuN soma-to-soma contacts.

Table 2 - Laser settings

Laser	Power	Exposure Time (ms)	EM Gain
Alexa Fluor 405	25%	250	150
Alexa Fluor 488	17%	50	120
Alexa Fluor 561	30%	85	100
Alexa Fluor 638	20%	80	200

Statistics

The data was normalized in three different ways: PV contacts were normalized to the total number of PV cells (PV contacts/PV cells *100), PV contacts were normalized to the total number of microglia (PV contacts/microglia *100), and NeuN contacts were normalized to the number of microglia (NeuN contacts/microglia*100). The mean of each was taken and graphed with the standard error. Two-way and One-way ANOVAs were also performed to check for any significance.

Results

To observe the effects of development on microglial hugging mice of the CX3CR1-EGFP (microglia are labeled with GFP) line were used. Brains sections are imaged and counted for the number of microglia, PV cells, PV-microglia contacts (figure 2A), and NeuN-microglia contacts (figure 2B). Some difficulty was experienced during the staining process. First, PV is not expressed by inhibitory neurons in mice until around P8, so all of the P1 and most of the P8 brains were not stained successfully. Second, some of the NeuN staining stained blood vessels instead of neurons (Figure 2C). This is likely due to problems with the mouse secondary antibody. Finally, some of the early staining did not stain entirely through the brain slice. Some of the microglia still, however, appeared in the typical hugging shape (figure 2D). Therefore, in these slices where the middle was poorly stained, some contacts could have been missed.

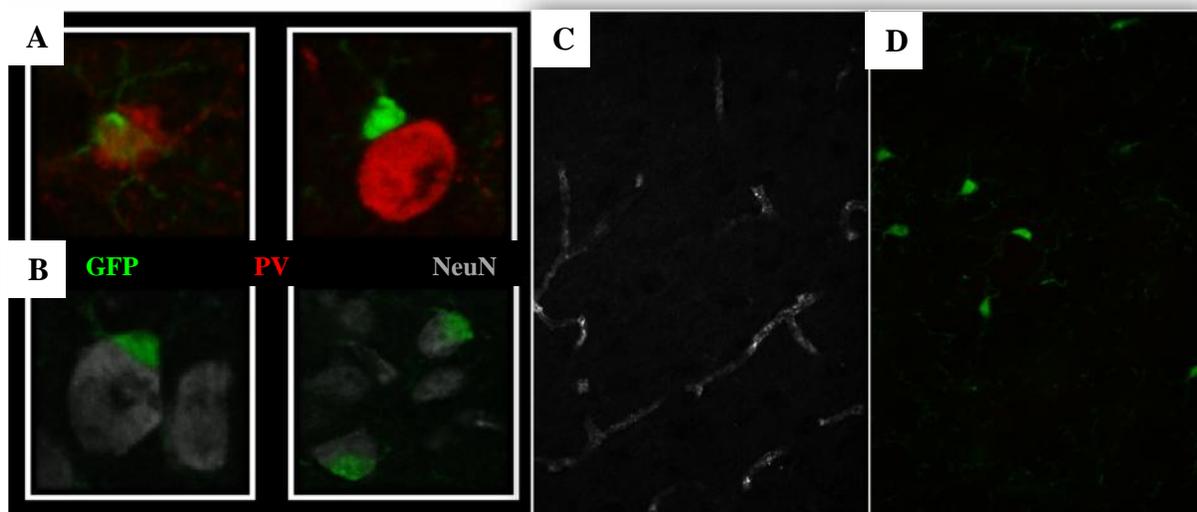


Figure 2 - Microscopy images. (A) Microglia (green) hugging PV-labeled cells (red). (B) Microglia (green) hugging NeuN (grey) cells (C) Example of failed NeuN staining. (D) Microglia (green) in hugging shape in unstained section.

Once the data was collected, it was normalized two separate ways. Normalizing to the number of PV cells allows to account for any unstained regions. Since microglia are labeled through the entire section the percent of microglia making contacts would be inaccurate due to unstained regions. However, percent microglia normalization allows for comparison between PV contacts and NeuN contacts. The normalization by number of PV cells was calculated by dividing the number of PV-microglia contacts of each image by the total number of PV cells. The mean of each age was taken and examined based on the cortical layer (figure 3A). The only significant change was between the prefrontal and somatosensory cortex in the p15 mice. This large reduction was not replicated in the other ages, however. For the total PV-microglia contacts throughout the cortex (figure 3B), there was a reduction in contacts between ages p15 and p21, while p21 and p61 appeared very similar.

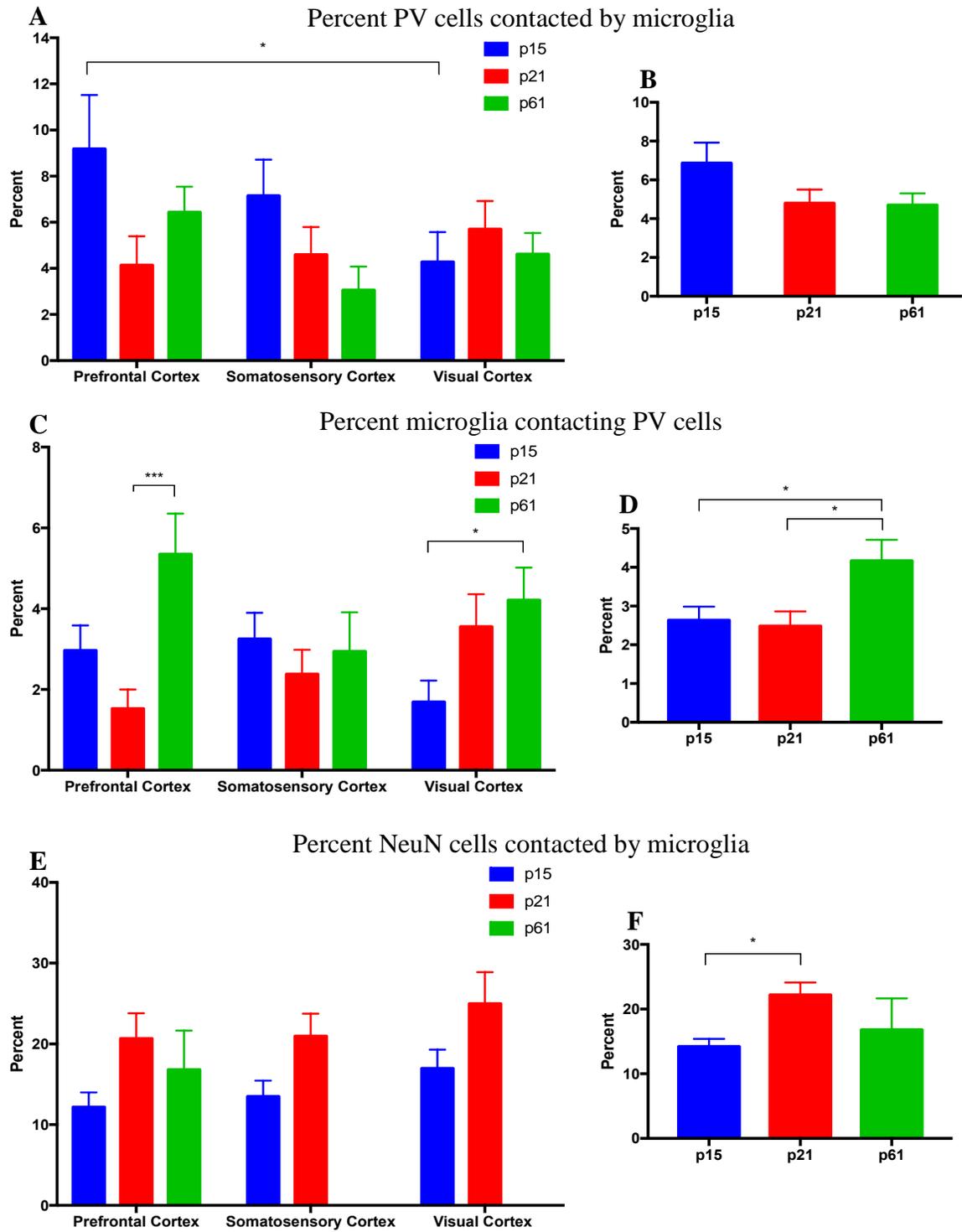


Figure 3 - Developmental effects on microglial hugging behavior. (A) Percentage of PV cells contacted by microglia by cortical region and (B) combined cortex. (C) Percentage of microglia contacting PV cells by cortical regions and (D) combined cortex. (E) Percentage of microglia contacting NeuN cells by cortical region and (F) combined cortex. Error bars represent standard error. * ($P < .05$), ** ($P < .01$), *** ($P < .001$), $N=2$.

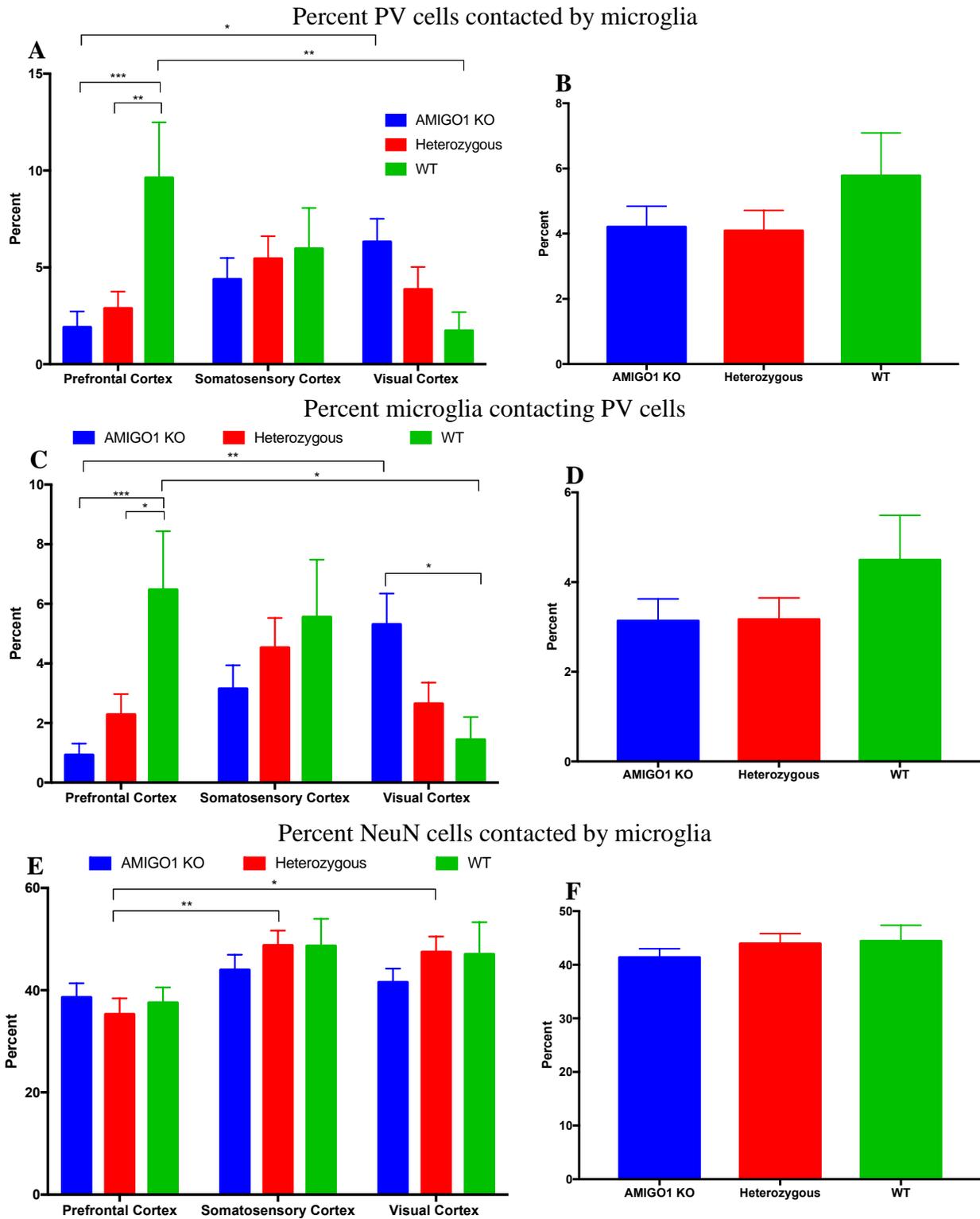


Figure 4 - AMIGO1 effects on microglial hugging behavior. (A) Percentage of PV cells contacted by microglia by cortical region and (B) combined cortex. (C) Percentage of microglia contacting PV cells by cortical regions and (D) combined cortex. (E) Percentage of microglia contacting NeuN cells by cortical region and (F) combined cortex. Error bars represent standard error. * ($P < .05$), ** ($P < .01$), *** ($P < .001$), $N=2$.

The data was then normalized to the number of microglia. Both PV-microglia and NeuN-microglia contacts were divided by the total number of microglia. There were significant increases in PV-microglia contacts in the prefrontal cortex from p21 to p61 and in the visual cortex from p15 to p61 (figure 3C), as well as a significant increase from both p15 and p21 to p61 with contacts in the all of the cortex (figure 3D). This data is slightly misleading, however, since most of the images where the middle was not stained well (figure 2D) appeared in the 015 and p21 ages. Therefore, these ages should have a lower percentage of microglia making contacts since the potential contacts in the unstained regions were unaccounted for. As for the NeuN contacts, an increase in percentage of microglia making contact with NeuN labeled cells from p15 to p21 appeared across all cortical layers (figure 3E). In addition, this proved to be a significant increase in hugging across the entire cortex (figure 3F). Much of the faulty NeuN staining (figure 2C) occurred in the p61 mice. As a result, only 10 images (all in the prefrontal cortex) were able to be quantified.

For the AMIGO1 experiments, all the mice used were p112 and CX3CR1-EGFP heterozygotes. Two knockouts for the AMIGO1 gene (KO), two heterozygotes (Het), and one wild type (WT) mouse was used. The procedure performed to stain and image these mice were the same as in the development experiment. When the data was normalized to the number of PV cells, a significant reduction in hugging behavior occurred in the prefrontal cortex from the WT to either Het or KO mice (figure 1A). This same sort of trend was seen, less drastically, in the somatosensory cortex as well. However, in the visual cortex, an opposite trend was observed with the KO mice having the most PV contacts and WT mice having the least. When looking over the entire cortex (figure 4B), both KO and Het mice appear to have a similar percent of PV cells being hugged, which is about 2% lower than the WT mice. Normalizing the PV-microglia contacts to the number of microglia gives the same trends across each cortical region (figure 4C) and the entire cortex as a whole (figure 4D). As for the NeuN-microglia contacts, there appeared to be little change between any of the genotypes. There was some change in hugging between cortical layers (Figure 4E), but all genotypes were very similar within the layers. The same was true for the entire cortex (figure 4F), where although there was a very miniscule increase in microglia contacting NeuN cells from KO to Het to WT mice, there was no significant changes.

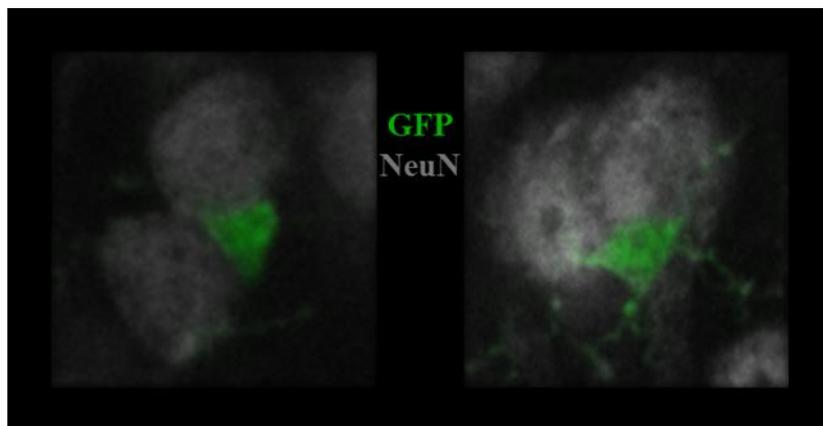


Figure 5 - “Double hugging” images. (A) Microglia (green) hugging two NeuN (grey) cells simultaneously in AMIGO1 knockout mice.

One interesting result that emerged from the AMIGO1 experiments was a strange, new phenotype of one microglia making soma-to-soma with two separate NeuN-labeled cells at the same time. This “double hugging” behavior was exhibited much more in the KO and Het mice than the WT mice. I recorded six separate occurrences in both the KO and Het mice, compared to one once in the wild type mice. In addition, during image analysis, there were several other occasions in the KO and Het mice where there might have been “double hugging”, but, due to unclear staining, it could not be confirmed. This phenomenon was also only observed once throughout the entirety of the development experiments and this example was seen with PV contacts.

Discussion

Through the developmental experiments it became apparent that, while not congruent, development of the healthy brain does have an effect on microglia “hugging” behavior. It was believed that as the brain gets older there would be more contacts with inhibitory cells while maintaining contact with excitatory neurons throughout. The results show an inverse of this, where the percent of PV-labeled cells being contacted by microglia fell with age (figure 3B) and the NeuN contacts increased (figure 3F). Despite some significant changes and potential trends, more replicates and experiments will need to be performed in order to determine a specific relationship between development and microglia “hugging” behavior.

In addition to more replicates there are certainly other ways to further characterize this relationship between microglial “hugging” and development. One way would be getting more time points. I was unable to collect data from either P1 or P8 time points due to staining issues. A potential solution to this could be using different transgenic mice. For example, I dissected some brains of the ‘DLXCre x Rosa26Tomato’ line during my experiments. In this line all of the inhibitory neurons are labeled with a red fluorescent protein. Using this line would allow for inhibitory cells to be labeled even from the earlier time points. I was unable to use these brains for my experiments because there were not enough mice available. Adding more time points throughout the developmental process would contribute in identifying any trend. Other potential future experiments could choose to focus on separate subsets of inhibitory neurons other than PV, such as SST (somatostatin) or CCK (cholecystokinin) inhibitory neurons or specifically focusing on a specific cortical layer, since it does appear there are significant differences in this behavior between them.

Little effect was observed in “hugging” behavior when the cells adhesion molecule, AMIGO1, was knocked out. The overall percentage of microglia contacting either neurons only changed by at most 2% between genotypes (figure 4D&F). One significant result collected however, is the steep reduction in PV-microglia contacts observed between WT and KO mice in the prefrontal cortex. This is meaningful because AMIGO1 KO has been associated, in the past, with schizophrenia related phenotypes (Peltola et al. 2016). A recent study from Anticevic et al. has found that schizophrenia patients exhibit hyper-connectivity of neurons in the prefrontal cortex (Anticevic et al. 2015). Given the phagocytic properties and synaptic pruning role of microglia, elevated connectivity experienced in the schizophrenic prefrontal cortex and the reduction of microglia contacts could prove to be related. Providing a potentially interesting point of study for schizophrenia.

The other interesting result from the AMIGO1 experiments was the emergence of the “double hugging” phenotype. This was the opposite of what was expected since I predicted knocking out AMIGO1, a cell adhesion molecule, would lead to a reduction of contacts. Since these experiments were performed with P112 mice, much older than any used in the development experiments, this phenotype could be resultant of the age of the mice, independent of AMIGO1. However, because the behavior is exhibited much more in the KO and Het mice I

am lead to believe that AMIGO1 KO is related. One potential explanation for this phenomenon could be that since AMIGO1 is involved in dendritic growth of neurons (Chen et al. 2011), knockout of the gene caused neurons to be closer to each other and consequently making it easier for microglia to make soma-to-soma contacts with two neurons at once. More research will be needed to further define microglial “double hugging”.

Overall there is still much left to be learned about the microglial “hugging” behavior. This study revealed evidence of a potential link between development of the brain and changes in this behavior. However, further studies and replicates will be needed to identify and definitive relationship between the two. In addition, two interesting new points of research arose from the AMIGO1 experiments. These being the possible connection of reduction in “hugging” in the prefrontal cortex and schizophrenia and the peculiar phenotype of “double hugging”. With future research microglial “hugging” could prove to be a key function of this versatile cell.

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